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Oral subchronic exposure to silver nanoparticles in rats

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ABSTRACT

Because of their extremely small size, silver nanoparticles (AgNPs) show unique physical and chemical properties, with specific biological effects, which make them particularly attractive for being used in a number of consumer applications. However, these properties also influence the potential toxicity of AgNPs. In this study, we assessed the potential toxic effects of an *in vivo* oral sub-chronic exposure to polyvinyl pyrrolidone coated AgNPs (PVP-AgNPs) in adult male rats. We also assessed if oral PVP-AgNPs exposure could alter the levels of various metals (Fe, Mg, Zn and Cu) in tissues. Rats were orally given 0, 50, 100 and 200 mg/kg/day of PVP-AgNPs. Silver (Ag) accumulation in tissues, Ag excretion, biochemical and hematological parameters, metal levels, as well as histopathological changes and subcellular distribution following PVP-AgNPs exposure, were also investigated. After 90 days of treatment, AgNPs were found within hepatic and ileum cells. The major tissue concentration of Ag was found in ileum of treated animals. However, all tissues of PVP-AgNPs-exposed animals showed increased levels of Ag in comparison with those of rats in the control group. No harmful effects in liver and kidney, as well as in biochemical markers were noted at any treatment dose. In addition, no hematological or histopathological changes were found in treated animals. However, significant differences in Cu and Zn levels were found in thymus and brain of PVP-AgNPs-treated rats.

1. Introduction

Recently, there has been an increased interest in nanoscience and nanotechnology (Seal and Karn, 2014; Formoso et al., 2015; Rai et al., 2015). Nanoparticles (NPs) are particles of very small size (1–100 nm) that consist of a core made of different materials such as metals, organic polymers or carbon, which can be covered with a coat (inorganic or organic molecules) to stabilize them in the environment (Christian et al., 2008). NPs can possess physical and chemical properties different from those with larger size making them desirable in materials science and biology. Nowadays, nanomaterials have a number of applications in the daily life (BSi Report, 2007; De Jong and Borm, 2008; Salata, 2004; Susan et al., 2009). Because of their extremely small size, NPs may have the ability to enter, translocate within, and damage living organisms. As result, an improved understanding of the potential risks and hazard assessments associated with exposure to nanomaterials is clearly necessary (Gwinn and Vallyathan, 2006; Lubick, 2008; Saiyed et al., 2011).

Silver nanoparticles (AgNPs) are among the most used nanoparticles in industry. It has been shown that AgNPs have the ability to release silver ions (Ag^{+}) in suspension partly because of its surface charge, particle size, or coating (Chernousova and Epple, 2013; Hadrup and Lam, 2014). Some studies have evaluated if the effects of AgNPs are a direct result of the NPs themselves, or rather due to the interaction with the released Ag ions (Lubick, 2008; Susan et al., 2009; Liu and Hurt, 2010; Behra et al., 2013). Ions released from AgNPs are believed to be the responsible for its antibacterial properties (Lansdown and Williams, 2007; Kumar et al., 2015). The strong antibacterial activity of these NPs makes them suitable for their use in a number of consumer products such as cosmetics, contraceptives, deodorants, and food products (Chen and Schluesener, 2008; Prabhu and Poulose, 2012; Brennan et al.,

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2015; Franci et al., 2015).

It has been also shown that exposure to AgNPs can lead to a variety of toxicological effects (Ahamed et al., 2010; EPA, 2010; Johnston et al., 2010). The special properties of NPs, which make them suitable for medical and consumer uses, are also responsible for their toxicity. Most of the current studies have been focused on *in vitro* models, suggesting that AgNPs possess cytotoxic effects providing inflammation, increased radical oxygen species (ROS) generation, mitochondrial dysfunction, DNA damage, and induction of apoptosis/necrosis cell death (AshaRani et al., 2009; Lima et al., 2012; Awasthi et al., 2013; Zhang et al., 2014).

Only a few *in vivo* studies have been performed. Most of them are focused on assessing the tissue distribution of different sizes of AgNPs after inhalation or intravenous exposure (Dziendzikowska et al., 2012). There are only a few studies through oral exposure (Loeschner et al., 2011; Van der Zande et al., 2012). Recently, some studies have investigated the neurotoxicological effects of AgNPs in brain. Apoptosis and neuronal degeneration after treatment at low doses of AgNPs, have been reported (Bagheri-Abassi et al., 2015; Xu et al., 2015). In turn, other studies showed that, depending on the route of administration, a different Ag concentration pattern in organs can be found (Susan et al., 2009). Following oral exposure, AgNPs can be absorbed across the gastrointestinal (GI) barrier, enter into the systemic circulation, and accumulate into different tissues. AgNPs have been found in kidney, liver, spleen, lung, brain and small intestine (EPA, 2010; Hadrup and Lam, 2014).

The current study was aimed at investigating tissue distribution, accumulation and excretion of PVP-AgNPs (20–30 nm) after oral subchronic administration during 90 days. Subcellular localization of PVP-AgNPs, biochemical, pathological and primary indicators of possible immune toxicity were also investigated. Moreover, we studied for the very first time, the effects of PVP-AgNPs accumulation on various metal (Fe, Mg, Zn and Cu) concentrations in different tissues.

2. Material and methods

2.1. Nanoparticles preparation

Polyvinyl pyrrolidone coated PVP-AgNPs (0.2 wt % PVP; Sky-Spring Nanomaterials, Inc., Houston, USA), with an average size 20–30 nm, were obtained as dry powder (Ag, 99.95%, PVP coated). PVP-AgNPs were resuspended in 0.9% saline and administered at concentrations of 0, 50, 100 or 200 mg AgNPs/kg/day. The main criteria followed for the selection of these doses was based on previous studies by Kim et al. (2010). PVP-AgNPs were dispersed by sonication on ice during 30 min at 35–40 W. The nanoparticles solutions were freshly prepared every day just before treatment.

2.2. Animals

Adult male Sprague Dawley rats (262 ± 17.70 g) were purchased from Charles River (Sant Germain-L'Arbresle, France). Animals were housed in a room equipped with automatic light cycles (12-h light/dark) and maintained at 22 ± 2 °C and 40%–60% humidity. Food (Panlab rodent chow, Barcelona, Spain) and tap water were offered *ad libitum* throughout the study. The experiment was approved by the Ethics Committee of Animals Research, "Rovira i Virgili" University (Tarragona, Spain).

2.3. Experimental design

After 10 acclimatization days, rats were weighed and randomly divided in four different groups (n = 12 per group). Each experimental group received 0, 50, 100 or 200 mg/kg/day of PVP-AgNPs. To evaluate the toxicological effects of PVP-AgNPs, animals were daily treated by gavage during 90 days (13 weeks), at a dose-volume of 4 mL/kg body weight either with vehicle (0.9% saline), or with the specific dose of PVP-AgNP, respectively. During the study period, the clinical signs and mortality of the rats were daily observed, while body weights and food intake were weekly recorded. For metal analysis, the week just before ending of the treatment, feces and urine of the animals were collected in individual metabolic cages. At the end of the experimental period, animals were weighed and anesthetized by an intraperitoneal injection of 75 mg/kg ketamine and 0.5 mg/kg medetomidine. Eight animals per group were used for hematological, biochemical assessment, and metal analysis. Blood was collected via the posterior cava, while liver, kidney, spleen, thymus, brain and small intestine were aseptically excised, weighed and stored at −20 °C for metal analyses. Four animals per group were used for histopathology and tissue specimen preparation for transmission electron microscope (TEM) evaluation.

2.4. Characterization of the nanoparticle suspension

The morphological characteristics of the PVP-AgNPs were analyzed by a JEOL JEM-1011 (JEOL, Tokyo, Japan) transmission electron microscope (TEM), operating at an acceleration range of voltages of 100–800 kV. The morphology of the PVP-AgNPs were analyzed by a carbon film-coated Cu grids in contact with a droplet of 4 mg/mL of PVP-AgNPs resuspended in 0.5% aqueous carboxymethylcellulose (Sigma Aldrich, San Louis, MO, USA). To reduce the risk of possible artifacts formation, all samples for TEM evaluation were prepared and analyzed on the same day in which the grids were prepared. The size of 200 particles was analyzed with a particle analysis tool to establish size distributions using the ImageJ software (Version 1.48).

2.5. Determination of the concentrations of Ag and other metals

Samples of liver, kidney, spleen, thymus, brain, ileum and urine were weighed/measured (0.5–1 g tissue or 300 μl urine) in a microsampling quartz, being 65% nitric acid (Suprapur, E. Merck) added to digest the samples. For feces, 0.5–1 g plus 0.25 ml of nitric acid 65% and 0.25 ml of hydrofluoric acid were added for digestion. The microsampling inserts were then introduced in Teflon vessels and put into a microwave oven Star D (Milestone, Sorisole, Italy) (Gómez et al., 2008). All materials were previously washed with 10% nitric acid in order to avoid any possible sample contamination. For quality control, NIST Standard Reference Material (Bovine liver 1577b; NIST, Gaithersburg, MD) was also measured in each assay. Ag, Cu and Zn concentrations were determinated by means of a computer-controlled sequential inductively coupled plasma spectrometer (ICP-MS, PerkinElmer Elan 6000), and Fe and Mg by means of a inductively coupled plasma optical emission spectrometry (ICP-OES, PerkinElmer Optima 8300). The recovery percentage of the reference material was higher than 93%. Detection limits were the following: 0.05 μg/kg for Ag, 0.010 μg/g for Fe and 0.10 μg/kg for Mg, 0.05 μg/g for Zn and 0.10 μg/kg for Cu.

2.6. Tissue specimen preparation for TEM evaluation

Ileum and liver of AgNPs-treated and control rats (n = 4) were aseptically excised, cut into ≈ 1 mm^3 pieces and fixed in 2% of glutaraldehyde solution in 0.1 M phosphate buffer at pH 7.0 for 24 h. Samples were washed twice with 0.1 M phosphate buffer being post-fixed with osmium tetroxide 1% solution in 0.1 M phosphate buffer for 2 h. Fixed samples were washed twice with 0.1 M phosphate buffer and dehydrated in a gradient series of

ethanol solution (30%, 50%, 90%, 96% and 100%). After 50% dehydration solution, samples were counterstained with uranyl acetate at 70% for 24 h at 4 °C in dark to continue dehydrating. Fully dehydrated tissues were embedded in epoxy resin, being blocks dried in an oven at temperature of 60 °C for 1 or 2 days. Ultrathin sections of 60 nm were cutted on Leica Ultracut E (Leica Geosystems S.L. Barcelona, Spain). The slices were transferred to copper TEM grids and observed with a JEOL 1011 (JEOL, Tokyo, Japan), which operates at 80 kV accelerating voltage.

2.7. Hematology and plasma biochemistry

Whole blood samples were collected into a 500 μl EDTA blood collection tubes and were applied to an automatic hematology analyzer ADVIA 120 (Siemens Diagnostics, NY, USA) to measure the following parameters: total white blood cell, red blood cell, hemoglobin, platelet, hematocrit, mieloperoxidase index, mean corpuscular volume, mean corpuscular hemoglobin and differential white blood cell count. For plasma biochemistry analysis, whole blood samples were collected into EDTA or heparin blood collection tubes. Plasma was obtained after low-speed centrifugation at 3000*g* for 10 min at 4 °C. Plasma was immediately separated and stored at −80 °C before analysis, or at −4 °C depending on the test assay. The following plasma biochemistry parameters were evaluated using a Cobas Mira automatic analyzer (Roche Pharmaceuticals, Basel, Switzerland) according to the instructions provided by the manufacturer: total protein, blood urea nitrogen (BUN), creatinine, albumin, alkaline phosphatase, aspartate transaminase and alanine transaminase (QCA kits, Spain).

2.8. Histopathological assessment

Liver, kidney, spleen, thymus, brain and ileum of different groups of rats (n = 4 per group) were removed, weighed and fixed in 4% formaldehyde, pH 7.4, for 24 h. Tissues were sliced and embedded in paraffin. Sections of 4-μm thickness were stained with hematoxylin-eosin for tissue morphology evaluation. Images were captured on a laser scanning confocal microscope (Nikon eclipse TE2000-E). For immunohistochemistry staining, the anti-Ki67 (39-9) antibody (Ventana Medical Systems, Tucson, Arizona, USA) was used.

2.9. Statistics

Statistical analysis was performed using the software Statistical Package for the Social Sciences (SPSS v.22). Homogeneity of variances was analyzed employing the Levene's test. If variances were homogenous, ANOVA was then used, followed by the Tukey's test to analyze all dose groups simultaneously. The Kruskal–Wallis test was used when variances were not homogeneous. Significance was set at $p < 0.05$.

3. Results

3.1. Nanoparticle characterization

The size and shape of the particles were observed by TEM. The images indicated a different frequency size distribution of AgNPs (Fig. 1). TEM showed an average particle core size of 25.24 ± 3.25 nm for the NPs of 20–30 nm, being the most abundant size together with NPs of 10–20 nm (15.16 ± 2.21). Information on the mean size and SD was calculated by measuring two hundred NPs in random fields of view (Fig. 1A). These results show that, in suspension, there are also abundant NPs of 10–20 nm, although size data provided by the manufacturer were 20–30 nm. The 75% of the particles had a core size <30 nm while the remaining particles (~25%) had core size raining from 30 to over 100 nm (Fig. 1B). Fig. 1C–F shows TEM images revealing that the AgNPs had a spherical shape. However, agglomerations and aggregations were observed.

3.2. Food intake, body weight and organ weights

Food intake was similar in the treated and non-treated groups, with the exception of the 50 mg/kg/day group, in which food intake per week was significantly lower than that in the control group (Table 1). No significant differences in body weight gain (Fig. 2) or organ-weight were observed after 90 days of treatment (Table 2).

3.3. Excretion of Ag

The excretion of Ag was assessed by measuring Ag concentrations in feces and urine of PVP-AgNPs treated and non-treated animals. The excretion of Ag through urine was lower than that through feces. Fig. 3 depicts an Ag significant dose-dependent accumulation in feces of exposed rats with respect to those in the control group, being the highest accumulation of Ag found in the 200 mg/kg/day group. The amounts of Ag found in urine of treated animals were low (<0.1 μg/g). Significant increased levels of Ag were noted in urine of rats in the 100 mg/kg/day of PVP- AgNPs.

3.4. Silver concentration and distribution

The accumulation and distribution of Ag in different tissues of PVP-AgNPs-exposed rats and those of the control are depicted in Fig. 4. After 90 days of treatment, rats treated at different doses of PVP-AgNPs showed significant higher levels of Ag in ileum, liver, kidneys, brain, thymus and spleen respect control animals. The highest Ag levels were found in the ileum of the animals, being 100 and 200 mg/kg/day groups those with the highest levels of Ag. However, a dose-dependent Ag accumulation between the different doses of PVP-AgNPs treated groups was not found. In brain, animals in the 200 mg/kg/day group showed no significant, but less Ag levels than those in the 50 and 100 mg/kg/day groups. The overall results revealed that the highest Ag accumulation was found in ileum, suggesting that only few amounts of Ag passed the GI tract barrier, and consequently, were transferred to other tissues.

