Gene Expression Changes in Rat Liver and Testes after Lung Instillation of a Low Dose of Silver Nanoparticles

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Abstract

The expression profile of genes involved in oxidative stress, metal toxicity, apoptosis/cell cycle, and protein folding was investigated in liver and testis of Sprague-Dawley rats at different time intervals after i.t. instillation of AgNP (20 nm, 50 µg/rat). At 7 days, selective changes in the expression of genes encoding oxidative stress-related enzymes, namely Gpx1, SOD, FMO2 and GAPDH were observed in hepatic and testicular tissues. Other genes implicated in oxidative stress (Txnrd1, Gas, Gsr), metal toxicity (Mtf1), apoptosis/cell cycle (casp3, p53), and protein-folding processes (Hsp70) were not modified. Gene expression was modulated by AgNPs in a tissue- and time-dependent manner. In particular, SOD was up-regulated in both tissues, but significant Gpx1, FMO2, and GAPDH overexpression was seen in testes only. No gene expression changes were seen in both tissues 28 days post-instillation. Unlike AgNPs, pulmonary treatment with AgNO3 (7 µg/rat), did not cause gene expression changes in both tissues at both time points studied, suggesting that dissimilar mechanisms are implicated in toxicity and/or biokinetics of nanoparticulate and ionic silver.

The results demonstrate subtle systemic changes involving selected oxidative stress-related genes in the liver and tests of animals exposed by pulmonary route to a low dose of AgNPs. These effects were apparently reversible as changes were observed at day 7 but not day 28. Recovery could possibly reflect either compensatory mechanism contrasting the initial toxicogenomic response to AgNPs or the silver removal from the tested organs. These findings may be of toxicological relevance in relation to possible health risks associated with occupational or consumer exposure to nanosilver.

Keywords: Systemic toxicity; Genomics; In vivo; Superoxide dismutase; Oxidative stress

Introduction

Nanosilver is increasingly used in a wide range of market sectors. Annual silver nanoparticle (AgNP) production was estimated at the level of 500 tonnes based on 2008 data and more than 200 products containing AgNPs are available to the public [1]. Nanosilver antimicrobials, in particular, are currently used in a variety of consumer products such as throat sprays, antiodor sprays and surface disinfectant agents that may be of toxicological relevance in relation to possible health risks associated with occupational or consumer exposure to nanosilver.

Studies in animals have indicated inflammatory changes in the lung after pulmonary application of AgNPs even at low doses. Mice treated with AgNPs by oropharyngeal aspiration (10 µg/mouse about 350 µg/kg bw) developed local inflammation with epithelial cell damage and activation of lung macrophages [11]. Similarly, Park et al. [12] described progressive inflammatory insult in the lung of mice in the 28-day period following a single i.t. instillation of AgNP (125-500 µg/kg bw, corresponding to 3-12 µg AgNPs/mouse).

There are also observations indicating that pulmonary toxicity of locally applied AgNPs may be accompanied by systemic changes resulting from the distribution of silver from the lung to multiple secondary organs. For example, a subchronic 90-day inhalation study in rats exposed to 18 nm AgNPs (49 to 515 µg/m³) revealed dose-dependent hepatic alterations with bile-duct hyperplasia, single-cell hepatocellular necrosis with increased cellular eosinophilia and shrunken condensed nuclei in the animals treated with the highest AgNP dose [13]. Hepatic inflammatory changes were also described in mice treated with AgNPs by oral route [14] or injection [15].

Biokinetic studies in animals given AgNPs by inhalation or lung instillation have shown distribution of silver to multiple organs, with increased silver levels in liver and testis [12,16-23]. Transfer of silver across the blood-testis barrier and predominant localization of silver in liver and testis of laboratory animals was also reported after administration of AgNPs by oral route [24] or injection [15,25]. Excessive testicular levels of silver were still measurable in the animals examined 8 weeks after treatment suggesting incomplete tissue elimination and some retention of the metal after exposure to AgNPs [24]. Crossing of blood-testis barrier by intravenously administered AgNPs has also been shown in rabbits at doses which did not affect the general health status of the animal, libido, serum testosterone, semen
volume and sperm concentration, but caused a dramatic decrease of sperm motility and curvilinear velocity [26].

In the present work, toxicogenomic methods were used to determine whether gene expression changes are induced in rat liver and testis by AgNPs given by lung instillation. The genes examined were selected genes related to biochemical end-points that, according to present knowledge, are implicated in nanosilver toxicity, namely oxidative stress (Gpx1, Sod, Gss, Fmo2, Gsr, Txnrd1, Gapdh), metal toxicity (Mt1), apoptosis/cell cycle (Casp3, p53), and protein folding (Hsp70).

Gene expression was examined 7 and 28 days after intratracheal (i.t.) instillation of a single dose (50 µg/rat) of AgNPs. The effect of AgNPs was compared with the response to AgNO3 used as a control for soluble silver ions.

Several reports have indicated oxidative stress as a mechanism playing an important role in cytotoxic effects of nanosilver [27,28]. AgNPs were shown to induce generation of reactive oxygen species and changes in enzyme activities that are associated with antioxidant defense systems such as glutathione peroxidase (Gpx), reduced glutathione (GSH), and superoxide dismutase (SOD). Changes involving metallothionein, heat shock protein 70 (Hsp70), glutathione S transferase (GST), and p53 have also been documented after nanosilver exposure [28-34].

Only few studies have been conducted regarding the effect of AgNPs on tissue-specific gene expression. In vitro experiments using a human lung epithelial cell line (A549) indicated modulation of more than 1000 genes, including members of the metallothionein, heat shock protein, and histone families in cells exposed to 12.1 µg/ml AgNPs (EC∞) for 24 hours. At this concentration, AgNPs also caused intracellular generation of reactive oxygen species and cell cycle alterations with no evidence of apoptosis or necrosis. Considerably fewer genes (133 genes) responded to an equivalent amount (1.3 µg/ml) of Ag⁺⁺ ions [34]. AgNP-induced modulation of oxidative-stress and tissue damage related genes was also documented by cell culture studies using Caco-2 and M-2 cells [35] as well as by in vivo studies in mice and rats. Modulation by AgNPs of inflammation and tissue damage-related genes was observed in the lung of ICR mice given a single dose of 500 µg/kg AgNPs (12-15 µg/mouse) by i.t. instillation [12]. A total of 261 genes were up-regulated and 103 genes down-regulated by over two fold in the lung 24 hours after treatment. Other toxicogenomic studies have been conducted in rats exposed to AgNPs for 12 weeks by inhalation at doses corresponding to AgNP concentrations from approximately 50 to 380 µg/m³. The results indicated gene expression changes in the kidney with 104 genes that were up- or down-regulated by more than 1.3-fold. The genes with the most significant change in their expression were genes involved in purine metabolism, the B cell and T cell receptor signaling pathway, and natural killer cell-mediated cytotoxicity. No genes related to apoptosis or cell cycle were modified by AgNPs in this study [36].

