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# 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*, *Gss*, *Gsr*), metal toxicity (*Mt1*), 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 AgNO<sub>3</sub> (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 testis 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 carry the potential for inhalation of silver-containing aerosols [2-5].

Humans may also come into contact with nanosilver in the occupational setting. During manufacturing, exposure of workers to AgNPs is most likely to occur via the respiratory tract [6,7]. Thus, absorption from the respiratory tract may represent an important route of exposure to AgNPs for workers and consumers.

Toxicity of inhaled AgNPs has been described in a multitude of reports and review articles [8]. However several open questions remain regarding toxicological and biokinetic aspects, for example the mode of action of inhaled AgNPs and the behavior of these nanoparticles compared with “conventional” silver aerosols (silver metal and ionic silver) [9,10].

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/m<sup>3</sup>) 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

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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 AgNO<sub>3</sub> 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<sub>20</sub>) 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<sup>(+)</sup> 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/m<sup>3</sup>. 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 (AgNO<sub>3</sub>) were purchased from Sigma-Aldrich (Milan, Italy).

### Physicochemical characterization of nanoparticles

A 1% water suspension of AgNPs (series PARNASOS<sup>®</sup> NAMA 39 1103 F01 1%) was supplied by Colorobbia S.p.A. (Vinci, Italy). The suspension presented a brown color and the following characteristics: 1 g/cm<sup>3</sup> density, 3 mPa/sec viscosity (25°C), < 0.50 PdI (polydispersity index) 6.5 pH, 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 acclimatize 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 (4RF21 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 AgNO<sub>3</sub> solution intratracheally to administer 7 µg/rat of AgNO<sub>3</sub> 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 AgNO<sub>3</sub> 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 Ostwald ripening stages. In this method, only three chemicals, including silver nitrate, oleylamine and liquid paraffin, are employed throughout the whole process. The higher boiling point of 300°C of paraffin affords a broader range of reaction temperature and makes it

possible to effectively control the size of AgNPs by varying the heating temperature alone without changing the solvent. Otherwise, the size of the colloidal AgNPs could be regulated not only by changing the heating temperature, or the ripening time, but also by adjusting the ratio of oleylamine to the silver precursor.

### Retrotranscription and Semi-quantitative PCR

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*, *Txnrd1*, *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 was amplified using 0.75 U of GoTaq DNA Polymerase (Promega Corporation, USA), 1 µM solution of specific primers, 5 µl of 5X Green GoTaq Reaction buffer, 0.5 µl of 25 mM MgCl<sub>2</sub> solution (Promega), 0.2 mM dNTPs mix in a final volume of 25 µl. PCR reactions were conducted in a GeneAmp® PCR System 2700 thermocycler (Applied Biosystem), at the following conditions: 94°C for 5 min followed by 25 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and final extension

Name		Sequence 5' - 3'	Tm	NCBI Accession Number
<i>β-actin</i>	F	CTGGTCGTACCACTGGC	55.79	NM_031144.2
	R	AGCCAGGGCAGTAATCTCC	59.25	
<i>glutathione peroxidase 1 (Gpx1)</i>	F	TTGAGAATGTCGCGTCC	56.53	NM_030826.3
	R	CAAGCCCAGATACCAGGA	57.07	
<i>glutathione synthetase (Gss)</i>	F	CCAGCGTGCCATAGAGAAC	59.41	NM_012962.1
	R	GCTGCTCCAGCGTGT	58.72	
<i>glutathione reductase (Gsr)</i>	F	CCATGTGGTTACTGCACTTC	56.59	NM_053906.2
	R	CTGAAGCATCTCATCGCAG	58.17	
<i>glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</i>	F	GTATGTCGTGGAGTCTACTG	50.33	NM_017008.3
	R	TTTAGTGGGCCCTCGGC	62.12	
<i>thioredoxin reductase 1 (Txnrd1)</i>	F	GTCTATGAGAATGCTTACGGG	56.49	NM_031614.2
	R	CCACGGTCTCTAAGCCAATA	57.85	
<i>flavin containing monooxygenase 2 (FMO2)</i>	F	TCACCTGGAGAAGCCAAC	58.36	NM_144737.2
	R	CGGTGATGGAGAAAAGTG	59.23	
<i>superoxide dismutase (SOD)</i>	F	AAGCATGGCGATGAAGG	58.22	NM_017050.1
	R	GAGACTCAGACCACATAGGGA	56.72	
<i>p53</i>	F	CAACACATGACTGAGGTCGT	57	NM_030989.3
	R	GGGTGAAATATTCTCCATCG	56.9	
<i>caspase 3 (Casp3)</i>	F	TGCTTACTCTACCGACCC	58.89	NM_012922.2
	R	CAACTACCTGATATCAAAGCTGAG	57.33	
<i>metallothionein 1 (Mt1)</i>	F	CTTACACCGTTGCTCCAGAT	57.83	NM_138826.4
	R	TGAGTTGGTCCGGAAATTAT	57	
<i>heat shock 70kD protein (Hsp70)</i>	F	AGTCGGAGAACGTGCAGG	59.98	NM_031971.2
	R	TGAGACCCTCGTCTCC	56.94	