3.5. Subcellular localization of silver in ileum and liver

In order to assess the subcellular localization of PVP-AgNPs in ileum and liver, a TEM was performed. TEM revealed that in treated rats PVP-AgNPs accumulated in different organ regions, as individual forms or as NPs aggregates (Fig. 5), while in untreated rats, cells did not show particle deposition. TEM images indicated that PVP-AgNPs accumulates in liver, showing that PVP-AgNPs cross the intestinal tract, and are translocated from the blood circulation to the liver. PVP-AgNPs clusters were observed in the mitochondria of liver cells in form of agglomerates (Fig. 5A). In intestinal cells, PVP-AgNPs were seen throughout the cytoplasm and organelles (Fig. 5B–D).

3.6. Fe, Mg, Zn and Cu concentrations and distribution

The effects of PVP-AgNPs administration on Fe, Mg, Zn and Cu levels were examined in ileum, liver, kidney, spleen, thymus and brain (Fig. 6). At the end of the experimental period, animals treated with PVP-AgNPs did not show significant differences in Fe and Mg levels in any of the analyzed tissues in comparison with those in the control group (Fig. 6A and B). In general terms, Mg

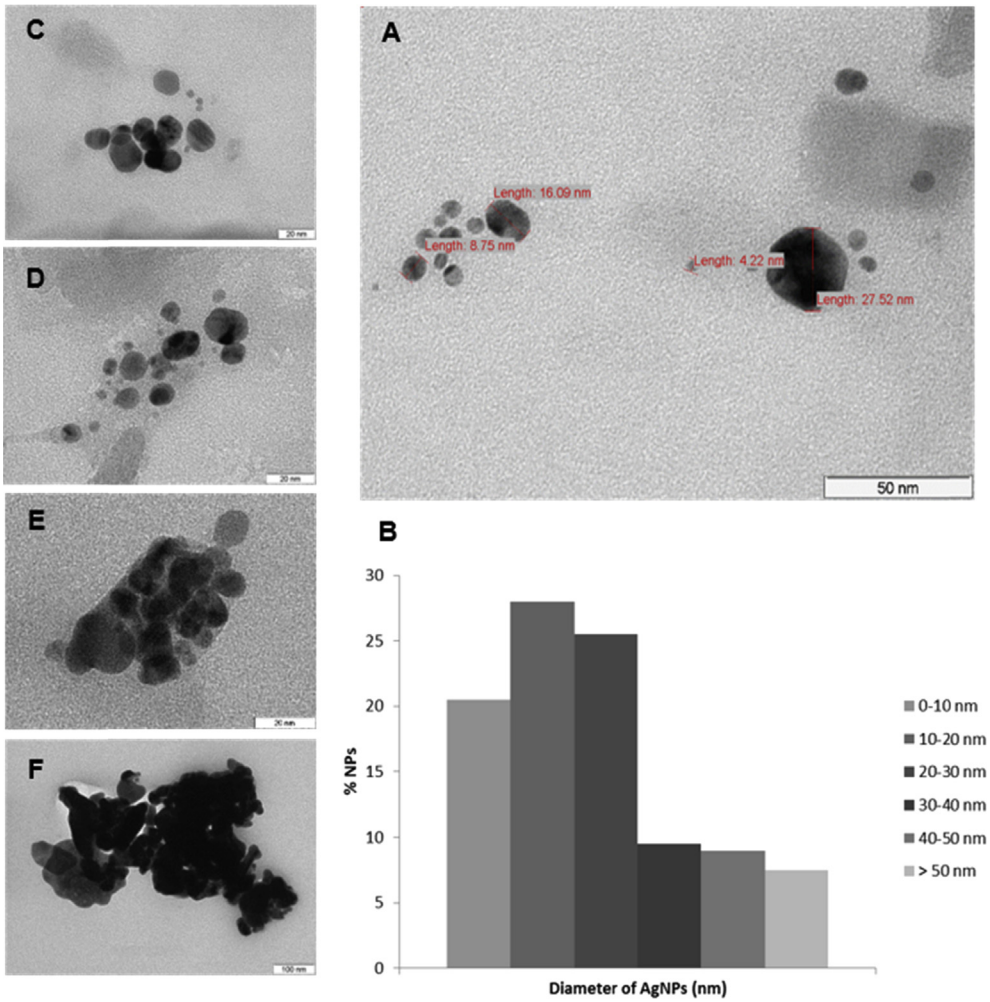

Fig. 1. NPs characterization. NPs characterization, size and shape by transmission electron microscopy (TEM). TEM images of representative single PVP-AgNPs show nearly spherical NPs and clusters of NPs. The scale bars are 20 nm (A, B, C), 100 nm (D) and 50 nm (E). Histogram of over two hundred single PVP-AgNPs measured by TEM showing the average diameter of NPs (F).

Table 1
Body weight gain (g/week) and food intake (g/week) in rats exposed to silver nanoparticles.

Variable	0 mg/kg/day	50 mg/kg/day	100 mg/kg/day	200 mg/kg/day
Final body weight (g)	457.82 ± 49.63	473.58 ± 58.40	475.83 ± 61.81	456.45 ± 39.52
Body weight gain (g/week)	13.26 ± 3.4	14.47 ± 3.62	16.54 ± 4.81	13.85 ± 3.19
Food intake (g/week)	92.86 ± 9.95	72.54 ± 3.68*	95.73 ± 3.04	95.27 ± 11.96

Results are expressed as mean ± SD (n = 12/group). * Different from the control group, $p < 0.05$.

levels were similar in all tissues, while Fe showed the highest levels in spleen. On the other hand, significant decreases of Zn and Cu levels were found in some tissues of treated groups (Fig. 6C and D). In thymus, a significant decrease of Zn was observed at 200 mg/kg/day of PVP-AgNPs. In brain, a significant decrease of Zn levels was noted at 100 mg/kg/day. However, increased levels of Zn were found at the highest dose, while in liver, a slight but not significant decrease of Zn levels, was found at 100 and 200 mg/kg/day (Fig. 6C). Cu levels were altered in kidney and brain. In kidney, PVP-AgNPs treatment decreased Cu levels, being the decrease significant at 50 mg/kg/day, while in brain, a significant decrease of Cu levels at 100 mg/kg/day was noted. As for Zn, increased levels of Cu were found in the 200 mg/kg/day group (Fig. 6D).

3.7. Blood hematology and biochemical analysis

The hematological parameters of exposed animals did not show significant changes in comparison to those of the respective to control groups (Table 3). Plasma biochemistry profiles were performed to assess hepatic and renal status. Alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) were used as indicators of hepatic function, BUN and creatinine were indicators of the renal function, while total plasma proteins and the albumin/globulin ratio were used to get information about liver and lymphocyte function. Table 4 summarizes enzymatic and biochemical parameters levels of the different PVP-AgNPs groups. No significant changes in BUN or creatinine levels in any group were observed, while (ALP), (AST) and (ALT) levels were not

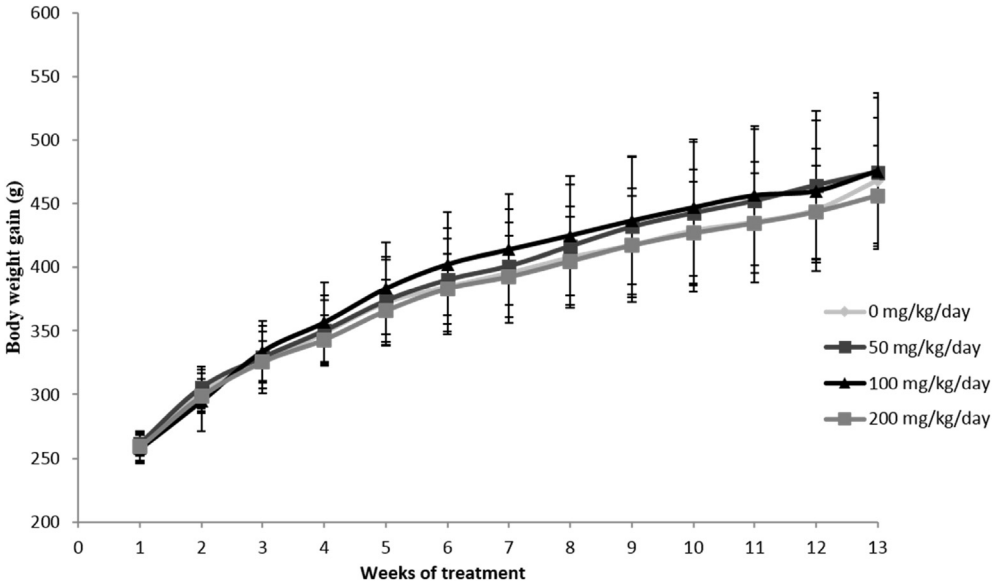

Fig. 2. Body weight changes during 90-day oral administration of silver nanoparticles.

Table 2
Organ weights and relative organ weights (g/kg BW) for rats orally treated with AgNPs for 90 days.

		0 mg/kg/day	50 mg/kg/day	100 mg/kg/day	200 mg/kg/day
Brain	g	2.07 ± 0.09	2.15 ± 0.17	2.13 ± 0.14	2.02 ± 0.18
	g/kg BW	4.43 ± 0.64	4.62 ± 0.47	4.68 ± 0.56	4.48 ± 0.36
Liver	g	10.68 ± 1.91	10.87 ± 2.27	11.56 ± 2.13	10.71 ± 1.27
	g/kg BW	24.37 ± 2.34	23.05 ± 1.49	21.54 ± 8.99	23.62 ± 1.66
Kidney	g	2.48 ± 0.28	2.76 ± 0.41	2.9 ± 0.31	2.6 ± 0.26
	g/kg BW	5.68 ± 0.40	5.90 ± 0.56	6.34 ± 0.61	5.79 ± 0.50
Spleen	g	0.68 ± 0.09	0.66 ± 0.09	0.72 ± 0.12	0.78 ± 0.32
	g/kg BW	1.57 ± 0.24	1.42 ± 0.19	1.57 ± 0.19	1.75 ± 0.78
Thymus	g	0.37 ± 0.12	0.39 ± 0.10	0.37 ± 0.10	0.39 ± 0.08
	g/kg BW	0.87 ± 0.23	0.72 ± 0.35	0.80 ± 0.13	0.87 ± 0.14
Jejunum	g	3.18 ± 1.03	3.20 ± 0.88	2.87 ± 0.55	3.15 ± 0.66
	g/kg BW	7.22 ± 2.03	6.93 ± 2.21	6.23 ± 0.91	6.94 ± 1.22
Ileum	g	3.04 ± 0.84	3.28 ± 0.85	3.12 ± 0.76	2.97 ± 0.47
	g/kg BW	6.89 ± 1.39	6.94 ± 1.01	6.84 ± 1.60	6.58 ± 1.03
Pancreas	g	1.05 ± 0.26	0.93 ± 0.22	0.96 ± 0.06	0.94 ± 0.15
	g/kg BW	2.39 ± 0.48	1.98 ± 0.33	2.11 ± 0.29	2.08 ± 0.29

Results are expressed as means ± SD (n = 8/group).

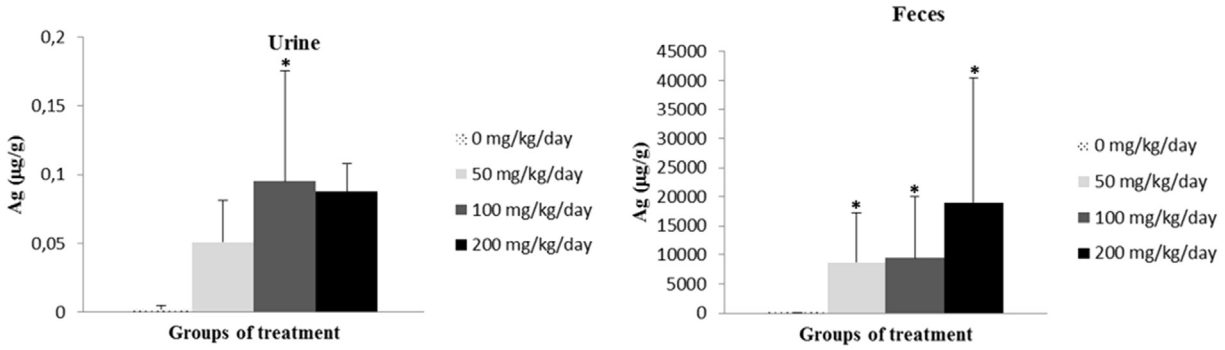

Fig. 3. Silver concentration in urine and feces. Silver concentrations after 90 days of oral administration of PVP-AgNPs. Statistically significant differences (mean ± S.D., $p < 0.05$) between PVP-AgNPs treated groups and control group are marked with asterisk (*). $N = 8$.

affected.

3.8. Histopathological evaluation

No significant morphological changes were observed in brain, thymus, spleen, ileum and kidney on postmortem evaluation.

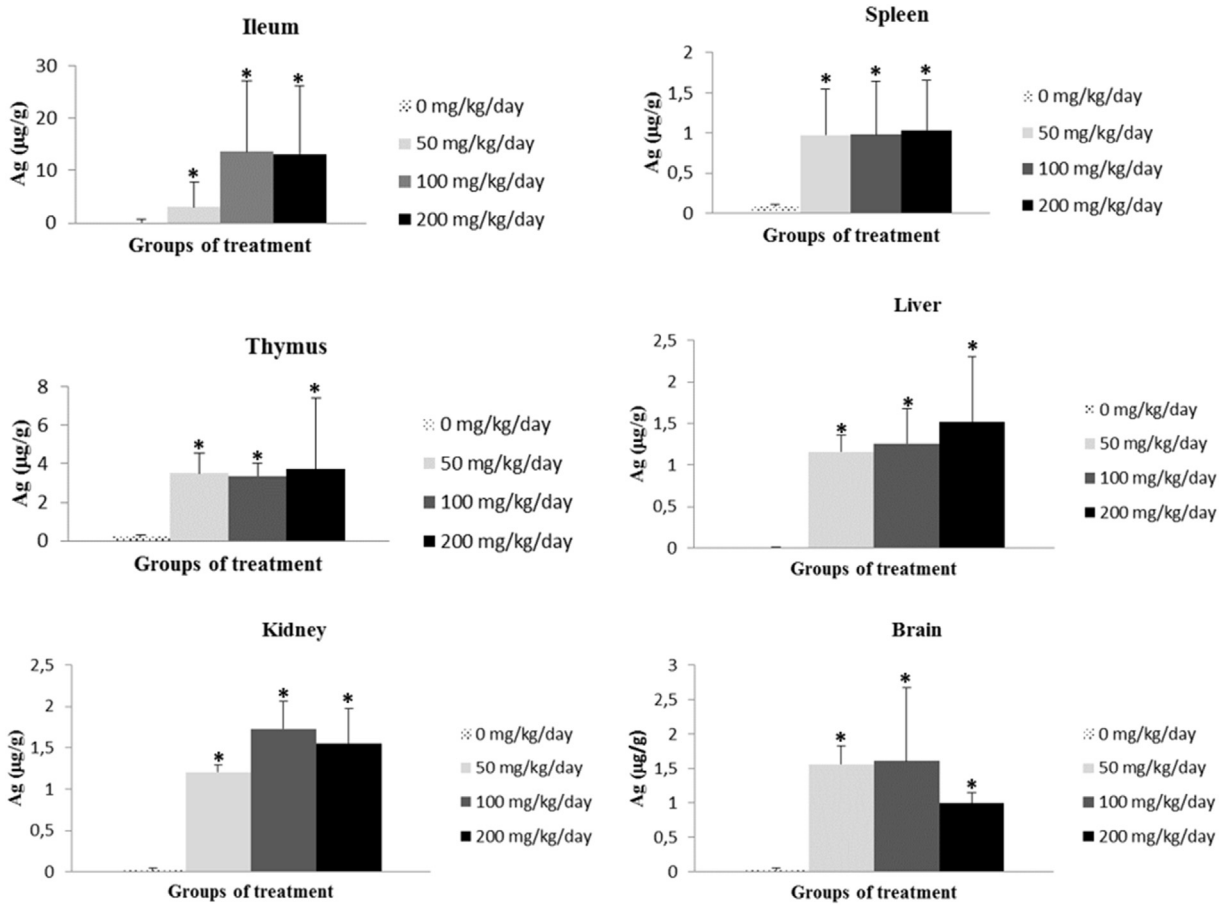

Fig. 4. Silver concentration in organs. Silver concentrations in rat organs after 90 days oral administration of PVP-AgNPs. Statistically significant differences (mean ± S.D., $p < 0.05$) between PVP-AgNPs treated groups and control group are marked with asterisk (*). Different letters on the bars (a and b) indicate significant differences between groups. $N = 8$.