The field of nanotechnology has grown rapidly over the past few years and has even ventured into the field of clinical medicine. Out of all kinds of nanoparticles, silver nanoparticles (AgNPs) seem to have attracted the most interests in terms of their potential application. Indeed, the widespread use of this precious metal in nano-size form from household paints to artificial prosthetic devices has imparted significant effects on our daily lives. Since the first issue in March 2005, this journal has published many quality papers on silver nanoparticles, both in basic science as well as in more clinically oriented subjects. In this virtual issue, several important papers over these past few years have been selected, which will provide readers with further and up-to-date understanding of synthesis, biological actions, and present applications silver nanoparticles in medicine.

Materials and Methods

Chemicals

All reagents, tissue buffers, chemicals and silver nitrate (AgNO3) were purchased from Sigma-Aldrich (Milan, Italy).

Physicochemical characterization of nanoparticles

A 1% water suspension of AgNPs (series PARNASOS NAMA 391103 F01 1%) was supplied by Colorobbia S.p.A. (Vinci, Italy). The suspension presented a brown color and the following characteristics: 1 g/cm³ density, 3 mPa/sec viscosity (25°C), < 0.50 PdI (polydispersity index) 6.5 pΗ, and 20 nm nominal hydrodynamic size diameter (supplier data).

Detailed physicochemical characterization of AgNPs was performed before their administration to animals. Dynamic light scattering (DLS) and scanning transmission electron microscopy (STEM) technique with energy dispersion X-ray (EDX) were used to determine shape, size distribution, morphology and crystal structure.

A Zetasizer Nano ZS90 (Malvern Instruments, Alfatest-Roma Italy) was used to determine the diameter size distribution and zeta potential of the AgNPs suspended in deionized water. Aliquots of the suspension (250 µl) were transferred to a disposable low volume cuvette and after equilibration to 25°C for 2 min. repeated measurements were performed using 12 runs of 70 s each.

AgNPs were imaged by STEM and energy dispersive x-ray spectroscopy (EDX) was used for point and line profile analysis. EDX was used to determine phase composition of the AgNPs.

STEM with high-angle annular dark-field (HAADF) imaging (CAMCOR, University of Oregon, Eugene, OR, USA) was used to determine elemental composition, shape, size distribution, and morphology of nanoparticles.

High-Resolution TEM (HRTEM) operating in Selected area electron diffraction (SAED) mode (a crystallographic experimental technique that can be performed inside TEM) was applied to obtain information, at small scales, on individual atoms of AgNPs and its defects.

Animals and ethics statement

Adult male Sprague-Dawley rats (12 weeks old) were purchased from Charles River Italia (Calco, Italy) and allowed to acclimate for at least 2 weeks before treatment. Throughout the experiment, animals were kept in an artificial 12 h light:12 h dark cycle with humidity at 50 ± 10%. Animals were provided rat chow (HRF21 diet) and tap water ad libitum.

All animal experiments were performed according to the guidelines of the Maugeri Foundation Animal Care and Use Committee and in compliance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes. The study’s objectives and procedures were authorized by the Veterinary Health Dept. of the Italian Ministry of Health (Rome) - License issued on 01.08.1994, ref. n. 90/94, and approved by the Ethic and Welfare Committee for Experiments on Animals of Maugeri Foundation.

Animal treatments

A group of twelve rats was treated with a single intratracheal (i.t.)
instillation of AgNPs (50 μg/rat). Separate groups of animals received
0.1 ml/rat AgNO₃ solution intratracheally to administer 7 μg/rat of
AgNO₃ corresponding to 4.4 μg Ag/rat; 10 rats, or 0.1 ml/rat of saline
(8 rats).

The different applied dosages between AgNPs and AgNO₃, rely on
previous studies [37,38] indicating that the proportion of silver in the
AgNPs (14 ± 4 nm in diameter) suspension not present as nanoparticles
was 11% of the total silver concentration and this (ionic form) fraction
remained stable for several months in suspension. Assuming that the
AgNPs used in our study released 11% of their silver content as ionic
silver, it is expected that the animals treated with 50 μg/rat of AgNPs
received approximately 6 μg/rat of silver ions.

Rats were anesthetized with pentobarbital sodium and i.t. instilled
with the test materials or saline. Intratracheal administration was
performed using a Teflon catheter inserted transorally into the trachea
lumen. To facilitate the procedure, a veterinary fiberoptic otoscope was
used to view the epiglottis and a speculum to hold the mouth open.
AgNPs suspension was vortexed immediately before the administration
to force nanoparticle dispersion and avoid formation of agglomerates.

Gene expression profile examination was conducted 7 or 28 days
after treatment. Rats were anesthetized by i.p. injection of 35% chloral
hydrate (100 microl/100 g bw). Liver and testes were removed, placed in
RNAlater buffer (Sigma) and stored for further analysis.

Nearly monodisperse AgNPs have been prepared in a simple
oleylamine-liquid paraffin system [16]. It was shown that the formation
process of AgNPs could be divided into three stages: growth, incubation
and Oatwald ripening stages. In this method, only three chemicals,
including silver nitrate, oleylamine and liquid paraffin, are employed
to force nanoparticle dispersion and avoid formation of agglomerates.