F = Forward Primer; R= Reverse Primer

Table 1: Primer used in this work.



GENE	NCBI	Probe ID	Amplicon	Exon
<i>Gpx1</i>	NM_030826.3	Rn00577994_g1	77	1-2
<i>SOD</i>	NM_017050.1	Rn00566938_m1	62	1-2
<i>Fmo2</i>	NM_144737.2	Rn00595179_m1	73	7-8
<i>GAPDH</i>	NM_017008.3	Rn01749022_g1	60	1-1
$\beta$ -actin	NM_031144.2	Rn01412977_g1	81	1-2

Table 2: Real-time probes used in this work.

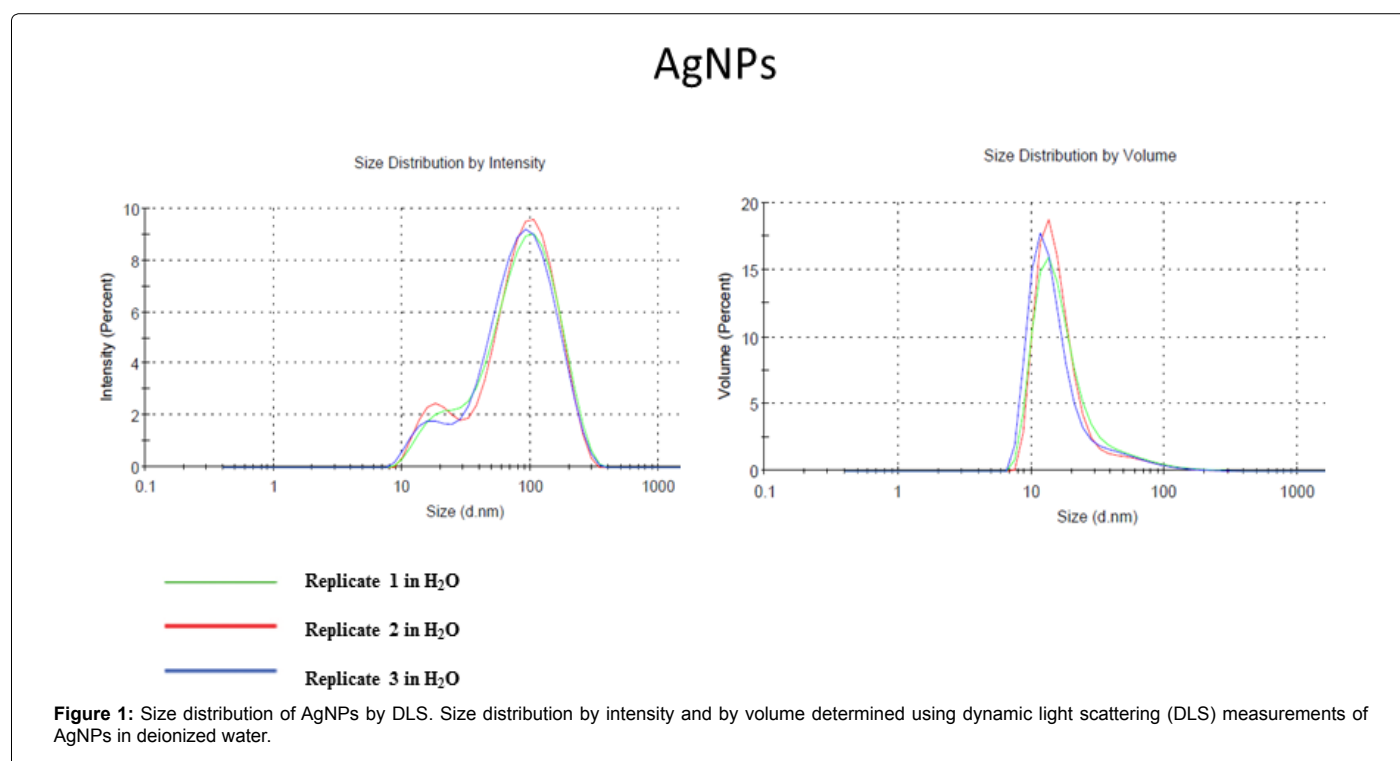


Figure 1: Size distribution of AgNPs by DLS. Size distribution by intensity and by volume determined using dynamic light scattering (DLS) measurements of AgNPs in deionized water.