However, in liver, a higher number of binucleated hepatocytes were observed at 100 and 200 mg/kg/day of PVP-AgNPs-exposed rats with respect to animals in the control group (Fig. 7). No signs of atrophy, necrosis, inflammation or fibrosis were observed in the hematoxylin-eosin staining evaluation. In liver sections, proliferating cells were detected by immunohistochemistry through the proliferation cell nuclear antigen Ki67. Ki67 single staining of liver samples resulted in variable amounts of cells with increased positive nuclear Ki67 staining per slide at 100 and 200 mg/kg/day of PVP-AgNPs. However, it was hard to detect if these cells were proliferating hepatocytes, leucocytes, Kupffer cells, or even other cell types in the field of interest (Fig. 8).

4. Discussion

In recent years, the interest on the potential toxicity of NPs has considerably increased as result of the uses of NPs in biomedicine (and biological applications), and due to the increased use of nanomaterials in consumer products (Formoso et al., 2015; Katz et al., 2015). AgNPs are among the most commercialized nanoparticles worldwide and Ag nanotoxicology has become an important area of research. Recent studies have investigated the toxicological effects of AgNPs *in vivo* and *in vitro*, showing that different doses, route of administration, and NPs size could lead to harmful effects in living organisms (AshaRani et al., 2009; Kovvuru et al., 2014; Li et al., 2013; Sarhan and Hussein, 2014).

Compared with other routes of administration, the intake of NPs has an important toxicologic potential for humans (Chun, 2009; Bergin and Witzmann, 2013; Yun et al., 2015). It has been shown that NPs can cross the GI tract barrier, and translocate into different tissues where NPs can exert their toxicity. However, there are only few studies evaluating toxic effects of oral AgNPs exposure (Kim et al., 2010; Loeschner et al., 2011; Van der Zande et al., 2012; Yun et al., 2015). On the other hand, there are no reported data on metal levels in tissues after AgNPs exposure. It is well known that metals such as Fe, Cu, Mg and Zn have an important role in biological systems and alterations of metal homeostasis in serum and tissues may cause different pathologies and nutritional effects (Kozlowsky et al., 2009; Bleackley and Macgillivray, 2011).

After 90 days of treatment, animals did not show significant reductions in organ weights and in the number of deaths. Likewise, no effects on body weight gain were found. Probably, either the route of administration, the dose used in this study, or both, were not appropriate to induce body weight gain alterations. These results are in agreement with those of a recent study in which the same route of administration and concentration of citrate-AgNPs (Yun et al., 2015) was given. Similar results were also reported by Hadrup and Lam (2014).

Most Ag excretion corresponded to feces, while only few amounts of Ag were excreted thought the urine. In feces, a dose-dependent increase of Ag was noted, being the highest levels of this metal found at 200 mg/kg/day. The possible aggregations and agglomerations of PVP-AgNPs formed in the GI tract did not facilitate AgNPs absorption, being most PVP-AgNPs excreted through feces. This agrees well with the results of previous studies (Loeschner et al., 2011; Van der Zande et al., 2012; Hadrup and Lam,

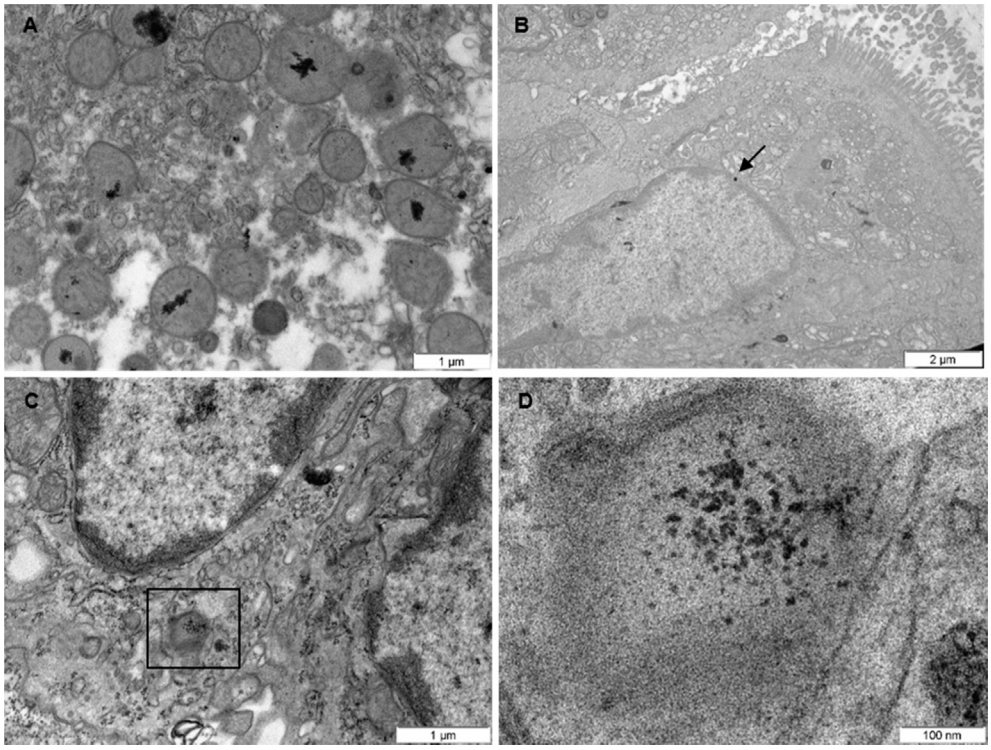

Fig. 5. TEM images of liver and ileum of treated rats. TEM images of 200 mg/kg/day PVP-AgNPs exposed rats after 90 days of oral treatment. A, liver; B, C, D, ileum.

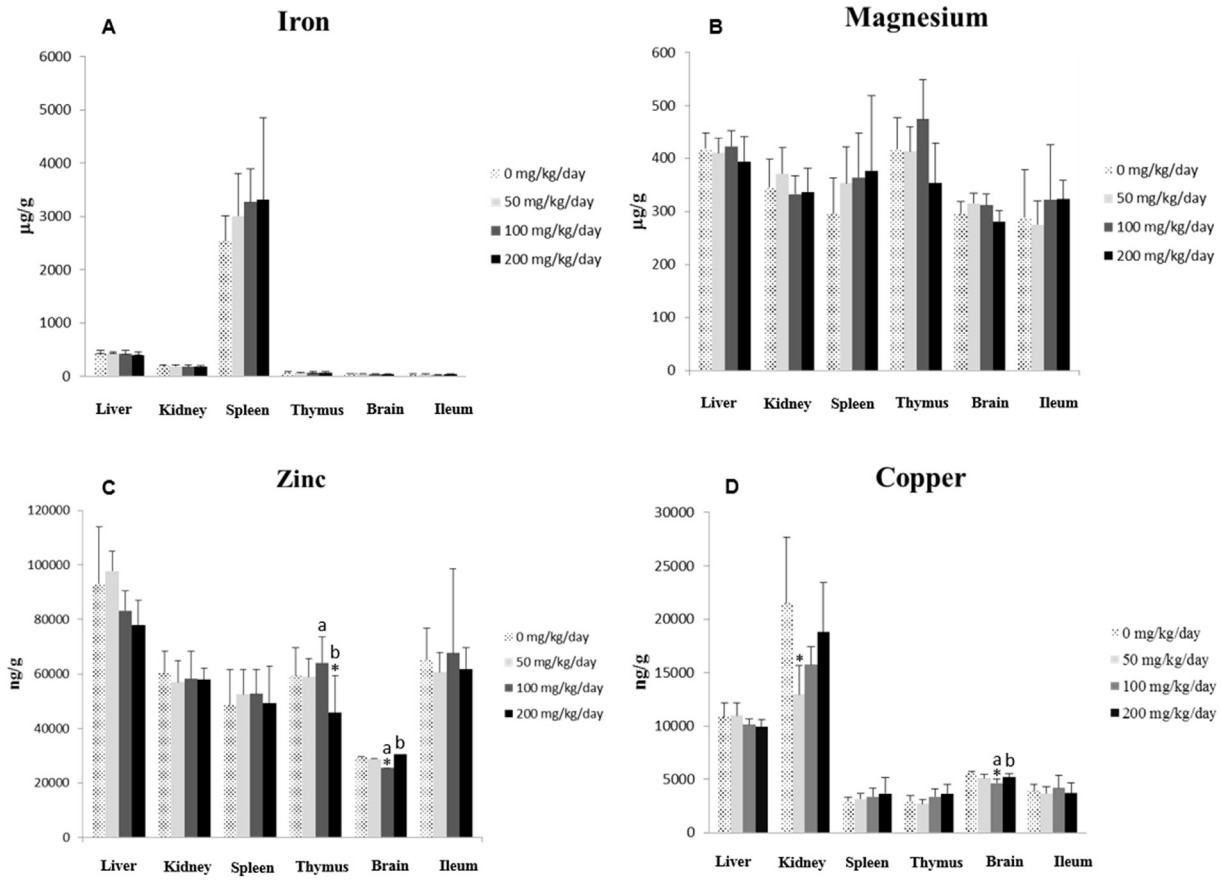

Fig. 6. Metals (Fe, Mg, Zn, Cu) levels in organs. Levels (mean ± S.D.) of (A) iron, (B) magnesium, (C) zinc, and (D) copper in liver, kidney, spleen, thymus, brain and ileum after 90 days of treatment. Different letters on the bars (a and b) indicate significant differences between groups. An asterisk (*) indicates a significant difference ($P < 0.05$) respect control group. $N = 8$.

Table 3
Hematological values for male rats after PVP-AgNPs 90-day orally administrated (n = 8 per group).

	0 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
WBC (×10E03 cells/μL)	3.95 ± 1.33	3.95 ± 1.91	4.54 ± 2.42	4.69 ± 1.65
RBC (×10E06 cells/μL)	8.43 ± 1.49	8.73 ± 1.67	9.30 ± 0.32	9.35 ± 0.53
HGB (g/dL)	16.43 ± 1.51	17.23 ± 2.15	17.62 ± 0.58	18.02 ± 1.18
HCT (%)	42.96 ± 8.20	44.69 ± 8.77	47.42 ± 1.50	49.20 ± 3.54
MCV (fL)	50.83 ± 1.19	51.11 ± 1.50	51.22 ± 0.72	52.55 ± 1.14
CHCM (g/dL)	34.17 ± 1.51	34.74 ± 0.84	34.22 ± 0.94	33.82 ± 0.52
PLT (×10E03 cells/μL)	738.8 ± 194.49	732.17 ± 216.59	1020.20 ± 192.14	856.200 ± 236.04
MPXI (no units)	10.94 ± 6.33	9.28 ± 7.64	9.22 ± 6.6	8.44 ± 2.18
% NEUT	20.30 ± 3.36	28.54 ± 12.97	29.38 ± 10.77	38.66 ± 18.34
% LYM	72.80 ± 5.96	67.88 ± 9.09	64.30 ± 11.79	64.42 ± 15.12
% MONO	1.79 ± 0.39	2.033 ± 0.77	2.48 ± 0.54	2.43 ± 0.88
% EOS	3.00 ± 1.34	3.96 ± 1.64	3.20 ± 1.48	3.96 ± 1.73
% BASO	0.3 ± 0.17	0.22 ± 0.11	0.12 ± 0.083	0.075 ± 0.09

WBC, white blood cells; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrits; MCV, mean corpuscular volume; CHCM, mean corpuscular hemoglobin concentration; PLT, platelets; MPXI, myeloperoxidase index; NEUT, neutrophils; LYM, lymphocytes; MONO, monocytes, EOS, eosinophils; BASO, basophils. Values are expressed as means ± SD.

Table 4
Plasma biochemistry profile for male rats after 90-day orally administrated with PVP-AgNPs (n = 8 per group).

	0 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
BUN (mg/dl)	19.23 ± 5.90	17.69 ± 3.92	14.94 ± 1.48	21.14 ± 3.56
TP (mg/dl)	5.94 ± 0.65	5.84 ± 0.64	5.79 ± 1.04	5.99 ± 0.55
ALB (g/dl)	3.22 ± 0.15	3.09 ± 0.081	3.22 ± 0.10	3.13 ± 0.22
A/G	1.35 ± 0.71	1.23 ± 0.49	1.13 ± 0.14	1.01 ± 0.10
ALP (IU-1)	78.86 ± 18.97	68.14 ± 11.91	63.27 ± 8.61	59.12 ± 7.01
AST (IU-1)	65.68 ± 25.45	65.21 ± 22.07	41.54 ± 13.04	52.89 ± 15.34
ALT (IU-1)	40.64 ± 16.15	34.07 ± 7.28	31.14 ± 7.51	30.46 ± 7.50
Creatinine (mg/dl)	0.69 ± 0.098	0.65 ± 0.024	0.64 ± 0.096	0.60 ± 0.05

BUN, blood urea nitrogen; TP, total proteins; ALB, albumin; A/G, albumin/globulin; ALP, alkaline phosphatase; AST, aspartate transaminase; ALT, alanine transaminase. Values are expressed as means ± SD.

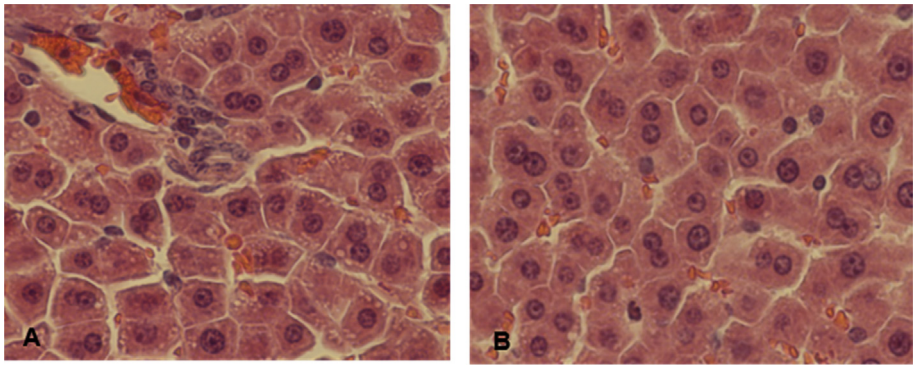

Fig. 7. Liver binucleation in treated rats. (A) 100 mg/kg/day PVP-AgNPs group of treatment demonstrating binucleation. (B) 200 mg/kg/day PVP-AgNPs group of treatment demonstrating binucleation.