For real-time PCR study, cDNA was obtained by reverse transcription
starting from 4 μg of total RNA combined with 1 μl of 50 μM OligodT
(Invitrogen™), 1 μl of 10 mM dNTPs (Promega Corporation, USA) and DEPC water to a final volume of 12 μl. The mixture was preheated at 65°C for 5 min to
denature secondary structures and then rapidly cooled to 4°C, adding
4 μl of 5X MMLV Reverse Transcriptase Reaction Buffer (Promega
Corporation, USA), 2 μl of 0.1 M DTT (Invitrogen™), 0.5 μl of 40 U/μl
Rnasin™ Plus RNase Inhibitor (Promega Corporation, USA) and 200 U
MMLV Reverse Transcriptase (Promega Corporation, USA). The RT
mix was incubated at 37°C for 50 min and then stopped by heating
at 70°C for 15 min. The cDNA stock was stored at -20°C for further
analysis.

Gpx1, Gss, Gsr, GAPDH, Txdn1, FMO2, SOD, p53, Casp3, Mt1 and
Hsp70 were selected as target genes for preliminary semi-quantitative
analysis [39-41]. β-actin was selected as endogenous gene.

Gene specific primers (Table 1) were designed using the software
Primer 3 [42,43] and synthesized by Invitrogen™. 1 μl of c-DNA
mix was incubated at 37°C for 50 min and then stopped by heating
and then rapidly cooled to 4°C, adding
4 μl of 5X MMLV Reverse Transcriptase Reaction Buffer (Promega
Corporation, USA), 2 μl of 0.1 M DTT (Invitrogen™), 0.5 μl of 40 U/μl
Rnasin™ Plus RNase Inhibitor (Promega Corporation, USA) and 200 U
MMLV Reverse Transcriptase (Promega Corporation, USA). The RT
mix was incubated at 37°C for 50 min and then stopped by heating
at 70°C for 15 min. The cDNA stock was stored at -20°C for further
analysis.

β-actin

**Table 1:** Primer used in this work.
at 72°C for 10 min. Amplification products were resolved on a 1% agarose gel in TAE buffer and stained with ethidium bromide, images were acquired by the Gel Doc 2000 system and band intensities were evaluated using Quantity One software (Bio-Rad).

### Real Time PCR and data analysis

Gene specific primers (Table 2) and TaqMan® probes for the genes selected by semi-quantitative analysis (Gpx1, GAPDH, FMO2, SOD, β-actin) were purchased from Applied Biosystems. cDNA synthesis was carried out starting from 4 μg of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystem), according to the manufacturer’s instructions.

Amplification was performed adding to 1 μl of cDNA, 0.5 μl of probe and 6 μl of Taqman Universal Master Mix (Bio-Rad) in a final reaction volume of 10 μl. Each sample was then split into two replicate of 5 μl. Reactions were conducted in a Bio-Rad CFX Connect™ thermocycler at the following conditions: 95°C for 10 min followed by 40 cycles at 95°C for 10 s and 60°C for 1 min. Real time amplifications were repeated two times for each gene and each sampling time. For each target gene (i.e., Gpx1, GAPDH, FMO2, SOD, β-actin) of each sample, β-actin was simultaneously processed as the endogenous control. The obtained 2-ΔCt values, where Ct is the Threshold cycle (i.e., the first cycle over the background), were recorded and data analysis was performed resorting to a Multiple Linear Regression. Time (i.e., 7 and 28 days) and organ (i.e., liver and testis) were taken as independent variables, 2-ΔCt as the dependent variable.

This citrate-based agent was selected because the weakly bound capping agent provides long term stability and is readily displaced by various other molecules including thiols, amines, polymers, antibodies, and proteins.

### Results

#### Animal body weight

The animals treated with AgNPs or AgNO₃ did not differ from controls in the rates of their body weight gain over the day-28 post-treatment period. The average body weight increase was about 40% in all groups.

#### Physicochemical characteristics of silver nanoparticles (AgNPs)

Dynamic light scattering (DLS) determination of the AgNP size distribution shows a preponderance presence of nanoparticles ranging from 10 to 20 nm (evaluation by volume) associated with few dispersed particles possessing larger diameter (evaluation by intensity) (Figure 1).

The zeta potential was -15 indicating nanoparticles dispersed in water would remain stable during storage. Although there was no tendency to agglomerate, the particle sample was vortexed prior to measurements.

| GENE   | NCBI          | Probe ID           | Amplicon | Exon |
|--------|---------------|--------------------|----------|------|
| Gpx1   | NM_030826.3   | Rn00577994_g1      | 77       | 1-2  |
| SOD    | NM_017050.1   | Rn00566938_m1      | 62       | 1-2  |
| Fmo2   | NM_144737.2   | Rn00595179_m1      | 73       | 7-8  |
| GAPDH  | NM_017008.3   | Rn01749022_g1      | 60       | 1-1  |
| β-actin| NM_031144.2   | Rn01412977_g1      | 81       | 1-2  |

Table 2: Real-time probes used in this work.
Additional techniques were used to better characterize the physicochemical properties of the AgNPs tested.

Quantitative scanning transmission electron microscopy (STEM) analysis showed that 25 nm nanoparticles were the predominant entity present in the suspension with a relatively narrow size distribution and no evidence of aggregation of the AgNPs tested (Figures 2A and 2C).

STEM with high-angle annular dark-field (HAADF) imaging showed AgNPs containing pure silver, as indicated by the energy dispersive x-ray (EDX) spectrum (Figure 2B). There were no peaks of impurities; the other minor elements detected being related to water background. Purity represents an important property of the test material.

Figure 3 shows the morphologies of AgNPs on atomic scale, as determined by High-Resolution Transmission Electron Microscopy (HRTEM). The AgNPs were mostly spherical in shape having a smooth surface and were well dispersed. HRTEM analysis indicated the presence of two populations of nanoparticles, their size being 10-20
Gsr, Txnrd1, FMO2, SOD, p53, Casp3, Mt1, Hsp70, treatments. Preliminary semiquantitative analyses examined Gpx1, Gss, samples to determine possible gene expression changes caused by gene expression profile evaluation and Figure 7). using real time PCR was specifically addressed to these genes. SOD, FMO2, and . Therefore, subsequent quantitative analysis GAPDH genes were mainly genes involved in oxidative stress, namely Gpx1, expression pattern observed in liver and testis 7 days after pulmonary actin as endogenous gene. The gene as candidate target genes, using β-... Coccini T, Gornati R, Rossi F, Signoretto E, Vanetti I, et al. (2014) Gene Expression Changes in Rat Liver and Testes after Lung Instillation of a Low Dose of Silver Nanoparticles. J Nanomed Nanotechnol 5: 227. doi: 10.4172/2157-7439.1000227

At day 7 post-instillation, the testis of rats treated with AgNPs, 50 µg/rat (Figure 5) showed up-regulation of all the genes considered (Gpx1, SOD, FMO2 and GAPDH). In contrast, at day 28 post-administration, testicular gene expression levels were not different in the AgNP-treated animals and control (Figure 5A). Similar, although less pronounced, changes in the gene expression profile was observed in liver (Figure 5B). Genes were up-regulated at day 7 following AgNP instillation while at day 28 gene expression was within basal levels.