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*,  $\beta$ -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*) of each sample,  $\beta$ -actin was simultaneously processed as the endogenous control. The obtained  $2^{-\Delta Ct}$  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,  $2^{-\Delta Ct}$  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 AgNO<sub>3</sub> 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.

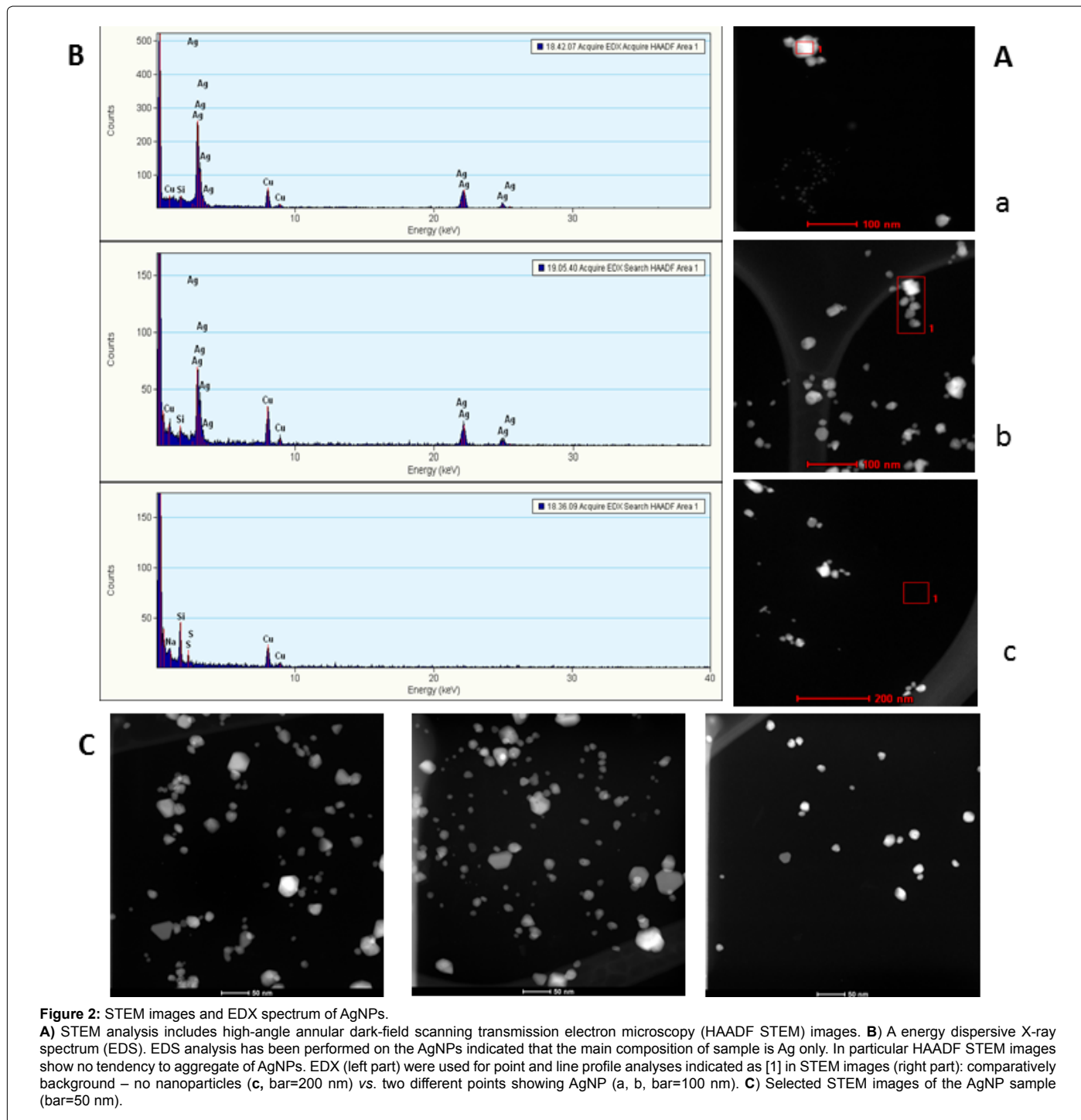
Additional techniques were used to better characterize the physicochemical properties of the AgNPs tested.