2014).

In general terms, after PVP-AgNPs treatment, Ag accumulation pattern in tissues was similar, showing the treated animals an increase of Ag levels in comparison with rats in the control group. The highest content of Ag in tissues was found in the small intestine (Ileum) with a significant increase in Ag concentration being found in all treated groups (50, 100 and 200 mg/kg/day). These results are also in accordance with the data reported by Loeschner et al. (2011). However, when different doses were compared, no significant differences were observed. It could be due to the increased excretion of Ag by feces at 200 mg/kg/day. The distribution of Ag inside illeum cells was also assessed by means of TEM. The images showed electron-dense accumulation of PVP-AgNPs in cell cytoplasm while individual granules were found within the intestinal villi, in agreement with Loeschner et al. (2011).

Once absorbed by the GI tract, Ag accumulation increased in all treated animals. However, no significant differences between the different doses of PVP-AgNPs were found. Even animals at 100 and 200 mg/kg/day had sometimes similar levels of Ag. It could be due to possible PVP-AgNPs agglomerations at the highest dose, which could make difficult the AgNPs absorption by the tissues. In kidneys, treated rats showed significant increased levels of Ag with respect to animals in the control group. However, histopathological evaluation and biochemical indicators of renal function were not altered in the exposed animals. It indicates that the Ag accumulated in kidneys was not sufficient to cause harmful effects in the renal function of treated animals. These results also agree with the results of previous studies in which rats were orally exposed to

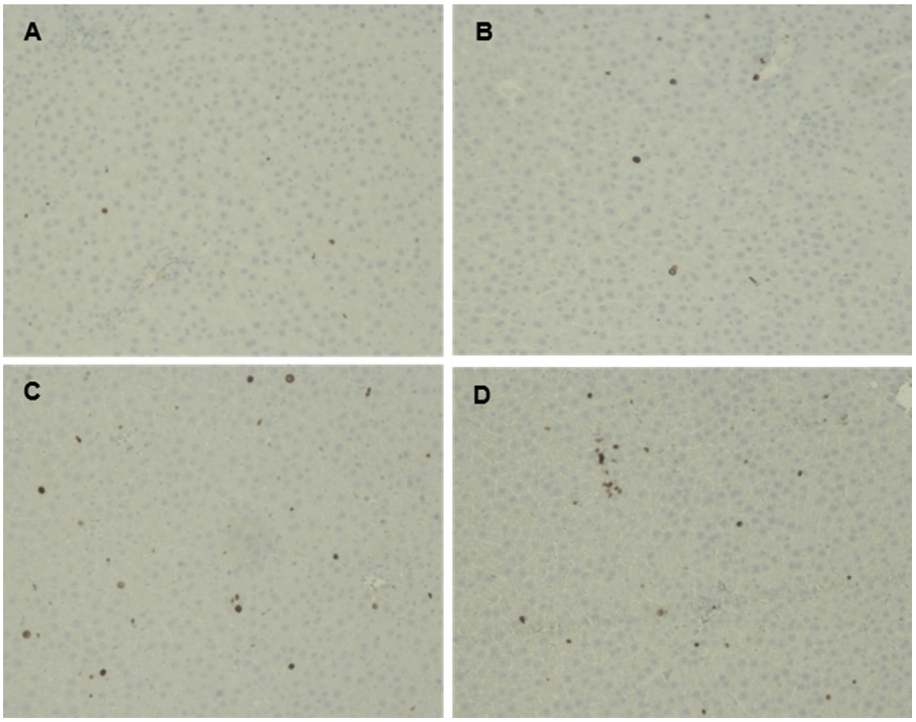

Fig. 8. Immunohistochemical staining of liver. Immunoreactive cells to antibody Ki-67 (40X) in (A) control rats, (B) 50 mg/kg/day PVP-AgNPs treated rats, (C) 100 mg/kg/day PVP-AgNPs treated rats and (D) 200 mg/kg/day PVP-AgNPs treated rats. Counterstained with hematoxylin.

different doses of AgNPs (Kim et al., 2010; Yun et al., 2015).

Some *in vivo* studies have observed the toxic effect of AgNPs in liver (Tiwari et al., 2010; Yun et al., 2015). In the present study, although a significant dose-dependent increase of Ag levels in liver of exposed animals was observed, significant alterations in enzymatic markers of liver damage were not noted. These results are in accordance with those of a previous study, where the authors showed that exposure to more than 125 mg/kg/day of AgNPs would cause slight liver damage (Kim et al., 2010). On the other hand, TEM images showed that, in some sections, the 200 mg/kg/day group, some electrondense AgNPs aggregates are located inside mitochondria. Previous investigations have observed that after intravenous exposure of AgNPs in rats, individual and cluster of AgNPs could accumulate inside liver cells (Tiwari et al., 2010; Dziendzikowska et al., 2012).

Although histopathological examination did not show harmful effects in liver of PVP-AgNPs treated groups, a higher number of binucleated cells was found at 100 and 200 mg/kg/day. In turn, a non-significant increased level of proliferating liver cells was also found in the same groups. Probably, the levels of Ag found at 100 and 200 mg/kg/day were not sufficient high to induce alterations in liver. Notwithstanding, AgNPs located into liver cells could start to induce a slight damage.

In the remaining tissues (brain, spleen and thymus), the different doses of PVP-AgNPs showed a similar Ag absorption pattern, indicating similar Ag levels in tissues. Possible aggregates formed at higher doses could prevent a proper absorption by this tissues. In turn, the levels of PVP-AgNPs absorbed by tissues were not sufficiently high to induce histological alterations, which is in agreement with the results of a recent study with similar AgNPs doses and route of administration (Yun et al., 2015).

Moreover, no significant effects of the different doses of PVP-AgNPs after 90 days of treatment were found in hematological parameters. Which is also in accordance with the results of previous investigations, which evaluated the toxic effects of an oral subchronic exposure of AgNPs in male rats (Kim et al., 2010; Yun et al., 2015).

In the present study, we determined for the very first time the levels of Fe, Mg, Zn and Cu in liver, kidneys, thymus, spleen and small intestine. No significant effects of PVP-AgNPs treatment on Mg or Fe levels were noted in any of the tissues evaluated. However, Zn and Cu levels were altered in some tissues. Cu levels decreased in brain at 50, 100 and 200 mg/kg/day, being significant at 100 mg/kg/day. In kidney, decreased levels of Cu were found in treated animals, reaching statistical significance at 50 mg/kg/day. Cu is a trace element required for different cellular process, being a cofactor for numerous enzymes and playing an important role in central nervous system. Cu is present in brain, being prominent in the hippocampus, basal ganglia, cerebellum, as well as, in the cell bodies of cortical pyramidal and cerebellar granular neurons. Alterated levels of Cu had been associated with different neurological disorders (Desai and Kaler, 2008).

Significant changes in Zn concentrations were observed in thymus and brain of PVP-AgNPs treated animals. In liver, there was a slight (no significant) decrease of Zn levels at 100 and 200 mg/kg/day, in comparison to the 50 mg/kg/day and control groups, which was probably due to the relative inter-animal variation. Zn is a trace element required as a catalytic component for more than 200 enzymes and is a structural constituent of many hormones, neuropeptides, and proteins. Changes in liver Zn levels are associated with liver pathologies. Some studies have reported a relation between low Zn concentrations and liver damage (Mohammad et al., 2012; Nangliya et al., 2015). A significant decrease in Zn concentration was also observed in thymus at 200 mg/kg/day. It has been shown that severe Zn deficiency causes alterations of lymphoid organs, thymic hypoplasia, as well as, the absence of germinal centers in lymph nodes (Prasad, 1983; Ozturk et al., 2004). In relation to brain, significant decreased Zn levels at 100 mg/kg/day were noted. These results indicate that the levels of Ag found in brain of treated animals were sufficient to induce changes in Cu and

Zn concentrations. Brain is a specialized organ that normally concentrates Cu, Zn and Fe in the neocortex. Alterations in metal levels have been linked with neurodegenerative diseases (Gómez et al., 2008). However, we did not found histopathological alterations in brain after PVP-AgNPs treatment. Similarly, Yun et al. (2015) did not report histopathological changes in rats orally given with 200, 500 and 1000 mg/kg/day of AgNPs for 13 weeks. There are no previous information showing biological interactions between AgNPs and metal accumulation in tissues. In the current study, we observed that accumulation of PVP-AgNPs could alter concentration of Zn in thymus and brain, and Cu in kidney and brain of treated rats. Although the highest levels of Ag were found in ileum of treated rats, being the AgNPs located in the same tissue by TEM, no significant changes in Fe, Mg, Zn and Cu levels were observed in the small intestine of treated animals. Furthermore, no histological changes in ileum were observed. It suggests that ileum is less sensible to the PVP-AgNP accumulation than other tissues. These results are not in agreement with the results reported by Kim et al. (2010), who found slight histological changes in small intestine of rats after AgNPs treatment. However, we did not evaluate if subchronic PVP-AgNPs treatment could affect intestinal microbiota as Williams et al. (2015) recently reported.

5. Conclusions

The results of this study show that oral subchronic exposure of adult Sprague Dawley rats to PVP-AgNPs caused accumulation of Ag in different tissues at doses of 50, 100 and 200 mg/kg/day. However, Ag accumulation was not sufficient (at any dose) to alter hematological parameters or to induce harmful effects in kidney, spleen, or ileum of treated rats. The ileum of treated rats was the tissue with the highest Ag levels. However, it seems to be less sensible to PVP-AgNPs effects at the current doses than other tissues. In liver, the AgNPs were located inside mitochondria and cytoplasm. Again, this accumulation was not sufficient to alter biochemical markers for hepatic status, or general tissue morphology. However, increased binucleated hepatocytes and increased proliferating cells were found, showing slight effects at cellular level.

We also evaluated for the very first time the interaction between AgNPs and metal homeostasis in various tissues. The results revealed that PVP-AgNPs could alter Zn and Cu levels in thymus and brain of treated animals, indicating that brain is more sensible than other tissues at the current PVP-AgNPs doses. Because Zn and Cu are essential for many metabolic and enzymatic functions, and deficiency of these elements has been associated with various diseases, further studies are required to clarify the interaction between AgNPs and trace elements concentration.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

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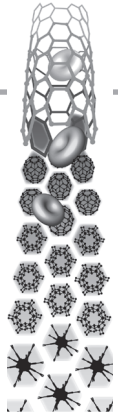

Toxicological studies on silver nanoparticles: challenges and opportunities in assessment, monitoring and imaging

Silver nanoparticles (Ag NPs) are becoming increasingly prevalent in consumer products as antibacterial agents. The increased use of Ag NP-enhanced products may lead to an increase in toxic levels of environmental silver, but regulatory control over the use or disposal of such products is lagging due to insufficient assessment on the toxicology of Ag NPs and their rate of release into the environment. In this article we discuss recent research on the transport, activity and fate of Ag NPs at the cellular and organismic level, in conjunction with traditional and recently established methods of nanoparticle characterization. We include several proposed mechanisms of cytotoxicity based on such studies, as well as new opportunities for investigating the uptake and fate of Ag NPs in living systems.

KEYWORDS: cell uptake ■ characterization methods ■ imaging ■ physiological sensing ■ silver nanoparticles ■ toxicology

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Silver nanoparticles (Ag NPs) have become increasingly popular as antibiotic agents in textiles and wound dressings, medical devices and appliances such as refrigerators and washing machines. They are traditionally defined as particles with overall dimensions below 100 nm, but the term 'nanosilver' is also becoming widely adopted, especially in the context of commercial products that contain nanomaterials with a large fraction of silver. The number of Ag NP-containing products has grown from less than 30 in 2006 to over 300 at the beginning of 2011 [301] and they are most often employed as bacteriostatic coatings for preventing infection or as deodorants. It is estimated that approximately 280 tons of Ag NPs were produced for use in commercial or industrial products and that number is expected to quadruple by 2015 [1]. However, an adequate assessment of the long-term effects of Ag NP exposure on human physiology and their release into the environment is lagging behind the rapid increase in the commercialization of Ag NP products. Most of the scientific literature on the toxicology of Ag NPs has only been published in the past decade [2–4]. Many of these studies have revealed Ag NPs to have noticeable toxicity against several cell lines as well as a number of aquatic organisms, but the mechanistic basis of these toxic effects is now an area of active research. In particular, the bioavailability of silver ions (Ag^+) from Ag NPs, considered by many as a major factor in Ag-mediated toxicity, remains poorly understood [5]. For example, certain algal species are more sensitive to Ag NPs than to free Ag^+, but the addition of cysteine (known to form complexes with Ag^+) reduces the toxic effects of both Ag sources [6]. Such studies underscore the need to understand the transport, uptake and degradation of Ag NPs under physiological conditions, to accurately assess the relative benefits and risks of using Ag NPs in commercial products.

Traditional methods in toxicology research and assessments have focused mostly on chemical agents and were not originally designed to encompass nanoparticles, so determining the toxicological effects of Ag NPs raises several challenges. For instance, Ag NPs and Ag-oxides have strong optical extinctions at visible wavelengths and can interfere with colorimetric assays such as the MTT assay, which is used to measure cell viability based on mitochondrial activity [7]. Another issue is the large variation in physicochemical characteristics, depending on the source and type of Ag NPs; variations in particle size, shape and surface chemistry can have significant impacts on toxicity. It is necessary to characterize Ag NPs both prior to use and also during the course of a study, because significant temporal changes will often occur during an experimental trial [8,9]. In fact, particle agglomeration is commonly observed in studies involving Ag NPs, particularly when diluted in media with high ionic strength (> 10 mM). This agglomeration can affect their bioavailability by reducing their rate of degradation or cell uptake, as larger aggregates are less efficiently internalized [8]. These variables impose significant challenges for designing

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in vivo toxicological studies, which must be supported by numerous control studies in order to identify the most important experimental parameters.

On the contrary, the strong optical properties of Ag NPs can be useful in the context of biosensing and biological imaging and offer excellent opportunities to study NP uptake and biodistribution, *in vivo* as well as *in vitro* [10,11]. Ag NPs support localized surface plasmons that give rise to resonant light scattering and other optical properties [12], and can support a variety of bioanalytical sensing and imaging modalities. Recent applications of Ag NPs include the detection of biomarkers in Alzheimer's disease [13], the targeted imaging of cancer cells [14] and the identification of pathogens by surface-enhanced Raman scattering [15]. The plasmon-enhanced optical activities of Ag NPs enable them to be tracked in real time without the need for additional labels, as well as a handle for evaluating their eventual degradation.

The goal of this article is to examine potential fate or exposure pathways of Ag NPs, to discuss recent findings related to the mechanisms of Ag NP-mediated toxicity and to present current and emerging methods for assessing the transport, uptake and fate of Ag NPs in biological systems. In relation to the latter, we include methods that have not yet been applied toward toxicological studies, but have strong potential to monitor individual Ag NPs in real time, particularly when comparing their effects to their ionic counterparts. This article is not intended to be fully comprehensive in any one area, but rather to highlight the most recent findings and novel approaches for measuring Ag NP uptake and toxicity. We conclude with a section covering the current gaps in knowledge and future research needs.