The animals treated with AgNO3 showed no relevant gene expression changes in both the testis and liver tissues at both day 7 and day 28 after dosing (Figure 6).

In control animals, the expression of the testicular and hepatic genes considered was identical at the day-7 and day-28 observation periods (Figures 5 and 6).

The raw data (means reported in Figures 5 and 6) were analyzed resorting to generalized linear model (GLM). The p-values of the pair wise comparisons are reported in Table 3 for both tissues. The results showed that effects of treatments were consistent across the tissue samples tested indicating reliable experimental conditions and results across the gene expression profile experiments.

In testis, statistical analysis confirmed the AgNP-induced up-regulation at day 7 and the consequent recovery of the expression at day 28 post-treatment. Significant p-values were observed for all the comparisons relating to rats examined at day 7, but not for those examined at day 28.

For the liver, AgNP-induced up-regulation was significantly confirmed for SOD only suggesting a less pronounced effect of AgNPs in liver compared to testis.

For a better comparison of the experiments, in Figure 6, values are visualized after normalization with their controls. In other words, the data reported in Figure 5 as 2-ΔCt are here expressed as 2-ΔΔCt. This normalization consists in subtracting the ΔCt of the control tissues from the ΔCt of the tissues of the animals exposed to NPs and ions.

The p-values obtained for all the comparisons between control animals examined at day 7 and those examined at day 28 post-instillation resulted greater than 0.05 confirming that controls at day 7 and at day 28 can be considered equivalent.

Similarly, pairwise comparisons between animals instilled with AgNO3 and controls did not show significant differences at both day 7 and day 28, confirming that no changes in the hepatic or testicular gene expression occurred at both time periods after AgNO3 treatment.

**Discussion**

In this study, induction of selected oxidative-stress related genes was shown to occur in rat liver and testis after a single treatment with AgNPs given by intra-tracheal instillation. The amount of AgNPs administered (50 µg/rat) represent a considerably low dose compared with other in vivo studies that examined systemic toxicological effects of nanosilver. In mice treated with a single dose of AgNPs by oropharyngeal aspiration (10 µg/mouse), Bezemer et al. [11] observed a strong inflammatory response in the lung, with epithelial cell damage and activation of lung macrophages. Similarly, Park et al. [12] described marked inflammatory changes in the lung of mice dosed with a single i.t. instillation of AgNP (125 to 500 µg/kg bw, corresponding to approximately 3-12 µg/mouse). In these studies, the extra-pulmonary effects of AgNPs were not examined.

Extra-pulmonary gene expression was up-regulated by the i.t.

nm (the predominant entity) and 40-50 nm, respectively. Presence of approximately 20 nm pure silver nanoparticles was also confirmed by selected area (electron) diffraction (SAED) pattern analysis (Figure 4 and Figure 7).

**Gene expression profile evaluation**

Real-time PCR experiments were performed on testis and liver samples to determine possible gene expression changes caused by treatments. Preliminary semiquantitative analyses examined Gpx1, Gss, Gsr, Txnrd1, FMO2, SOD, p53, Casp3, Mt1, Hsp70, β-actin, and GAPDH as candidate target genes, using β-actin as endogenous gene. The gene expression pattern observed in liver and testis 7 days after pulmonary instillation of AgNPs differed from control. The differentially expressed genes were mainly genes involved in oxidative stress, namely Gpx1, SOD, FMO2, and GAPDH. Therefore, subsequent quantitative analysis using real time PCR was specifically addressed to these genes.

At day 7 postinstillation, the testis of rats treated with AgNPs, 50 µg/
instilled AgNPs in a gene type-, organ-, and time-dependent manner. AgNPs modulated a group of selected genes that are involved in oxidative stress, namely Gpx1, SOD, FMO2 and GAPDH. Other genes which are similarly important in oxidative stress processes (TxnrD1, Gss, and Gsr) as well as genes that are known to be involved in metal toxicity (mt1), apoptosis/cell cycle (casp3, p53), and protein folding mechanisms (Hsp70) were not modified by pulmonary treatment with AgNPs.

There were differences between liver and testis in the gene modulation patterns associated with nanosilver administration. SOD was up-regulated by AgNPs in both tissues whereas significant up-regulation of Gpx1, FMO2, GAPDH occurred in testes only. Induction of these genes in hepatic tissue was minimal possibly indicating higher susceptibility of testis to gene modulatory effects of AgNPs compared with liver. The response to AgNPs was also time-dependent. Gene expression changes were observed in the organs of animals examined 7 days post-administration whereas 28 days after dosing the gene expression profiles in liver and testis did not differ from controls.
No statistically significant gene expression changes were found in hepatic and testicular samples obtained from the animals treated with a corresponding dose of AgNO₃.

Physicochemical characterization studies indicated presence of two populations of AgNPs, with size ranging from 10 to 20 nm (the majority entity) and 40-50 nm (minor item), respectively in the suspension used for product instillation.

Particles were shown to contain no impurities. They were stable in suspension, as indicated by STEM analysis, with no tendency to agglomerate.

No attempts were made in our study to determine the release of silver from NPs. According to literature data [24] likely referring to data reported by Bouwmeester et al. [35], AgNPs suspensions used for toxicological studies contain from 6 to 17% of silver in ionic form. Moreover, Loeschner et al. [38] in their in vivo AgNP investigation, quantified the proportion of silver in the AgNP (14 ± 4 nm) suspension not present as nanoparticles. The determination indicates that 11% of the total silver concentration was not present as nanoparticles despite purification of the AgNP suspension after synthesis, this in agreement with a former study from Kittler et al. [37]. Moreover, the Ag ion component remained stable over a time period of at least nine months.