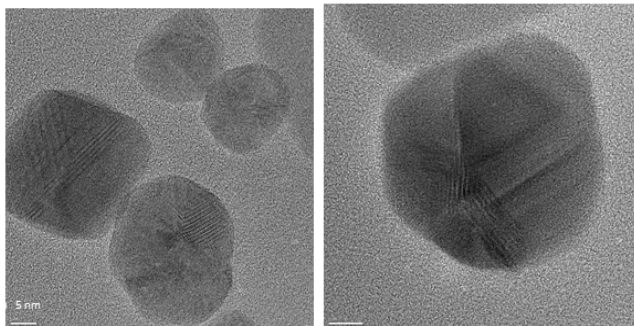
Quantitative scanning transmission electron microscopy (STEM) analysis showed that 25 nm nanoparticles were the predominant entity present in the suspension with a relatively narrow size distribution and no evidence of aggregation of the AgNPs tested (Figures 2A and 2C).

STEM with high-angle annular dark-field (HAADF) imaging showed AgNPs containing pure silver, as indicated by the energy

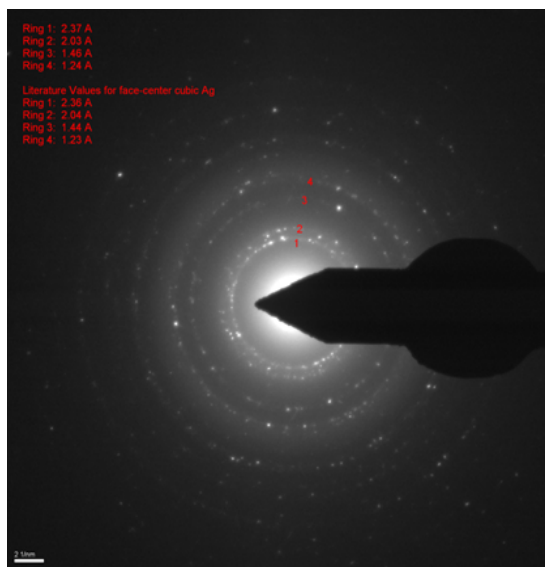
dispersive x-ray (EDX) spectrum (Figure 2B). There were no peaks of impurities; the other minor elements detected being related to water background. Purity represents an important property of the test material.

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





**Figure 3:** High-Resolution TEM (HRTEM) images. HRTEM images simultaneously give information, on atomic scale, in real space (in the imaging mode) and reciprocal space (in the diffraction mode). At these small scales, individual atoms of AgNP and its defects can be resolved (bar = 5 nm).



**Figure 4:** Selected area electron diffraction (SAED) pattern of the AgNPs. SAED pattern indicates pure AgNP: four diffraction rings and each coincides with a diffraction ring of pure Ag (21 nm).

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*,  $\beta$ -actin, and *GAPDH* as candidate target genes, using  $\beta$ -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 post-instillation, the testis of rats treated with AgNPs, 50  $\mu$ g/

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 AgNO<sub>3</sub> 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  $2^{-\Delta\Delta Ct}$  are here expressed as  $2^{-\Delta\Delta Ct}$ . This normalization consists in subtracting the  $\Delta Ct$  of the control tissues from the  $\Delta Ct$  of the tissues of the animals exposed to NPs and ions.