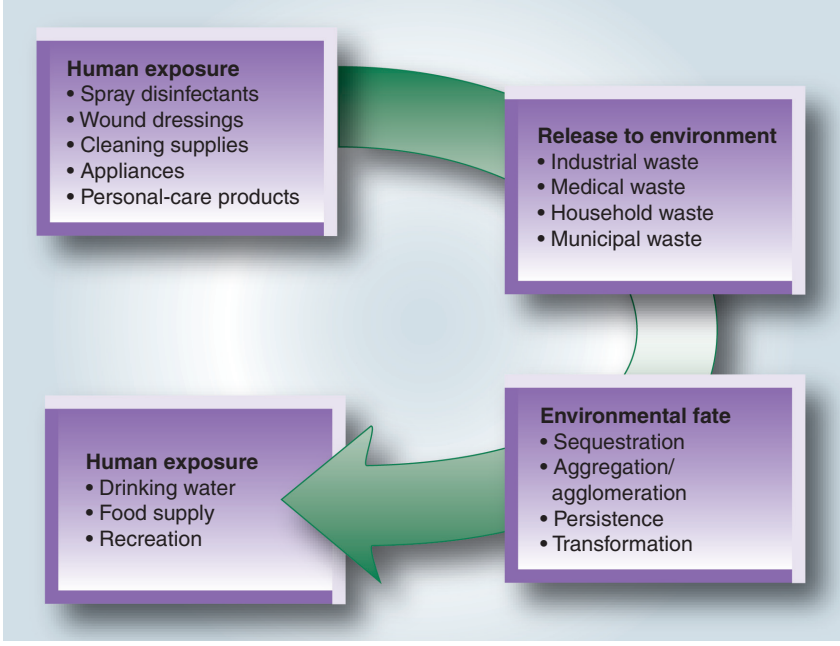

Figure 1. Potential human and environmental exposure routes for silver nanoparticles.

Environmental fate & exposure pathways of Ag NPs

Silver ions have long been known for their antimicrobial properties. Early Romans used Ag^+ to disinfect potable water and Ag^+ was the most common antimicrobial agent until the large-scale development of synthetic antibiotics in the mid 20th century. Nevertheless, Ag^+ remains a common biocide in household products, biomedical instruments, drinking water filters and appliances [16–18]. A comprehensive review by Silver summarizes the many uses and misuses of silver-containing products and the downstream biological impact of Ag^+ on prokaryotic and eukaryotic cells [19].

Silver nanoparticles have notable biocidal activity, due in part to the sustained release of Ag^+, but further enabled by its surface and photocatalytic properties that can facilitate oxidative damage in nearby cells [20–24]. Ag NPs are significantly more toxic than Ag^+ to prokaryotic cells and have been shown to be effective bactericides at nanomolar concentrations, compared with micromolar levels for Ag^+ [25–27]. For these reasons, Ag NPs have been incorporated into numerous textile products and surface coatings as a bacteriostat. Unfortunately, these commercial activities have resulted in the unintended but worrisome consequence of Ag NPs moving into the ecosystem [23,28,29], raising recent concerns over the bioaccumulation of Ag NPs and the increased risk of human exposure (Figure 1) [30].

There are no detailed studies of the release of Ag NPs from medical or household sources. In large part, this is due to the lack of tools capable of measuring the various types of Ag NPs, which includes metallic nanoparticles [31], Ag-zeolites [32], Ag–dendrimer complexes [33] and many other forms reviewed by Marambio-Jones and Hoek [34].

Blaser and coworkers predicted that Ag^+ and Ag NPs comprise up to 15% of the biocidal compounds released from the plastics and textile industries entering waterways [35]. If Ag NPs enter municipal wastewater treatment plants, it has been estimated that approximately 7% leave the facility bound in sludge [35]. However, the subsequent fate of the Ag NP is largely unknown

and depends on the sludge management approach used by individual municipalities (e.g., landfilling, incineration and land application).

As discussed by Marambio-Jones and Hoek, the increased use of Ag NPs in medical devices, clothing, household water filters, contraceptives, antibacterial sprays, cosmetics, detergents, cooking utensils, cell phones, computers and children's toys is likely to result in an increase in the concentration of Ag NP discharge to our ecosystems [34]. Some studies have noted the potential for bioaccumulation of Ag NPs in various organisms, such as biofilms [36] and *Mytilus edulis* [37], a marine mussel. One study found that *Daphnia magna*, an aquatic invertebrate, accumulated nanoparticles from aqueous as well as foodborne exposure [38]; however, to date no large-scale bioaccumulation studies have been conducted.

Recent studies have investigated the effects of acute Ag NP exposure to humans from commercial products, such as disinfectant sprays [201,202], wound dressings [39], kitchen utensils [40] and cleaning supplies [41]. However, there is only anecdotal information concerning the fate of Ag NPs released by direct industrial discharge, wastewater treatment effluent, and medical or consumer waste, and no systematic studies on their environmental and toxicological impact. While there is some evidence to suggest that Ag^+ released from Ag NPs is rendered less toxic by complexation with organic ligands and sulfur [42], there remain large gaps in knowledge on the chemistry and biochemistry of Ag NP biodegradation, which is necessary to address the toxicological impact of Ag NPs and Ag^+ introduced by anthropogenic activities [43].

Assessments of Ag NP release into the environment

Current models indicate that environmental Ag^+ and Ag NPs comprise nearly 15% of the biocidal compounds released as a point source from the plastics and textile industries [35]. Ag NPs have also been identified in the effluent waste from mining, photographic and electronic industrial processes [28,29]. Although there are currently no published data concerning the fate of Ag NPs in hospital waste, the potential for accumulation of Ag NPs from this industry is large considering the increased use of Ag NPs in wound dressings [39], surgical equipment [44], catheters [45,46] and even paint on the walls of hospital rooms [47]. It is estimated that the amount of Ag NPs released into aquatic ecosystems is currently on the order of 10–100 ng/l, a level that is likely to be exceeded within the next decade [48].

Silver nanoparticles are commonly discharged into the environment as a point-source pollutant and thus may be collected in municipal wastewater treatment plants (WWTPs) [26]. However, approximately 7% of Ag NPs entering WWTPs will be accumulated in sludges that are later deployed as agricultural fertilizers [35]. A majority of the Ag NPs in sludge remain in the upper soil layers and can potentially enter surface waters via runoff or groundwater tables [49]. The fate and transport of Ag NPs is also complicated by the fact that while some materials can be complexed as silver sulfide [42], removed by precipitation [50] or agglomeration [21,51], other forms may pass through WWTPs unaltered [35] and/or released as Ag^+ [28].

It is important to note that in addition to the potentially adverse effects of increased biological exposure to Ag NPs, there is a risk that an increased release of Ag NPs into the environment may stimulate a rise in bacterial strains with heightened resistance to silver [19]. At present, most studies have focused on planktonic microbes [24,29,49] and very little information is available to outline the effect of Ag NPs on aquatic or terrestrial microbial populations [17]. One study did find a high sensitivity of a crucial soil microbe, *Bradyrhizobium canariense*, to Ag NPs [52]. However, there is a large body of literature describing the penetration of chemical toxins into bacterial biofilms [24,53,54], with suggestions that such environmental pressures may encourage the development of 'persister cells' with high levels of antimicrobial resistance [55]. In fact, the increased resistance to antimicrobials [53] and community-based defense mechanisms [56] are hallmarks of microbial biofilms, a topic that has been studied for decades [57]. The relationship between Ag NPs and increased Ag^+ resistance has not been studied, perhaps hindered in part by limitations in technologies to enable such investigations.

Mechanisms of Ag NP toxicity

Silver nanoparticles are frequently touted as being highly effective as antimicrobial agents while being nontoxic to mammals. However, numerous *in vitro* studies have demonstrated the toxic effects of Ag NPs on rat liver (BRL3A) and neuronal cells [58,59], human lung epithelial cells [60] and murine stem cells [61]. Ag NPs have also exhibited toxicity in aquatic organisms, including vertebrates [11,62–64]. There is also strong evidence that microorganisms and plants are capable of concentrating nanoparticulate materials, which increases the potential for

Ag NPs to accumulate in the food chain [65]. It should be noted, however, that many of the studies to date have used concentrations of Ag NPs that are much higher (>1 ppm) than what could be considered environmentally relevant. So far, most of the information relevant to the mechanisms of Ag NP toxicity has been derived from *in vitro* studies. Only a handful of mechanistic studies have been conducted *in vivo*, reflecting a significant gap in knowledge on a topic of increasing concern to the environment as well as human health.

The mechanisms of Ag^+ toxicity are well understood, with many studies published over the past 50 years [302]. There is a general consensus that mitochondria are a primary target of Ag^+ and are vulnerable to the 'permeability transition pathway', characterized by the formation of proteinaceous pores in mitochondrial membranes. In rat liver mitochondria, this increased permeability results in mitochondrial swelling, aberrant metabolism and eventually cellular apoptosis [66]. The lowest observed adverse effect level for Ag^+ in mammalian cells has been reported to be in the range of 222 to 362 mg Ag/kg-day [67,68].

By contrast, there is less agreement on the factors that enable Ag NPs to deliver toxic effects to cells and organisms. In addition to size-dependent physical properties that can affect the release rate of Ag^+, Ag NPs also exhibit size-dependent mechanisms of cell uptake that greatly influences their bioavailability. This mode of action has been referred to as the 'Trojan Horse' mechanism [23,24]: Ag NPs that permeate cell membranes can produce higher levels of intracellular Ag^+, causing cytotoxic and genotoxic effects by the disruption of cell transport and local depletion of glutathione and other antioxidants [58,69–71]. Ag NPs smaller than 5 nm can passively penetrate cell walls and membranes, while larger NPs are generally internalized by endosomal mechanisms [22,23]. Therefore, considerable attention should be directed toward the transport and fate of Ag NPs, in order to better understand its toxicological effect on cells and organisms.

In vitro studies

The cytotoxic effects of Ag NPs have mostly been characterized in terms of oxidative stress, DNA damage and modulation of cytokine production. The cell uptake of Ag NPs can stimulate the production of radical oxygen species (ROS), resulting in oxidative stress and genotoxic effects. ROS are produced owing to a disruption in the flux of ions and electrons across the mitochondrial membrane; if produced in sufficiently high amounts, ROS can induce cell death by either apoptosis or necrosis [58,66,72–74]. Studies have shown Ag NP toxicity to be both size and shape dependent; for example, one study with alveolar macrophages indicated that Ag NPs with a mean size of 15 nm induced the greatest loss in mitochondrial activity [73]. However, contradictory data exists on the influence of Ag NP size and mitochondrial toxicity [58], suggesting that such effects may be case or species dependent, so a wider range of studies are needed before any generalities can be assumed.

With regard to genotoxicity, Ag NPs can damage DNA (in this case from human lung fibroblasts, IMR-90 and human glioblastoma cells, U251) indirectly by increasing ROS production or by decreasing ATP production (related again to mitochondrial damage), which impairs energy-dependent DNA repair mechanisms [74]. Direct DNA damage by Ag^+ (released by Ag NPs) or by Ag NPs themselves have also been reported [75–78]. The latter case has been measured in mouse embryonic and fibroblast cells, indirectly by the increased expression in DNA repair proteins (Rad51 and H2AX) and an upregulation of p53, a cell-cycle checkpoint protein [79].

Silver nanoparticles have been reported to have both stimulatory and suppressive effects on the production of cytokines associated with the inflammatory response and again are likely to be dependent on case, dose and cell type. For instance, alveolar macrophages exposed to Ag NPs responded with an increase in the production of proinflammatory response mediators (TNF-α, MIP-2 and IL-1β) [73] and human epidermal cells exposed to Ag NPs produced an increase in IL-1β, IL-6, IL-8 and TNF-α [7]. By contrast, human mesenchymal stem cells exposed to Ag NPs exhibited both declines (IL-6 and IL-8) and increases (IL-8) in proinflammatory factors [80]; the latter was only observed when cells were exposed to less than 5 μg/ml Ag NP.

Recently, a fourth chemometric of Ag NP-induced toxicity has been reported. Rat coronary endothelial cells exposed for 24 h to high doses of Ag NPs (100 μg/ml) responded with an increased production of nitric oxide, which also increased cell proliferation [81]. At lower doses (<10 μg/ml), only a decrease in mitochondrial function was observed. Nitric oxide is known to have an important role in the cardiovascular system, suggesting another direction for the biological effects of Ag NPs.

In vivo studies

Compared with *in vitro* studies, significantly less information is available on the potential mechanisms of toxicity of Ag NPs from *in vivo* studies. As reviewed below, exposure of laboratory rodents to Ag NPs has resulted in a myriad of toxicological responses, including effects on circulatory, respiratory, central nervous and hepatic systems. Effects on dermal tissues have also been reported after topical administration of Ag NPs.

Ingestion or inhalation of Ag NPs results in their transport to the circulatory system [22,82]. Only one whole animal study is available on the effects of Ag NPs on hematological parameters. Mice injected with Ag NPs responded with a decrease in platelet aggregation [83]. The mechanisms of such a response remain unknown. With regard to adverse respiratory effects of Ag NPs, only one complete study has been reported [84]. In this study, rats were exposed to 18–19 nm Ag NPs at a concentration of 0.7–2.9 × 10^6 particles/cm^3 for 90 days. Lung function, measured as tidal volume, minute volume and peak inspiration flow, was impaired in the highest concentration tested. Inflammatory responses (total protein, alveolar wall thickening and macrophage infiltration) were also increased in some animals [84]. A second, independent study indicated a rapid clearance of silver from rat lungs after an acute (6 h) inhalation exposure to Ag NPs, but an autopsy revealed the translocation of Ag NPs to the brain a week after initial exposure [85]. A pharmacokinetic study of Ag NPs injected into the bloodstream also confirmed their movement through the blood–brain barrier with subsequent accumulation in the brain, accompanied by neuronal degeneration and necrosis [86].

Recent evidence indicates that liver and bile ducts are targets of toxicity for Ag NPs: Ag^+ has been found to accumulate in liver following exposure to Ag NPs [82,84,87,88]. Histopathological analyses of liver and bile ducts of mice after Ag NP exposure also revealed vacuolization and hepatic focal necrosis, hyperplasia of bile ducts, increased infiltration of inflammatory cells and dilation of central veins. Increases in the expression of genes involved in apoptotic and inflammatory pathways have also been detected in mice livers exposed to Ag NPs [75].

Toxic effects were also observed during zebrafish development. Mortality, heart rate and hatching rate were all impacted by Ag NPs. Each of these end points was affected in a dose-dependent manner (5–100 µg/l Ag NPs). Changes in morphology, edema and an overall slowing of development were also detected. In this study, uniform distribution of the nanoparticles within the zebrafish embryos was also observed [89].

A single study is available on the morphological alterations of skin cells following exposure to Ag NPs [7]. In this study, pigs were topically dosed with Ag NPs (20–50 nm, 0.34–34 µg/ml) for 14 days. The highest doses caused edema, epidermal hyperplasia and focal inflammation.

In summary, most of the information available on the mechanisms of toxicity and associated effects of Ag NPs comes from *in vitro* studies, with only limited information from *in vivo* studies. Three main mechanisms of toxicity of Ag NPS have been proposed: oxidative stress, DNA damage and cytokine induction. Results from *in vivo* studies have shown that exposure of Ag NPs can result in effects in different major organs. It is important to mention that the studies summarized here used different formulations of Ag NPs (most were generated in the laboratory and some were purchased commercially). Very few studies have evaluated the mechanisms and associated toxicity effects of Ag NPs 'leached' from current commercial products.