The antioxidant genes (Cu/Zn-SOD and gpx1) upregulated by AgNPs are genes encoding enzymes that are linked to metabolic pathways of considerable toxicological importance. Copper/zinc superoxide dismutase (SOD) catalyzes the dismutation of superoxide to hydrogen peroxide and molecular oxygen, glutathione peroxidase (Gpx) can act as a hydrogen peroxide (H₂O₂)-removing enzyme. Both these enzymes are scavengers exerting a protective role against oxidative damage in mammalian cells [44]. FMO and GAPDH genes also were upregulated in the liver and testis of rats receiving AgNPs by pulmonary route. Flavin-containing monoxygenases (FMO), a family of NADPH and FAD-dependent enzymes, are involved not only in oxidative metabolism of xenobiotics but also in control of the cellular redox potential [45]. In the nematode Caenorhabditis elegans model, AgNP-induced up-regulation of the FMO-2 gene was associated with oxidative stress [46]. Similarly, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was recently identified as a major target protein in oxidative stress [47-48].

The observed changes in the expression of oxidative-stress related...
genes may likely represent a process occurring as part of the initiating events in AgNP-induced toxicity, although the mRNA concentration changes have been presently emphasized as proxies for abundance of corresponding proteins without concurrent measurements of oxidative stress (e.g., oxygen species assays, GSH/GSSG quantification) or individual protein (Gpx1, FMO2, SOD and GAPDH) expression. Studies have indicated a variety of cellular alterations such as protein oxidation, lipid peroxidation, inflammatory changes, membrane disruption, signaling pathways and mitochondrial dysfunction, DNA damage, and apoptosis as common events typically associated with AgNP-induced oxidative stress [9,27,39,49]. The up-regulation of oxidative stress-related genes encoding SOD and Gpx enzymes may be an adaptive mechanism by which hepatic and testicular cells can contrast the action of reactive oxygen species generated and initial cytotoxic effects caused by AgNPs.

It is unclear from our results if the gene expression changes observed after administration of AgNPs reflected a direct action of NPs, the action of silver ions released from the nanoparticles or both. It has been suggested that the ionic silver released from the particle surface is the actual species that accounts for the toxicity of AgNPs [35]. Studies have indeed demonstrated a certain release of ions from the surface of AgNPs [24,38] that remained stable over several months.

In our experiments, unlike AgNPs, AgNO3 caused no changes in hepatic and testicular gene expression, possibly reflecting differences in the mechanisms of toxicity and/or pharmacokinetics between AgNPs and AgNO3.

With AgNO3 given at low dose levels, much of the absorbed Ag ions are expected to precipitate with chloride or phosphate anions in biological media or become strongly bound in the form of inert ions are expected to precipitate with chloride or phosphate anions [24,38]. Consequently, there would be no passage of biologically active Ag ions from the lungs to systemic organs and the toxicological impact of the irreversibly bound silver would be negligible.

In our study, the dosage of AgNPs administered to animals was considerably low. Therefore, it seems unlikely that the molecular changes observed in testis and liver were exclusively dependent on Ag ion dissolution from the NPs.

The gene expression changes may have resulted, at least in part, from a direct action of AgNPs translocated from lung to liver and testis. In principle, there are various mechanisms by which locally administered AgNPs may translocate throughout the body and affect at distance secondary organs. For example, AgNPs dissolved in body fluid may generate systemically distributed Ag ions or AgNPs may interact with some proteins and be distributed in the body throughout protein metabolism [50].

Although tissue silver concentrations were not measured in our study, deposition of silver in liver and testis was documented in animals given AgNPs by different administration routes, including inhalation, intratracheal instillation, oral gavage, or intraperitoneal injection [12,17,18,21,23,51,52]. Comparative studies in rats treated orally with Ag particles, or nanoparticulate silver indicated almost identical organ distribution pattern of the metal, supporting the concept that the metal organ deposition is not due to the whole particles being deposited, but rather due to the ionic silver contained in the suspension or ionic silver that is released from the particle surface [24,36].

However, other experiments in rats treated subcutaneously with a large dose of AgNPs (62.8 mg/kg) demonstrated that, even 24 weeks after dosing, amounts of silver were present in liver also in the form of particles, as shown by TEM analysis [53].

In fact, the respective roles of NPs and ions in AgNP toxicity remains a complex issue [54]. The rate of silver ion release from the surface of AgNPs in suspension is dependent on the particle size, temperature, pH, and oxygen concentration [27]. It is not completely clear how dissolved silver ions and the particulate form interact with cell components. Gene expression studies in the nematode Caenorhabditis elegans showed up-regulation of the FMO-2 gene and oxidative stress that were more pronounced with AgNPs than AgNO3 [46] indicating that AgNP toxicity cannot be explained solely by the presence of ions released into solution by the particles [55].

In recent studies, AgNPs were shown to cause inflammatory changes in the lung when given to laboratory animals by respiratory route [11,13,19]. Our work confirmed these observations (manuscript in preparation). However, the most relevant finding was the occurrence of gene expression changes in testis and liver, indicating a possible role of these organs as toxicity targets for AgNPs given at low dose intratracheally. Testis was apparently more susceptible than liver to AgNP-induced changes in gene expression. While SOD was up-regulated in both tissues, only in testes Gpx1, FMO2, and GAPDH were significantly up-regulated 7 days after AgNP instillation.

Susceptibility of liver and testis to effects of inhaled or ingested silver has been indicated by previous reports describing adverse effects such as generation of reactive oxygen species, inflammation, morphological alterations, changes in enzyme activities, autophagy-related changes, cell apoptosis/necrosis and DNA damage to laboratory animals given large doses of silver [13,14,18,52,53,55-57]. Rats treated with AgNPs (49 to 515 µg/m³) for 13-week showed bile-duct hyperplasia and hepatocelelular necrosis [13]. Other experiments in rats indicated testicular changes after intravenous administration of AgNPs (5 or 10 mg/kg), with dose-dependent decrease in epididymal sperm count, DNA damage in germ cells, and histological changes in seminiferous tubules morphology [58]. AgNPs applied to testicular cell preparations caused concentration dependent cytotoxicity as indicated by apoptosis, necrosis and decreased cell proliferation [59].