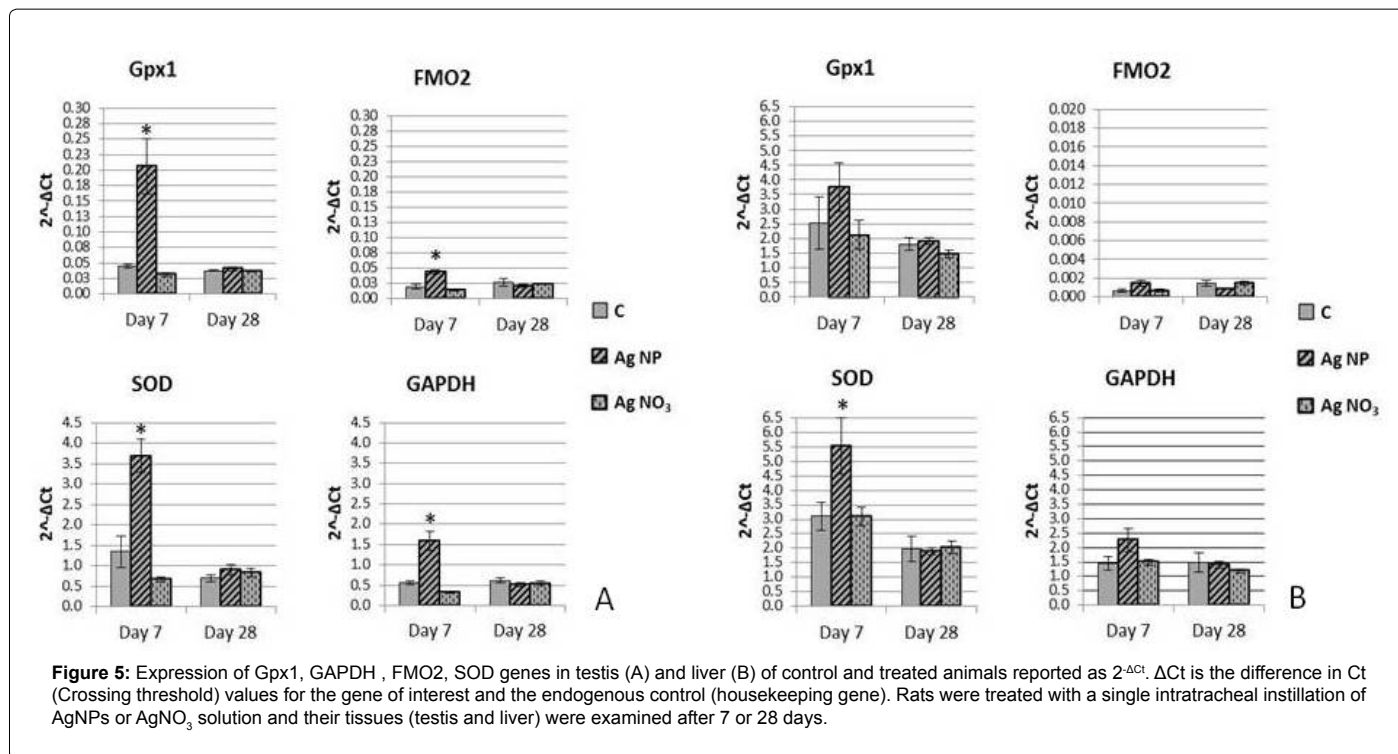
The p-values obtained for all the comparisons between control animals examined at day 7 and those examined at day 28 post-instillation resulted greater than 0.05 confirming that controls at day 7 and at day 28 can be considered equivalent.

Similarly, pairwise comparisons between animals instilled with AgNO<sub>3</sub> 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 AgNO<sub>3</sub> treatment.