Physicochemical assessment techniques

The physicochemical properties of Ag NPs are relevant to their toxicology; namely size, shape and surface chemistry. For example, a number of studies have correlated the size and shape of Ag NPs with their bactericidal properties [17,23,90,91]. A variety of methods are available to quantify these properties, several of which are summarized in TABLE 1; other approaches have been described in recent articles and reviews [92,93]. It has been noted, however, that while the physicochemical characterization of Ag NPs is necessary for a comprehensive analysis of its biological uptake and interactions with cells, such measurements are not sufficient for predicting nanotoxicological effects [94]. Indeed, this issue should be viewed in the opposite direction: toxicological studies are critical for correlating the physicochemical properties of nanoparticles and their interactions with living systems.

Transmission electron microscopy

Transmission electron microscopy (TEM) is the most common method of characterizing NP size and shape [91,95]. TEM images of NPs are typically acquired in brightfield mode, based on the contrast generated by electron scattering from heavy atoms, then subjected to image analysis software to obtain statistical distributions in

Table 1. Methods for characterization of silver nanoparticles.

Method of characterization	Attribute measured	Advantages	Disadvantages	Ref.
Transmission electron microscopy	Size, shape	Measures absolute size and shape	Preparation subject to artifacts	[18,80,84–86]
Dynamic light scattering	Size	Measures effect size	Polydispersity and irregular shapes skew results	[80,84,87–91]
Atomic force microscopy	Size, shape	Measures effect size and shape	Limited by cantilever tip size and particle surface chemistry	[88,92–95]
Zeta potential	Surface charge	Indicates stability and surface chemistry	Affected by capping agents and shape	[25,86,87,89,96,97]
Brunauer Emmett Teller	Specific surface area	Measures total area available for interaction	No size distribution	[84,85,98–100]

particle size and ellipticities. Individual NPs can also be assessed for uniformity in shape or lack of thereof [17,96]. However, TEM analysis is limited by sample size (typically <200 particles) and also by variability in sample preparation methods, which are typically performed by casting and drying droplets of NPs in solution onto polymer-coated grids. This practice can skew the true size distribution of particles in suspension, as well as their state of agglomeration [97]. For these reasons, size analysis by TEM is best conducted in conjunction with other methods that characterize particles in their equilibrium states.

Dynamic light scattering

Dynamic light scattering (DLS) is a method of particle size analysis based on light scattering and the Brownian motion of particles in solution [91,95,98]. Whereas TEM defines particle size by differences in electron scattering, DLS measures the hydrodynamic radius of particles based on their rates of translational diffusion. DLS measurements will often produce larger values than those obtained by TEM, because they include the influence of the organic surface coating in the size estimates and the particle size distribution includes aggregates as well as individually dispersed particles. For these reasons, DLS is considered by many to be a more accurate estimate of the effective size of particles in solution [99,100]. However, DLS also has some drawbacks: the values are dependent on particle concentration, due to its sensitivity to aggregation effects, and it cannot provide an accurate assessment of particle shape without considerable parameterization. Furthermore, nonspherical particles are subject to additional motional behaviors that are easily misinterpreted. For example, DLS measurements of monodispersed gold nanorods can produce two peaks, corresponding either to rotational diffusion [101] or to an anisotropic orientation relative to the light source [102].

Atomic force microscopy

Scanning probe microscopies, such as atomic force microscopy, can provide complementary size and surface analysis of NPs bound to substrates, in liquid states as well as in air. Nanometer-sized probes are mounted on cantilevers and rastered across the sample, recording changes in forces as the probe tip interacts with the surface [103]. The lateral resolution of atomic force microscopy is lower than that of TEM due to limitations in tip size and shape; on the other hand, it is highly sensitive in the z-direction and is especially useful for depth profiling with nanometer resolution [104]. The cantilevers can also be functionalized to increase its sensitivity to surface properties and has been used to map electrostatic and chemical interactions [105,106]. Topological analyses based on such interactions do not necessarily reflect the true dimensions of nanostructured features, but rather an effective size based on physicochemical interactions [99].

Zeta potential

Another important factor in the transport and fate of Ag NPs is their electrokinetic or zeta potential, measured in millivolts. This is typically defined by the electrostatic double layer surrounding each NP, which in turn is influenced by surfactant coatings and the ionic strength of the supporting medium [107]. NPs can form stable suspensions in aqueous solutions when their zeta potentials (typically negative) are above 30 mV. This is usually the case for particles dispersed in low ionic strength solutions, but their zeta potentials are reduced at higher ionic strength because the cations are more closely associated with the particle surface, which reduces the electrostatic double layer [98,108] and can lead to agglomeration [26]. It should be noted that the zeta potential is not the sole factor in NP stabilization; particles can also be sterically stabilized by organic surface coatings, independent of surface charge [100].

Brunauer Emmet Teller

Specific surface area is yet another size-dependent factor in NP toxicity [96,109,110]. The specific surface area of NPs is much greater than that of their bulk counterparts and is an especially important issue in the case of aerosolized particles, which can enter the body through inhalation. The BET method is the most common method of quantifying exposed surface and has been used to measure the specific surface area of Ag NPs [95,109]. This method is based on the absorption of gas molecules onto the surface of the target analyte at a specified pressure. The specific surface area is then obtained as the ratio of the total surface area to the weight of NPs (in m^2/g) [111].

Real-time physiological sensing

The unique properties of Ag NPs have been found to interfere with some of the more traditional toxicological assays. To accommodate this interference researchers in the field of nanotoxicology have had to incorporate new techniques or modify existing techniques. Some of these techniques were recently reviewed [2]; however, this article will focus on the use of microsensors for real-time physiological sensing and the incorporation of advanced imaging techniques into toxicological studies.

A logical first step for understanding the mechanisms of Ag NP toxicity is to compare the adverse effects with that of its ionic counterpart. The differential responses to Ag^+ and Ag NPs can provide insights into the relationship between cell/tissue physiology and any size-dependent phenomena attributable to Ag NPs. Such information can be expected to be useful for guiding future regulations, as there are no rules currently in place to control the commercialization of Ag NP enhanced products [112]. However, efforts to obtain this knowledge is limited by available technologies for monitoring physiological changes during Ag NP exposure. While semiquantitative physiological assessment methods are widely employed and still very useful, one major drawback is that they are destructive (i.e., organisms need to be sacrificed). This imposes significant limits on the amount and quality of information that can be obtained from an individual specimen during experimental trials.

Over the last few decades the use of electrochemical microsensors and nanosensors has become more prominent for biological research applications [113]. Used in both intracellular and extracellular applications, these sensors have allowed us to measure analytes related to metabolism, stress and cell communication/signaling in ways which were not previously conceivable. Traditional use of nano/microsensors has involved penetration into cells/tissues or extracellular measurements along the surface of cells/tissues. Intracellular micro- and nano-sensors have been shown to cause membrane damage and cytotoxicity, respectively [114]. While recent developments in intracellular nanosensors have allowed them to be used in minimally invasive formats [115], most extracellular micro/nanosensors are still used invasively due to low signal-to-noise ratio and a lack of multidimensional spatial resolution.

One extracellular technique that has alleviated these problems is the self-referencing microsensor technique. This sensor modality significantly improves signal-to-noise ratio and provides direct measurement of dynamic analyte flux, increasing spatial resolution with minimal sacrifice of temporal resolution [116]. While the use of microsensors in self-referencing modality has been around for decades [117], its potential for real-time physiological sensing has yet to be fully realized. The operation of sensors in self-referencing mode involves the oscillation of a single microsensor between two points, separated by a constant distance via computer-controlled stepper motors. Flux information can be obtained in real time by measuring Ag concentrations at each point, based on Fick's first law of diffusion. The combination of a dc-coupled amplification scheme and oscillatory movement of a single electrode produces significant noise filtration and an increase in signal-to-noise ratio [116].

While there are currently no microsensors for the direct detection of Ag NPs, other sensors can be employed for monitoring physiological responses to Ag NPs. For instance, Ag^+ can affect the uptake of H^+ and various ions (e.g., Na^+ and K^+), simple sugars (e.g., glucose) and metabolic analytes necessary for cellular growth and development [118]. Self-referencing microsensors can be used to monitor the real-time flux of glucose [119,120], glutamate [121], indoleacetic acid [122] and hydrogen peroxide. The latter case has been used to detect an increase in H_2O_2 efflux after exposing an excised, murine spinal cord to citrate-stabilized Ag NPs at 1 ppm (FIGURE 2). This release of H_2O_2 was most likely the result of oxidative stress induced by Ag NPs, in agreement with previous experiments [123,124]. While others have noted net increases in H_2O_2 efflux due to Ag NP exposure, the self-referencing microsensor technique provides a higher resolution of temporal

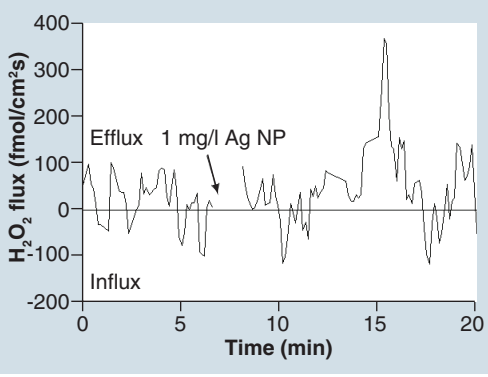

Figure 2. Real-time hydrogen peroxide efflux from a murine spinal cord segment exposed to 1 mg/l silver nanoparticles. The gap in the graph is a result of having to reposition the sample and probe after the addition of Ag NPs. A spike in efflux is visible approximately 7 min after the addition.
Ag NP: Silver nanoparticles.
Reproduced with permission from [Stensberg *et al.*, Unpublished Data].

and spatial data not obtainable with previous techniques. This high degree of resolution enables the use of metrics such as time to response, duration of response, peak efflux and total efflux, measured as integrated flux (Table 2) [122].

Amperometric sensors have also been used with enzymes that produce electroactive species generated from redox reactions. For example, glucose oxidase converts glucose into gluconic acid and H_2O_2, which can be measured by amperometry [125]. Enzyme-based biosensors can also be used in the self-referencing modality near cell/tissue surfaces and measure the effect of Ag NPs on physiological transport of various analytes.

Another type of microsensor that can be used in a self-referencing modality is the ion-selective electrode (ISE), which can detect a vast array of biologically relevant cations [125]. With regard to Ag NP and Ag^+ toxicity, self-referencing ISEs have been used to evaluate toxicity based on the aberrant flux of Na^+ and H^+ [118]. For example, H^+ efflux was measured from *Daphnia magna* embryos dosed with silver nitrate (Figure 3A) and Ag NPs (Figure 3B). Increases in time to response, peak response and total integrated flux were observed for Ag NP exposure when compared with embryos exposed to Ag^+ (Table 3).

A micro-ISE for Ag^+ has been recently developed and demonstrated in self-referencing mode, with detection limits below 100 nm [126]. The sensor can monitor the rate of Ag^+ uptake without interference from Ag NPs (Figure 4) and is thus particularly useful for separating the physiological effects of Ag^+ from those of Ag NPs. In particular, any physiological response that does not correlate directly with Ag^+ influx can be attributed specifically to Ag NP toxicity. The ability to noninvasively segregate the effects of Ag NPs and Ag^+ on physiological transport will be crucial for establishing a mode of action for Ag NPs.

Finally, optical microsensors have been used in a self-referencing modality [127,128]. These have some advantages over electrochemical sensors, as they are relatively unaffected by electrical or mechanical noise. The self-referencing optical microsensors have been used to measure real-time O_2 flux as a metric for physiological stress in fathead minnows, exposed to several environmental contaminants [129]. However, preliminary data on *Daphnia magna* embryos exposed to Ag NPs indicate little or no effect on O_2 consumption (Figure 5 & Table 4).

Biological imaging with Ag NPs

Silver nanoparticles exhibit a strong optical activity due to plasmon resonance, an electrodynamic phenomenon based on the excitation of conduction electrons at specific frequencies of light [12]. These plasmon resonances enhance the detection and tracking of Ag NPs by a number of optical imaging methods, from simple light scattering to multiphoton luminescence, surface-enhanced Raman scattering (SERS) and *in vivo* biomedical imaging modalities. With major exception to the darkfield imaging studies, most of these cases can be considered exploratory in their use of Ag NPs, but also demonstrate their potential utility in the design of *in vitro* and

Table 2. Summary of H_2O_2 efflux from exposure of murine spinal cord to 1 mg/l citrate stabilized silver nanoparticles.

Treatment	H_2O_2 iFlux (fmol cm^{-2})	Peak response (fmol $cm^{-2}s^{-1}$)	Time to response (min)	Duration of response (min)
Control (DMEM)	7907	N/A	N/A	N/A
1 mg/l Ag NPs	32381	364.2	5.2	5.7

iFlux values for the nanoparticle dose were much larger than the control value (control was integrated over the same time span as the duration of response) [122].
Ag NP: Silver nanoparticles; iFlux: Integrated flux.
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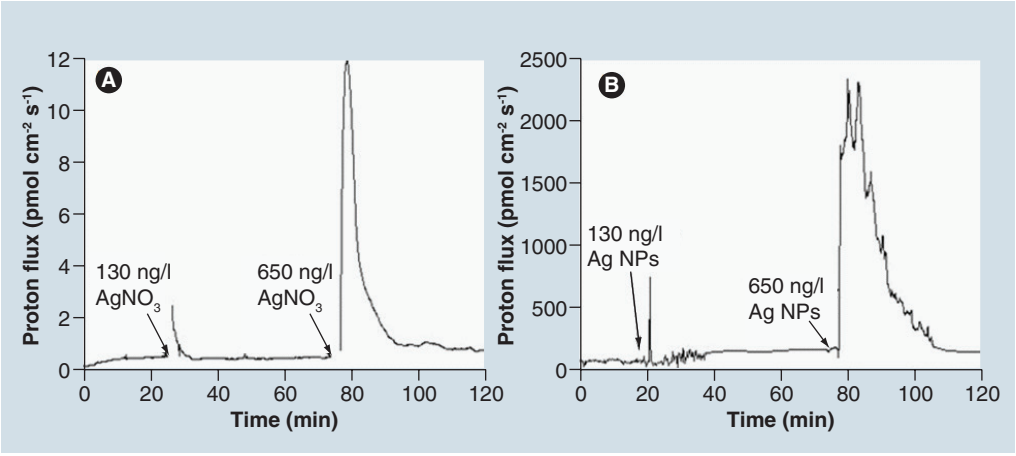

Figure 3. Proton efflux from *Daphnia magna* embryos when dosed with 130 and 650 ng/l (A) $AgNO_3$ and (B) silver nanoparticles. Note the differences in scale on the Y axis between the two graphs.
Ag NP: Silver nanoparticles.
Reproduced with permission from [Stensberg *et al.*, Unpublished Data].

in vivo studies that address downstream toxicological effects. Ultimately, the accumulation of toxicological data will determine the scope and limitations in developing Ag NPs as imaging agents for clinical use. On the other hand, these novel imaging tools present opportunities for tracing the various pathways and fates of Ag NPs in biological systems.