The results of our study suggest that the hepatic and testicular effects induced by nanosilver were reversible. Indeed, over-expression of genes encoding antioxidant enzymes was observed in these organs at day 7 but not day 28 after i.t. instillation of AgNPs. This would indicate recovery from the early gene expression changes caused by AgNPs possibly due to compensatory mechanisms or elimination of silver from the organs tested. In rats treated by inhalation with elemental silver (15 nm) nanoparticles [21], silver was rapidly cleared from the lung with only 4% of the initial burden remaining in lung tissue by day 7 after cessation of exposure. Rapid removal of the metal from lung was also observed in rats treated with 50 µg of agglomerate ultrafine elemental Ag by i.t. instillation [21]. In rats examined 17 days after i.v. administration of AgNPs, Ag concentrations rapidly decreased in organs including liver and testes; the peak Ag concentrations was measured at day 6 and total elimination of silver was observed by day 17 [60]. However, other studies in animals treated with high oral doses of AgNPs (several milligrams per day) indicated tendency of silver to be retained in tissues for long periods, excessive concentrations of the metal being measurable in the testis even months after dosing [24,51].

In summary, this study indicated AgNP-induced over-expression of four oxidative stress related genes in systemic organs (liver and
testis) of animals exposed to a low concentration of Ag-nanoparticles by pulmonary route. No AgNP-induced changes occurred in these organs regarding other genes similarly linked to mechanisms that are important in silver toxicity such as apoptosis and metallothionein genes. Unlike AgNPs, ionic silver given as AgNO₃ caused no gene expression changes in testis and liver.

The induction of antioxidant genes found in 7 days after instillation of AgNPs may reflect a first defense response line that is activated in the target tissues to contrast the initial AgNP-induced changes in oxidative stress pathways.

How toxicologically relevant are the effects caused by inhaled silver nanoparticles in the testis and liver is unclear. Imbalance between factors promoting oxidative stress and antioxidant capacity has generally been described as a condition of toxicological concern which can play an important role in pathophysiology of several disease states that involve the liver [61] and testis [62]. In particular, a pro-oxidative insult inflicted to testis may be of special concern given the inherent vulnerability of testis to oxidative stress. Spermatozoa membranes are rich of polyunsaturated fatty acids this making them very susceptible to oxidative damage. Generation of reactive oxygen species and oxidative stress processes have been shown to exert a deleterious impact on spermatogenesis and a key role in mechanisms leading to sperm function impairment and male infertility [62].

The occurrence of oxidative stress-related changes caused by a low dose of AgNPs given via the respiratory tract should also be considered with respect to possible health risks associated with occupational exposure to AgNPs [63]. The American Conference of Governmental Industrial Hygienists [64] recommends a threshold limit value-time weighted average (TLV-TWA) of 100 µg/m³ and 10 µg/m³ for metallic silver and soluble silver compounds, respectively. These threshold limits were derived from classical toxicological studies in which the unique physical and chemical properties of silver nanoparticles were not fully considered. Indeed, in accordance with the results of our study, a large number of literature data, indicate a number of biological and toxic effects caused by AgNPs that are peculiar of their nanoscale properties.

More information is needed to determine if the currently established ACGIH standards for occupational exposure to silver compounds are also adequate to protect workers and consumers from any potential harmful exposures to AgNPs.

On the other hand, generalization of our findings requires caution for different reasons. First, our study examined the response to AgNPs at only two time points post-administration, this precluding a precise characterization of the observed gene expression changes with respect to time course and recovery. Second, the gene modifications were only investigated after one single dose and therefore it is unclear whether they reflected a transient non-specific stress response to AgNPs or the starting point of progressive changes leading to frank organ toxicity. Third, it remains to be established if the AgNP-induced changes were specifically dependent on the size of the particles tested. Size can be relevant in the cytotoxic effects of AgNPs. Smaller AgNPs can induce more oxidative stress, DNA damage and lethality than larger particles when compared at mass doses. Furthermore, the high surface area to volume ratio of smaller AgNPs may cause enhanced release of toxic Ag⁺ ions from their surface, in comparison with larger AgNPs [27].

Further dose-response and long-term exposure studies may contribute to determine more precisely the role of AgNPs-induced oxidative damage in toxicological effects and health hazards associated with nanosilver exposure.

Conclusions

This in vivo research study demonstrates subtle systemic changes involving selected oxidative stress-related genes in secondary target organs (such as testis and liver) of animals exposed by pulmonary route to a low dose of silver nanoparticles (AgNPs, 20 nm diameter). The AgNP-induced hepatic and testicular effects were reversible as gene expression changes were observed at day 7 but not day 28 after instillation.

The present findings, in accordance with a large number of literature data, support the evidence of the capability of AgNPs to cause biological and toxic effects that are peculiar of their nanoscale properties, and may contribute to provide information needed to determine if the currently established ACGIH standards (for occupational exposure to silver compounds) are also adequate to protect workers and consumers from any potential harmful exposures to AgNPs.

Declaration of Interest

The authors declare that they have no competing interests. The authors alone are responsible for the content and writing of the paper.