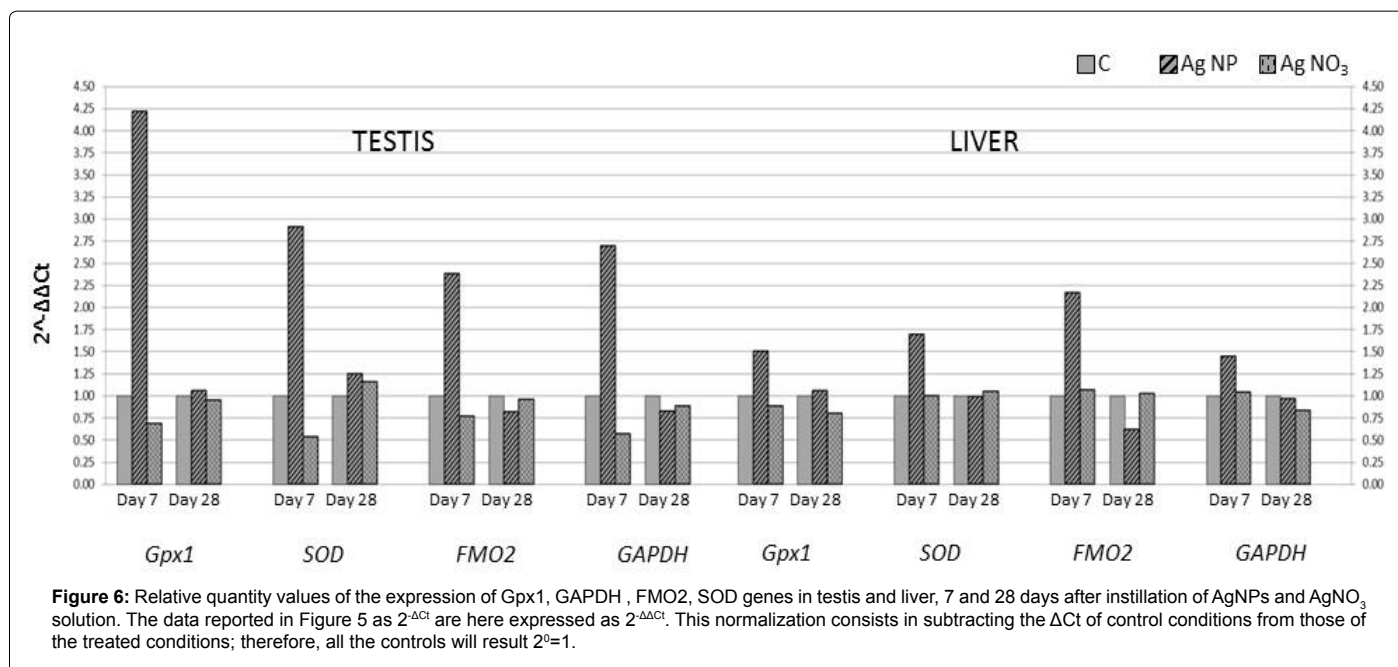
### Discussion

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  $\mu$ g/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  $\mu$ g/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  $\mu$ g/kg bw, corresponding to approximately 3-12  $\mu$ g/mouse). In these studies, the extra-pulmonary effects of AgNPs were not examined.

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



**Figure 5:** Expression of *Gpx1*, *GAPDH*, *FMO2*, *SOD* genes in testis (A) and liver (B) of control and treated animals reported as  $2^{-\Delta Ct}$ .  $\Delta Ct$  is the difference in Ct (Crossing threshold) values for the gene of interest and the endogenous control (housekeeping gene). Rats were treated with a single intratracheal instillation of AgNPs or AgNO<sub>3</sub> solution and their tissues (testis and liver) were examined after 7 or 28 days.



**Figure 6:** Relative quantity values of the expression of *Gpx1*, *GAPDH*, *FMO2*, *SOD* genes in testis and liver, 7 and 28 days after instillation of AgNPs and AgNO<sub>3</sub> solution. The data reported in Figure 5 as  $2^{-\Delta Ct}$  are here expressed as  $2^{-\Delta\Delta Ct}$ . This normalization consists in subtracting the  $\Delta Ct$  of control conditions from those of the treated conditions; therefore, all the controls will result  $2^0=1$ .

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.

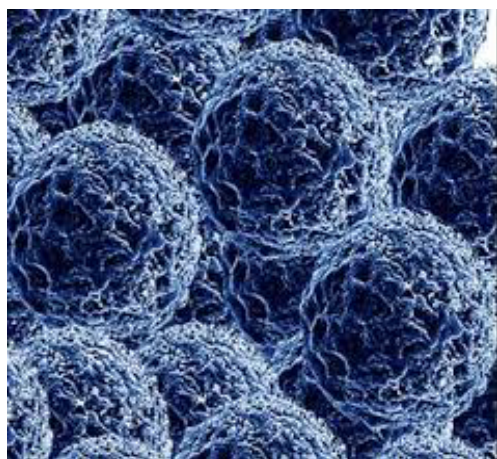
There were differences between liver and testis in the gene

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.