Ag NPs in optical darkfield microscopy

Colloidal Ag NPs are widely recognized as optical labels for biosensing and imaging applications based on light scattering [12]. Ag NPs below 50 nm typically support strong extinctions between 400 and 500 nm (blue–green region of the visible spectrum), although individual NPs as small as 2.6 nm can be detected under optimal conditions [130]. The plasmon resonances can shift toward longer wavelengths if the particles are larger than 50 nm or are anisotropic in shape [131]. This wavelength-dependent scattering enables Ag NPs to be distinguished according to their physical characteristics allowing independent tracking of NP uptake rate as a function of size or shape. Xu and coworkers have investigated the uptake of Ag-coated gold NPs by several different organisms using darkfield optical imaging [132]. In one such study involving *Pseudomonas aeruginosa*, an opportunistic bacterial pathogen, the active uptake and efflux of single NPs as large as 80 nm in diameter were monitored with transport times ranging from minutes to hours, depending on the particle size [10]. Differences in NP efflux activities could also be discerned between various strains of *Pseudomonas* and was attributed to the existence of yet-unidentified membrane pumps. Remarkably, the bacteria were proficient at excreting NPs of all sizes and their viabilities were unaffected by the low (picomolar) level of Ag-coated NPs used in this study.

Darkfield imaging has also been used to investigate the dynamics of Ag NP uptake by zebrafish embryos [11,132]. Early-stage embryos (8–64 cells) were exposed to unfunctionalized

Table 3. Summary of proton flux from exposure of *Daphnia magna* embryos to $AgNO_3$ and silver nanoparticles.

Treatment	H^+ iFlux (pmol cm^{-2})	Peak response (pmol cm^{-2} s^{-1})	Time to response (min)	Duration of response (min)
Control (hard water)	1015	N/A	N/A	N/A
130 ng/l $AgNO_3$	1313	2.4	<1	6.4
650 ng/l $AgNO_3$	5831	11.8	<1	17.3
130 ng/l Ag NPs	1552	698.4	3.4	2.1
650 ng/l Ag NPs	25,363	2452.6	3.3	27.4

iFlux values for the high nanoparticle dose was much larger than the comparable dose of $AgNO_3$ [122].
Ag NP: Silver nanoparticles; iFlux: Integrated flux.
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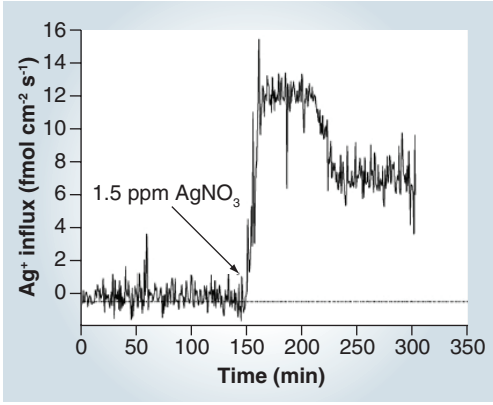

Figure 4. Real-time ionic silver flux measured at the surface of a *Pseudomonas aeruginosa* biofilm exposed to 9 µM (1.5 ppm) silver nitrate.
Reproduced with permission from [126].

Ag NPs of various sizes (mean diameter 11.6 nm) and at subnanomolar concentrations (10^{10} to 4×10^{11} NPs/ml). Single-particle tracking confirmed that most Ag NPs were sufficiently dispersed to diffuse freely into the embryo by passage through the chorion pore canals, followed by their penetration into the inner mass (Figure 6). Single-particle tracking also revealed changes in the diffusion coefficients of Ag NPs over time, attributable to local gradients in viscosity as the particles continued to permeate through the embryonic tissue. However, the passive transport of Ag NPs into the chorionic space could be affected by the clogging of pores caused by particle aggregation, as determined by the localized accumulation of NPs with a redshift in scattering. With respect to organismic toxicity, Ag NPs did not appear to have a detrimental effect on the development of embryos exposed to particle concentrations below 0.08 nM and Ag NPs could be found in all parts of the developed zebrafish embryo at 120 h postfertilization. However, embryos exposed to higher concentrations of Ag NPs experienced a high death rate, accompanied by multiple developmental malformations whose frequency of occurrence increased with Ag NP levels (Figure 7). It is worth noting that a complementary study using similarly sized Au NPs indicated far fewer adverse effects on embryonic development, meaning that the developmental abnormalities induced by Ag NPs are not simply due to particle size [133].

Darkfield optical imaging is equally useful for obtaining insights into the cytotoxic effects of Ag NPs in mammalian cells [134]. Unfunctionalized Ag NPs were found to inhibit the growth of L929 cells (derived from a murine fibrosarcoma) at a concentration of 0.46 nM (22 µg/ml). Darkfield microscopy indicated that the amount of Ag NPs increased in both the cytoplasm and nuclei over time, with the relative concentration several fold higher in the former versus the latter. Most cells exhibited abnormal morphologies after a 72 h exposure with either oversized nuclei or multiple nuclei, all of which contained higher quantities of DNA than cells with normal nuclei. This suggests that while Ag NPs may be directly responsible for inhibiting cytokinesis, they do not interfere with DNA replication.

Ag NPs as fluorescent & nonlinear optical labels

Recent advances in synthesis and optical analysis have enabled researchers to determine that Ag NPs can also be luminescent and provide excellent contrast under various types of fluorescence imaging. Small (<2 nm) Ag nanoclusters (NCs)

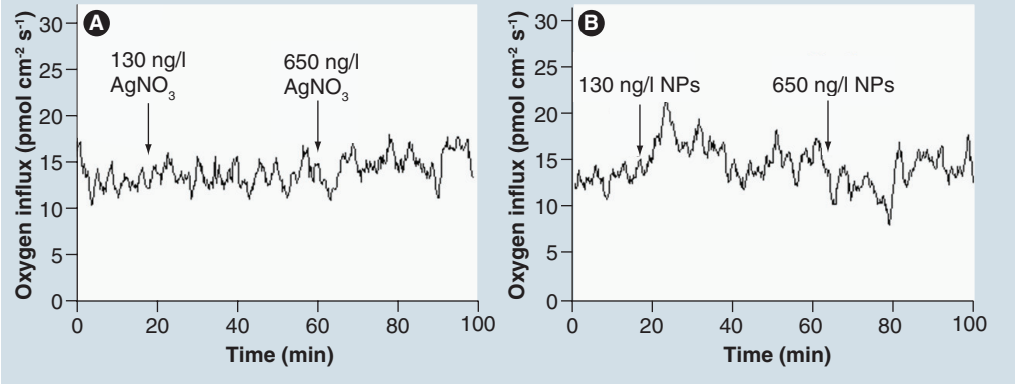

Figure 5. Proton efflux from *Daphnia magna* embryos when dosed with 130 and 650 ng/l (A) $AgNO_3$ and (B) silver nanoparticles. No notable differences were observed between the two treatments.
NP: Nanoparticle.
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Table 4. Summary of oxygen flux from exposure of *Daphnia magna* embryos to $AgNO_3$ and Ag NPs.

Treatment	O_2 iFlux (pmol cm^{-2})	Peak response (pmol cm^{-2} sec^{-1})
Control (hard water)	244	N/A
130 ng/l $AgNO_3$	265	16.1
650 ng/l $AgNO_3$	288	17.3
130 ng/l Ag NPs	265	22.1
650 ng/l Ag NPs	260	16.2

Integrated influx values (iFlux) were calculated over the same timespan at points before and after each dose. Time to response and duration of response values were not discernable [122].
Ag NP: Silver nanoparticles; iFlux: Integrated flux.
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have an intrinsic capacity to produce photoluminescence, similar to semicondutor quantum dot NPs, whereas colloidal Ag NPs can produce luminescence under two-photon excitation conditions at plasmon resonance. Ag NPs can also contribute to fluorescence imaging by providing a local electromagnetic field to increase the rate of emission of nearby fluorophores, a mechanism known as surface-enhanced fluorescence. These imaging modalities are readily applicable toward toxicological studies, although such studies have yet to be reported.

The luminescent properties of Ag NCs were first reported by Dickson and coworkers [135]. Ag NCs can be synthesized by several different methods, including biosynthesis in live cells, and for *in situ* fluorescence imaging studies [136], and are easily functionalized with biomolecular ligands. For example, Ag NCs have been synthesized in the presence of avidin-conjugated DNA, then used as fluorescent labels of biotinylated NIH 3T3 cells [137] (Figure 8A & 8B). The Ag NCs are well suited for *in vitro* fluorescence imaging and are superior to conventional dye molecules in both emission intensity and photostability. Variations in chemistry enable Ag NCs to be used as fluorescent labels for various cellular components, such as actin, microtubule filaments and specific surface proteins [138]. The emission wavelength of Ag NCs is highly size dependent and can even be tuned to near-infrared (NIR) wavelengths. For example, Ag NCs synthesized in the presence of oligocytosine DNA can be used as NIR-active biomarkers to monitor their transfection of live cells [139].

Colloidal Ag NPs are also capable of photoluminesence, although their quantum yields are much smaller than that of Ag NCs. Nevertheless, they can still be used as fluorescent labels if the excitation intensity is sufficiently high. For instance, 36-nm Ag NPs encapsulated in polymer shells have been used as drug delivery

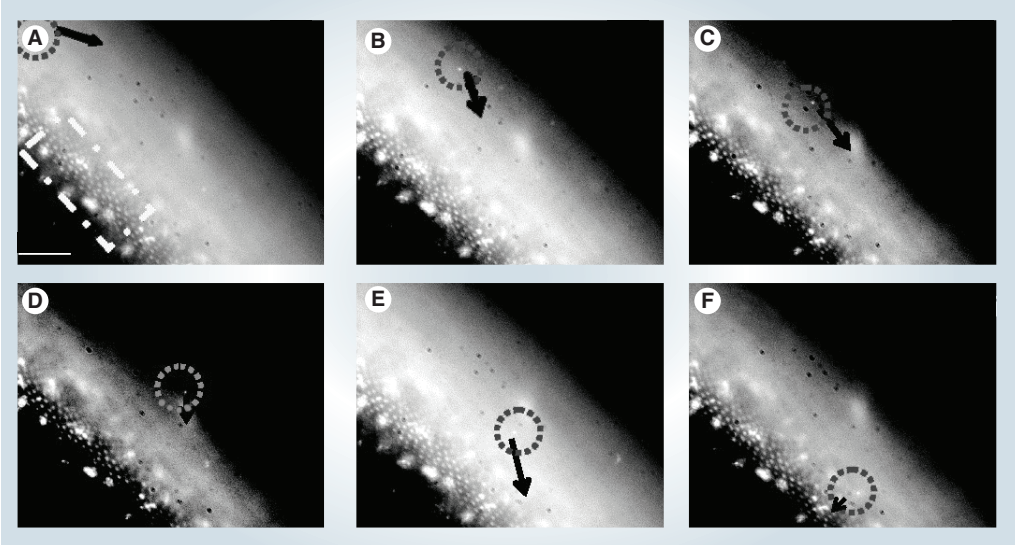

Figure 6. Single-particle tracking (A–F) of a silver nanoparticles (in dashed circle) toward the chorionic space of a zebrafish embryo, using optical darkfield microscopy. Rectangular outline in **(A)** includes chorionic pore channels; scale bar = 15 µm.
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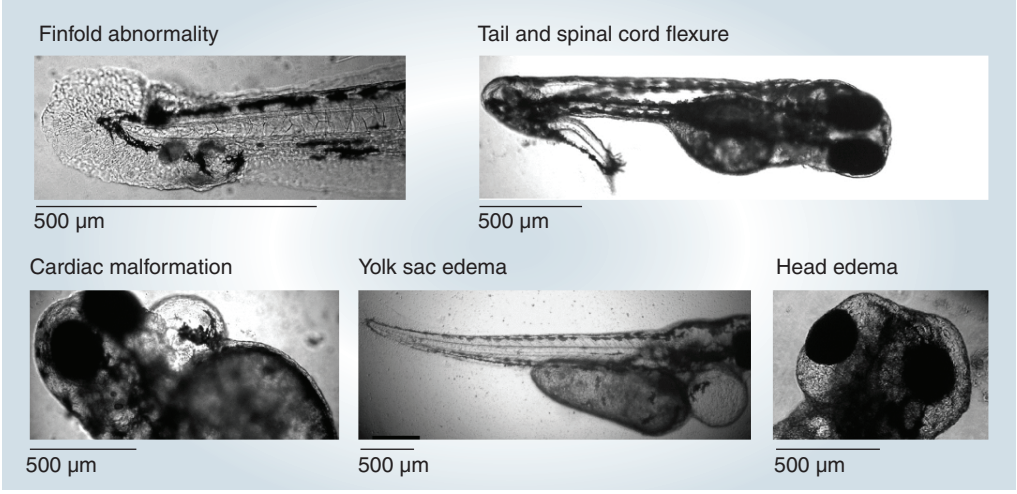

Figure 7. Selected images of zebrafish larvae with various developmental deformities.
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vehicles and tracked by fluorescence imaging inside of B16F10 cells [140]. Metallic nanoshells with 50-nm silica cores and 10-nm Ag shells have also been reported as fluorescent labels for detecting CXCR4 chemokine receptors on the surfaces of T lymphocytes [141].

Metal nanoparticles can also produce a two-photon excited luminescence (TPL) by ultrafast pulsed laser excitation. This has been particularly well studied in NIR-resonant NPs, such as Au nanorods [142], but the TPL activity of Ag NPs has also been reported with applications toward biological imaging [143,144]. For example, TPL has been used to monitor the nonspecific uptake of Ag–Fe_3O_4 NPs into macrophages (Figure 8C & 8D) [145]. Ag NPs as small as 10 nm could produce strong TPL signals with femtosecond NIR laser excitation, whereas the magnetic component allowed cells to be manipulated by external magnetic field gradients.

Third-harmonic generation (THG) is another nonlinear optical technique for imaging Ag NPs, one that is more efficient than TPL because it does not require excited states for energy conversion. Ag NPs are ideal contrast agents for THG owing to their large third-order susceptibility and the overlap of their plasmon resonance with the tripling of the NIR frequencies used to excite THG signals [146]. THG imaging has been applied toward *in vitro* cancer cell detection, using antibody-labeled Ag NPs incubated with mouse bladder carcinoma cells (Figure 9) [14]. While TPL and THG are excellent imaging tools for *in vitro* studies involving Ag NPs, their short working distances are a drawback for whole-animal imaging, so their application toward nanotoxicology is best served at the cellular level.

Surface-enhanced fluorescence & Raman imaging

Although colloidal Ag NPs are less efficient than Ag NCs as fluorescent markers, they can indirectly support fluorescence imaging by enhancing the emission rates of nearby dye molecules by a process termed surface-enhanced fluorescence. Ag NP–dye conjugates have been demonstrated as fluorescent probes for cellular imaging by conjugating fluorescently labeled lectins onto 20-nm Ag NPs, which were then incubated with HEK 293A cells (Figure 8E) [147,148]. Cells labeled with the Ag NP-coupled probes produced fluorescence signals 20–30-times brighter than those labeled with organic dyes alone (Figure 8F & 8G). Further studies demonstrated that the lifetime of the coupled Ag NP–dye is significantly modified compared with uncoupled dye molecules, which extends the application of Ag NP–dye conjugates to fluorescence lifetime imaging (Figure 8H & 8I).