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References

1. (2014) ENRHES Project final report: Engineered nanoparticles: Review of Health and Environmental Safety Joint Research Centre.
2. Chen X, Schluesener HJ (2008) Nanosilver: a nanoproduct in medical application. Toxicol Lett 176: 1-12.
3. Quadros ME, Marr LC (2011) Silver nanoparticles and total aerosols emitted by nanotechnology-related consumer spray products. Environ Sci Technol 45: 10713-10719.
4. Becker S (2013) Nanotechnology in the marketplace: how the nanotechnology industry views risk. J Nanopart Res 15: 1426-1427.
5. http://www.nanotechproject.org/inventories/consumer/analysis_draft/
6. Miller A, Drake PL, Hinz P, Habjan M (2010) Characterizing exposure to airborne metals and nanoparticle emissions in a refinery. Ann Occup Hyg 54: 504-513.
7. Lee HJ, Mun J, Park JD, Yu UJ (2012) A health surveillance study of workers who manufacture silver nanomaterials. Nanotoxicology 6: 667-669.
8. Hansen SF, Baun A (2012) When enough is enough. Nat Nanotechnol 7: 409-411.
9. Christensen FM, Johnston HJ, Stone V, Aitken RJ, Hankin S, et al. (2010) Nano-silver - feasibility and challenges for human health risk assessment based on open literature. Nanotoxicology 4: 284-295.
10. Lem KW, Hsu SH, Lee DS, Ibqal Z, Sund S, et al. (2012) Waste Minimization for the Safe Use of Nanosilver in Consumer Products - Its Impact on the Eco-Product Design for Public Health. Public Health - Methodology, Environmental and Systems Issues.
11. Bezenzer GF, Bauer SM, Oberdörster G, Breyssse PN, Pieters RH, et al. (2011) Activation of pulmonary dendritic cells and Th2-type inflammatory responses on instillation of engineered, environmental diesel emission source or ambient air pollutant particles in vivo. J Innate Immun 3: 150-166.
12. Park EJ, Choi K, Park K (2011) Induction of inflammatory responses and gene expression by intrastracheal instillation of silver nanoparticles in mice. Arch Pharm Res 34: 299-307.
13. Sung JH, Ji JH, Park JD, Yoon JU, Kim DS, et al. (2009) Subchronic inhalation toxicity of silver nanoparticles in mice. Toxicol Sci 108: 452-461.
14. Cha K, Hong HW, Choi YG, Lee MJ, Park JH, et al. (2008) Comparison of acute responses of mice livers to short-term exposure to nano-sized or micro-sized silver particles. Biotechnol Lett 30: 1893-1899.
15. Wang Z, Qu G, Su L, Wang L, Yang Z, et al. (2013) Evaluation of the biological fate and the translocation through biological barriers of silver nanoparticles in mice. Curr Pharm Res 19: 6691-6697.

16. Chen D, Xi T, Bai J, Wang J (2009) Nanosilver subchronic toxicity and silver distribution in different rat tissues. J Clin Rehabil Tissue Eng Res 13: 3181-3184.

17. Ji JH, Jung JH, Kim SS, Yoon JU, Park JD, et al. (2007) Twenty-eight-day inhalation toxicity study of silver nanoparticles in Sprague-Dawley rats. Inhal Toxicol 19: 857-871.

18. Kim YS, Kim JS, Cho HS, Rha DS, Kim JM, et al. (2008) Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats. Inhal Toxicol 20: 575-583.

19. Stebouanova LV, Adamcakova-Dodd A, Kim JS, Park H, O’Shaughnessy PT, et al. (2011) Nanosilver induces minimal lung toxicity or inflammation in a subacute murine inhalation model. Part Fibre Toxicol 8: 5.

20. Sung JH, Ji JH, Yoon JU, Kim DS, Song MY, et al. (2008) Lung function changes in Sprague-Dawley rats after prolonged inhalation exposure to silver nanoparticles. Inhal Toxicol 20: 567-574.

21. Takanaka S, Karg E, Roth C, Schultz H, Ziesenis A, et al. (2001) Pulmonary and systemic distribution of inhaled ultrafine silver particles in rats. Environ Health Perspect 109: 547-551.

22. Xue Y, Zhang S, Huang Y, Zhang T, Liu X, et al. (2012) Acute toxic effects and gender-related biokinetics of silver nanoparticles following an intravenous injection in mice. J Appl Toxicol 32: 890-899.

23. Song KS, Sung JH, Ji JH, Lee JH, Lee JS, et al. (2013) Recovery from silver-nanoparticle-exposure-induced lung inflammation and lung function changes in Sprague-Dawley rats. Nanotoxicology 7: 169-180.

24. Van der Zande M, Vandenbriel RJ, Van Doren E, Kramer E, Herrera Rivera Z, et al. (2012) Distribution, elimination, and toxicity of silver nanoparticles and silver ions in rats after 28-day oral exposure. ACS Nano 6: 7427-7442.

25. Lee Y, Kim P, Yoon J, Lee B, Choi K, et al. (2013) Serum kinetics, distribution and excretion of silver in rabbits following 28 days after a single intravenous injection of silver nanoparticles. Nanotoxicology 7: 1120-1130.

26. Castellini C, Ruggieri S, Mattioli S, Bernardini G, Macchioni L, et al. (2014) Long-term effects of silver nanoparticles on reproductive activity of rabbit buck. Syst Biol Reprod Med 60: 143-150.

27. Kim S, Ryu DY (2013) Silver nanoparticle-induced oxidative stress, genotoxicity and apoptosis in cultured cells and animal tissues. J Appl Toxicol 33: 78-89.

28. Völker C, Oetken M, Oehmann J (2013) The biological effects and possible modes of action of nanosilver. Rev Environ Contam Toxicol 223: 81-106.

29. Oberdörster G, Maynard A, Donaldson K, Castranova V, Fitzpatrick J (2005) ILSI Research Foundation/Risk Science Institute Nanomaterial Toxicity Screening Working Group. Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Part Fibre Toxicol 2: 8.

30. Lewinski N, Colvin V, Dreze R (2008) Cytoxicity of nanoparticles. Small 4: 26-49.

31. Ahamed M, Posgai R, Gorey TJ, Nielsen M, Hussain SM, et al. (2010) Silver nanoparticles induced heat shock protein 70, oxidative stress and apoptosis in Drosophila melanogaster. Toxicol Appl Pharmacol 242: 263-269.

32. Sur I, Altunbek M, Kahraman M, Culha M (2012) The influence of the surface chemistry of silver nanoparticles on cell death. Nanotoxicology 23: 375-392.

33. Luther EM, Schmidt MM, Diendorf J, Epple M, Dringen R (2012) Upregulation of metallothioneins after exposure of cultured primary astrocytes to silver nanoparticles. Neurochem Res 37: 1639-1648.

34. Foldbjerg R, Irving ES, Hayashi Y, Sutherland DS, Thorsen K, et al. (2012) Global gene expression profiling of human lung epithelial cells after exposure to nanosilver. Toxicol Sci 130: 145-157.