	C7	NP7	Ag7	C28	NP28	Ag28
C7		0.143 <b>6.04E-03</b> 0.027 0.035	0.639 0.994 0.917 0.951	0.464 0.242 0.069 0.950	0.461 0.142 0.584 0.849	0.224 0.207 <b>0.031</b> 0.468
NP7	<b>6.62E-05</b> <b>2.38E-06</b> <b>2.84E-06</b> <b>8.70E-06</b>		<b>0.043</b> <b>3.68E-03</b> <b>0.024</b> <b>0.029</b>	<b>0.039</b> <b>4.87E-04</b> 0.924 0.060	<b>0.019</b> <b>3.80E-05</b> 0.054 <b>0.012</b>	<b>0.006</b> <b>1.08E-04</b> 0.984 <b>3.88E-03</b>
Ag7	0.700 0.095 0.258 0.208	<b>1.04E-05</b> <b>1.30E-08</b> <b>6.06E-08</b> <b>1.29E-07</b>		0.735 0.224 0.070 0.993	0.787 0.120 0.639 0.786	0.419 0.184 <b>0.029</b> 0.405
C28	0.868 0.158 0.151 0.820	<b>1.21E-04</b> <b>2.19E-07</b> <b>5.53E-04</b> <b>5.41E-05</b>	0.857 0.940 <b>0.015</b> 0.166		0.906 0.928 0.133 0.809	0.715 0.950 0.913 0.464
NP28	0.903 0.269 0.714 0.767	<b>2.14E-05</b> <b>5.46E-08</b> <b>2.87E-06</b> <b>1.60E-06</b>	0.780 0.523 0.118 0.302	0.951 0.632 0.237 0.610		0.564 0.856 0.063 0.547
Ag28	0.809 0.196 0.268 0.931	<b>1.54E-05</b> <b>3.39E-08</b> <b>2.24E-05</b> <b>2.80E-06</b>	0.879 0.666 <b>0.023</b> 0.213	0.961 0.766 0.613 0.751	0.898 0.834 0.425 0.823	

**Table 3:** p-values of pair wise comparisons. Values of gene expression of liver are reported above the diagonal. The corresponding values for testis are reported below the diagonal. The set of four values correspond to the expression of *Gpx1*, *SOD*, *FMO2* and *GAPDH*, respectively. C7: animals instilled with control solution and sacrificed at day 7; NP7: animals instilled with NP suspension and sacrificed at day 7; Ag7: animals instilled with AgNO<sub>3</sub> solution and sacrificed at day 7; C28: animals instilled with control solution sacrificed at day 28; NP28: animals instilled with NP suspension and sacrificed at day 28; Ag28: animals instilled with AgNO<sub>3</sub> solution and sacrificed at day 28. (**p-value** <0.05; **p-value** <0.01).



**Figure 7:** Microscopic view of silver nanoparticles.

No statistically significant gene expression changes were found in hepatic and testicular samples obtained from the animals treated with a corresponding dose of AgNO<sub>3</sub>.

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 (H<sub>2</sub>O<sub>2</sub>)-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 monooxygenases (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, AgNO<sub>3</sub> caused no changes in hepatic and testicular gene expression, possibly reflecting differences in the mechanisms of toxicity and/or pharmacokinetics between AgNPs and AgNO<sub>3</sub>.

With AgNO<sub>3</sub> 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 complexes with albumin and other macromolecules. In particular, rapid neutralization of silver ions likely occurs in the lung of animals instilled with AgNO<sub>3</sub>. Consequently, there would be no passage of biologically effective silver from lung 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 ionic silver 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 [24,38].

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 AgNO<sub>3</sub> [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/m<sup>3</sup>) for 13-week showed bile-duct hyperplasia and hepatocellular 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 AgNO<sub>3</sub> 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/m<sup>3</sup> and 10 µg/m<sup>3</sup> 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<sup>+</sup> 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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