35. Bouwmeester H, Pootman J, Peters RJ, Wijma E, Kramer E, et al. (2011) Characterization of translocation of silver nanoparticles and effects on whole-genome gene expression using an in vitro intestinal epithelium coculture model. ACS Nano 5: 4091-4103.

36. Dong MS, Choi JY, Sung JH, Kim JS, Song KS, et al. (2013) Gene expression profiling of kidneys from Sprague-Dawley rats following 12-week inhalation exposure to silver nanoparticles. Toxicol Mech Methods 23: 437-448.

37. Kittler S, Greulich C, Diendorf J, Koller M, Epplle M (2010) Toxicity of silver nanoparticles increases during storage because of slow dissolution under release of silver ions. Chem Mater 22: 4548-4554.

38. Loescher K, Hadrup N, Qvortrup K, Larsen A, Gao X, et al. (2011) Distribution of silver in rats following 28 days of repeated oral exposure to silver nanoparticles or silver acetate. Part Fibre Toxicol 8: 18.

39. Rahman MF, Wang J, Patterson TA, Saini UT, Robinson BL, et al. (2009) Expression of genes related to oxidative stress in the mouse brain after exposure to silver-25 nanoparticles. Toxicol Lett 187: 15-21.

40. Wang J, Rahman MF, Duhart HM, Newport GD, Patterson TA, et al. (2009) Expression changes of dopaminergic system-related genes in PC12 cells induced by manganese, silver, or copper nanoparticles. Neurotoxicology 30: 926-933.

41. Gopinath P, Gogoi SK, Sanpui P, Paul A, Chattopadhyay A, et al. (2010) Signaling gene cascade in silver nanoparticle induced apoptosis. Colloids Surf B Biointerfaces 77: 240-245.

42. Koresaar T, Remm M (2007) Enhancements and modifications of primer design program Primer3. Bioinformatics 23: 1289-1291.

43. Untergasser A, Cutcutache I, Koresaar T, Ye J, Faircloth BC, et al. (2012) Primer3—new capabilities and interfaces. Nucleic Acids Res 40: e115.

44. Halliwell B (1994) Free radicals and antioxidants: a personal view. Nutr Rev 52: 253-265.

45. Krueger SK, Williams DE (2005) Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism. Pharmacol Ther 108: 357-387.

46. Eom HJ, Ahn JM, Kim Y, Choi J (2015) Hypoxia inducible factor-1 (HIF-1)-flavin containing monooxygenase-2 (FMO-2) signaling acts in silver nanoparticles and silver ion toxicity in the nematode, Caenorhabditis elegans. Toxicol Appl Pharmacol 270: 106-113.

47. Hwang NR, Yim SH, Kim YM, Jeong J, Song EJ, et al. (2009) Oxidative modifications of glycerolaldehyde-3-phosphate dehydrogenase play a key role in its multiple cellular functions. Biochem J 423: 253-264.

48. Nicholls C, Li H, Liu JP (2012) GAPDH: a common enzyme with uncommon functions. Clin Exp Pharmacol Physiol 39: 674-679.

49. Miura N, Shinohara Y (2009) Cytotoxic effect and apoptosis induction by silver nanoparticles in HeLa cells. Biochem Biophys Res Commun 370: 733-737.

50. Donaldson K, Stone V, Tran CL, Kreyling W, Borm PJ (2004) Nanotoxicology. Occup Environ Med 61: 727-728.

51. Lee JH, Kim YS, Song KS, Ryu HR, Sung JH, et al. (2013) Biopersistence of silver nanoparticles in tissues from Sprague-Dawley rats. Part Fibre Toxicol 10: 36.

52. Lee TY, Liu MS, Huang LJ, Lue SL, Lin LC, et al. (2013) Bioenergetic failure correlates with autophagy and apoptosis in rat liver following silver nanoparticle intraperitoneal administration. Part Fibre Toxicol 10: 40.

53. Tang J, Xiong L, Wang S, Wang J, Lu L, et al. (2009) Distribution, translocation and accumulation of silver nanoparticles in rats. J Nanosci Nanotechnol 9: 4924-4932.

54. Limbach LK, Wick P, Manser P, Grass RN, Bruinink A, et al. (2007) Exposure of engineered nanoparticles to human lung epithelial cells: influence of chemical composition and catalytic activity on oxidative stress. Environ Sci Technol 41: 4158-4163.

55. Hadrup N, Lam HR (2014) Oral toxicity of silver ions, silver nanoparticles and colloidal silver—a review. Regul Toxicol Pharmacol 68: 1-7.

56. Kim YS, Song MY, Park JD, Song KS, Ryu HR, et al. (2010) Subchronic oral toxicity of silver nanoparticles. Part Fibre Toxicol 7: 20.

57. Tiwari DK, Jit T, Behari J (2011) Dose-dependent in-vivo toxicity assessment of silver nanoparticle in Wistar rats. Toxicol Mech Methods 21: 13-24.

58. Gromadzka-Ostrowska J, Dziendzikowska K, Lankoff A, Dobrzyńka M, Instanes C, et al. (2012) Silver nanoparticles effects on epididymal sperm in rats. Toxicol Lett 214: 251-258.

59. Asare N, Instanes C, Sandberg WJ, Refnes M, Schwarze P, et al. (2012) Cytotoxic and genotoxic effects of silver nanoparticles in testicular cells. Toxicology 291: 65-72.
60. Lankveld DP, Oomen AG, Krystek P, Neigh A, Troost-de Jong A, et al. (2010) The kinetics of the tissue distribution of silver nanoparticles of different sizes. Biomaterials 31: 8350-8361.

61. Jaeschke H, McGill MR, Ramachandran A (2012) Oxidant stress, mitochondria, and cell death mechanisms in drug-induced liver injury: lessons learned from acetaminophen hepatotoxicity. Drug Metab Rev 44: 88-106.

62. Sikka SC (2001) Relative impact of oxidative stress on male reproductive function. Curr Med Chem 8: 851-862.

63. van Broekhuizen P, van Veen W, Streekstra WH, Schulte P, Reijnders L (2012) Exposure limits for nanoparticles: report of an international workshop on nano reference values. Ann Occup Hyg 56: 515-524.

64. http://www.nanotechproject.org/news/archive/silver/