The plasmon resonances of Ag NPs can also be applied toward imaging modalities based on SERS. Close-packed or aggregated metal NPs form electromagnetic 'hot spots' that can enhance Raman signals by several orders of magnitude, to the extent that their emissions are comparable to fluorescence. Bacteria adsorbed onto Ag nanostructures can be detected by their characteristic Raman vibrational spectra using SERS microscopy, with limits of detection as low as ten bacteria/ml [149,150]. SERS-active Ag NP 'tags' can also be prepared using appropriate surface chemistries, often through biophysical interaction with proteins or other biomolecular species. For instance, Ag NPs conjugated to ligands bearing cyano groups (C≡N) have been used to image membrane receptor clustering on

Hela cells through protein-mediated NP aggregation [151]. Alkyne- and carborane-functionalized Ag NPs can also produce large SERS signals and provide characteristic Raman signatures for antibody-targeted cell imaging [152,153]. SERS is even useful for detecting low-molecular-weight species and has been used to monitor adrenergic signaling to cardiac myocyte cells, which control

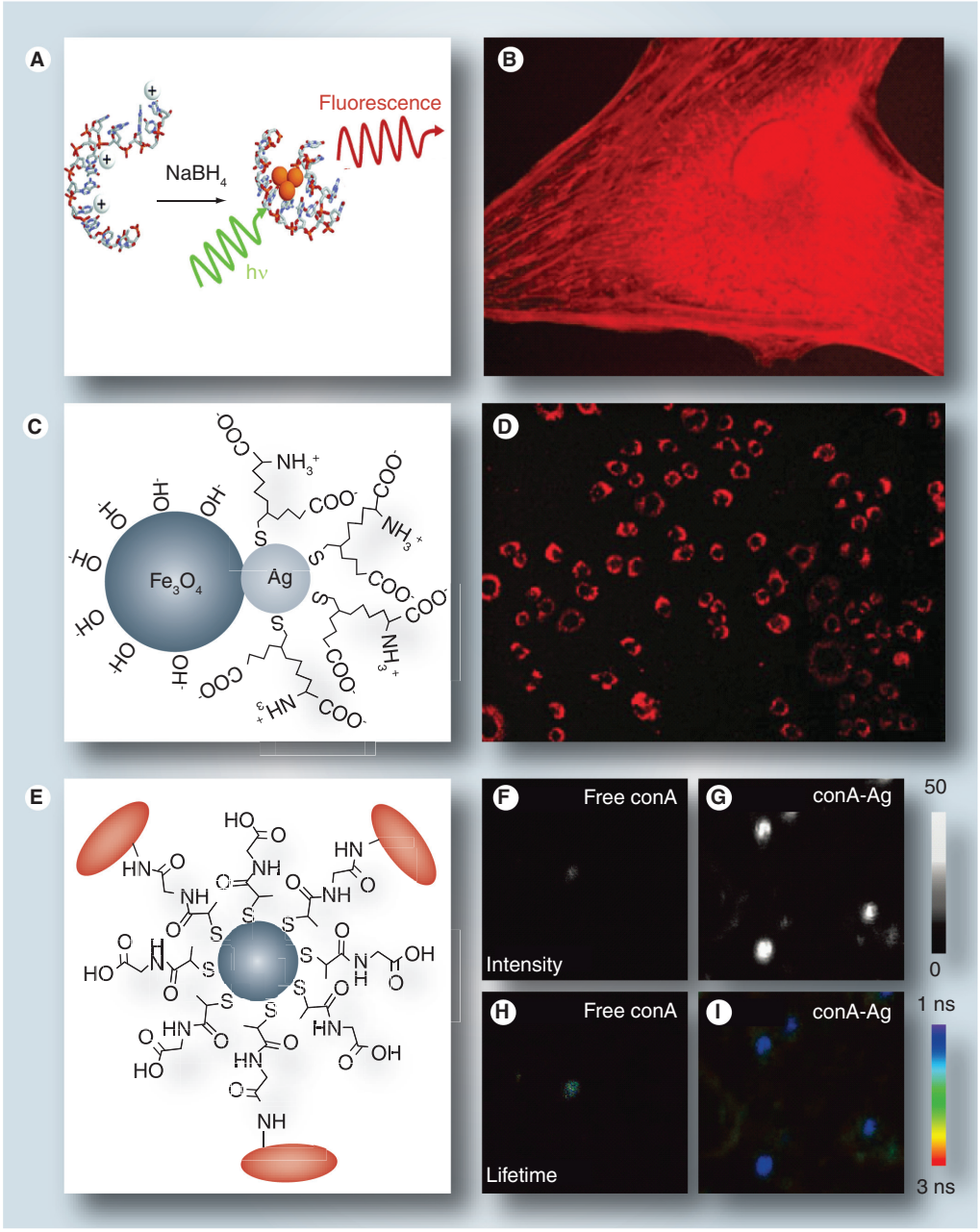

Figure 8. Silver nanoclusters and nanoparticles as fluorescent contrast agents. (A & B) linear fluorescence; **(C & D)** two-photon excited luminescence; **(E–I)** surface-enhanced fluorescence imaging. **(A)** Production of DNA-encapsulated silver nanoclusters (Ag NCs); **(B)** fluorescence imaging of live NIH 3T3 cells with anti-actin silver nanoclusters (Ag NCs); **(C & D)** Ag–Fe_3O_4 nanoparticle (NP) heterodimer as a contrast agent for two-photon excited luminescence imaging in macrophage cells; **(E)** plasmon-coupled Ag NP–dye probe; **(F & G)** demonstration of 20–30-fold enhancement in fluorescence intensity, in the presence of Ag NPs; **(H & I)** Ag NP-enhanced fluorescence lifetime imaging.
(A & B) Reproduced with permission from [137].
(C & D) Reproduced with permission from [145].
(F & G) Reproduced with permission from [147].
(H & I) Reproduced with permission from [148].

the contraction and subsequent beating of heart muscles [154]. This ability to track molecular signatures may prove useful for cellular toxicology studies involving Ag NPs, by correlating their presence with nearby stress-induced metabolites or biomarkers.

Ag NPs as contrast agents in biomedical imaging

Near-infrared-resonant Ag nanostructures, such as Ag nanoshells, have been investigated as optical contrast agents for photoacoustic imaging and other clinically relevant imaging modalities. Emelianov and coworkers proved the concept by injecting submicron Ag nanoshells with silica cores into porcine pancreas, with detection by multimodal ultrasound and photoacoustic imaging [155]. The Ag nanoshells not only increased photoacoustic contrast, but also enabled greater imaging depth into the tissue (FIGURE 10A & 10B).

Colloidal Ag NPs have also been examined as contrast agents for computed tomography, based on their large x-ray absorption coefficients. Dendrimer-stabilized Ag NPs (16 nm) were injected subcutaneously under mouse skin tissue and determined to attenuate x-ray transmission at levels comparable to iodine-based x-ray contrast agents used in clinical settings [156]. Ag NPs (12 nm) have also been functionalized with the radiotracer ^{125}I, followed by systemic administration in Balb/c mice for pharmacokinetic studies (FIGURE 10C & 10D) [157]. The *in vivo* biodistribution of these Ag NPs were evaluated by single-photon emission computerized tomography imaging, which indicated the spleen and liver as the primary organs of NP uptake 24 h after injection (41.5 and 24.5% ID/g, respectively). This study provides useful information about the near-term *in vivo* accumulation of Ag NPs, complementary to toxicity studies discussed earlier.

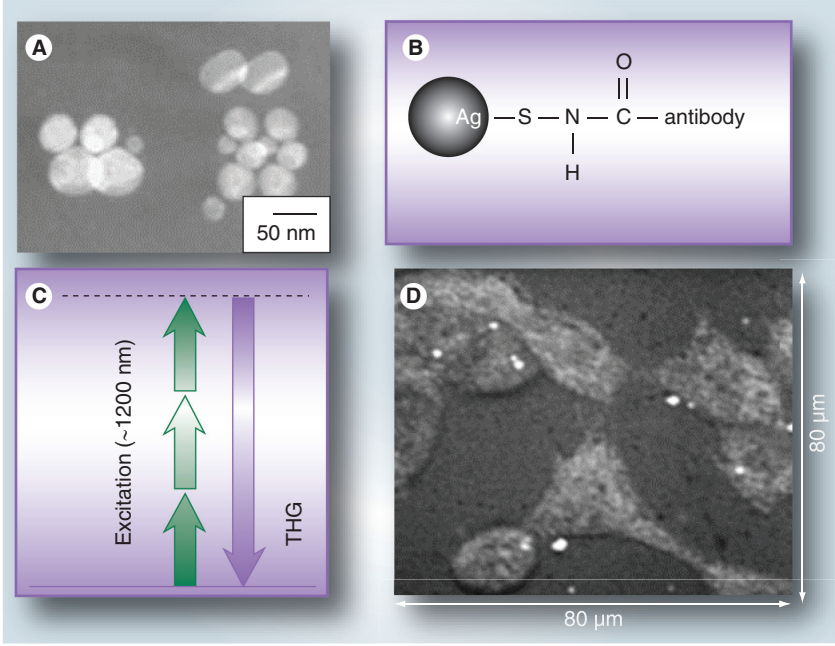

Figure 9. Silver nanoparticles used as contrast agents for third-harmonic generation imaging. (A) TEM images of silver nanoparticles (Ag NPs). **(B)** Ag NPs conjugated with anti-Her2 antibodies. **(C)** Transition state for THG. **(D)** THG image of mouse bladder carcinoma cells (MBT2) marked with antibody-labeled Ag NPs. THG: Third-harmonic generation.
Reproduced with permission from [14].

Conclusion

It is expected that the number of applications for Ag NPs will continue to grow, but there is still much that needs to be understood with respect to their fate and accumulation in the environment and their potential long-term effects on humans and other organisms. Recent studies have shown that the release of Ag NPs into the environment is increasing, yet there are large gaps in our understanding of how these particles are transported through ecosystems and migrate into the food chain and their consequences on human health.

At the cellular level, a variety of mechanisms of Ag NP toxicity have been reported, including ROS generation, DNA damage and cytokine induction during *in vitro* studies. The few *in vivo* studies that have been conducted so far indicate the potential for adverse effects at the organismic level, with vulnerabilities in the circulatory, respiratory, central nervous, hepatic and dermal systems. Many more studies are needed to determine the biodistribution and subsequent toxicity of Ag NPs using *in vivo* systems. These future studies should include modeling of the toxicological impact of Ag NPs leached from textiles, a major source of anthropogenic silver. The physicochemical properties of Ag NPs are also important factors and should be monitored during the course of a toxicological study to assess the effects of any physical changes on NP uptake and bioavailability.

While Ag NPs present some challenges for traditional toxicological assays, they also have unique qualities that enable entirely new approaches to examine their toxicological impact on cells and organisms. This includes the use of self-referencing microsensors for real-time physiological sensing and novel imaging modalities that take advantage of the strong plasmon resonances produced by Ag NPs, permitting their tracking in a label-free manner. These recently developed tools can easily be incorporated into experimental designs that will enhance the quality of risk assessment of Ag NPs.

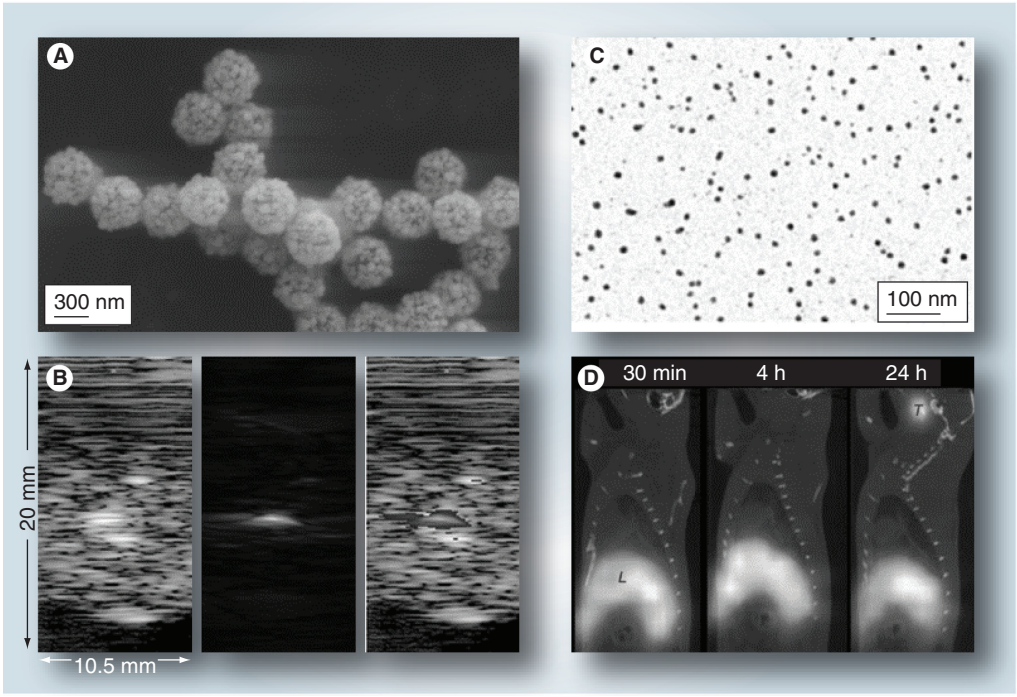

Figure 10. Silver nanoparticles as contrast agents in biomedical imaging. (A) Scanning electron microscope image of Ag nanoshells. **(B)** Ultrasound (left), photoacoustic (middle) and merged image of Ag nanoshells in porcine pancreas (right). **(C)** Transmission electron microscope image of ^{125}I-labeled 12-nm silver nanoparticles. **(D)** CT-SPECT images of ^{125}I-labeled Ag NPs in rats at different time points after intravenous administration.
(A) Reproduced with permission from [155].
(B) Reproduced with permission from [157].

Future perspective

The commercialization of Ag-enhanced products with antibacterial properties is increasing at an accelerated pace. The number of toxicological studies involving Ag NPs continues to grow as well, but most of these are performed *in vitro* on cell cultures, with lower-order lifeforms or with embryonic organisms. Toxicological assessments of Ag NPs on higher-order organisms lag far behind and may be limited by the availability of appropriate tools for *in vivo* characterization and assessment. The movement of Ag NPs from

Executive summary

Environmental exposure
- Silver nanoparticle (Ag NP)-enhanced commercial products have become a major source of environmental silver.
- Almost no data exists on the environmental concentrations of Ag NPs.

Mechanisms of toxicity
- Reported mechanisms of Ag NP toxicity include DNA toxicity, cytokine induction and oxidative stress.
- Some *in vivo* studies have reported Ag NP toxicity in respiratory, circulatory, central nervous, hepatic and dermal systems.

Nanoparticle characterization methods
- Nanoparticle characterization contributes toward our understanding of nanotoxicology, but does not define it.
- Standard NP characterization methods can be applied, including transmission electron microscopy, dynamic light scattering and zeta-potential measurements.

Physiological sensing
- More attention should be shifted to real-time quantitative analysis (e.g., self-referencing microsensors), which offer high spatial and temporal resolution.
- Electrochemical or optical self-referencing sensors can be used to monitor analyte fluxes that may be affected by Ag^+ or Ag NP exposure.

Nanoparticle-based imaging
- Ag NPs exhibit strong plasmon resonances at visible wavelengths and can support a wide variety of optical imaging modalities.
- Biomedical imaging modalities are highly useful for characterizing the *in vivo* biodistribution of Ag NPs, with high impact on toxicological studies.

consumer-related activities through the environment also remains poorly understood and is in need of more comprehensive studies.

One important goal for future nanotoxicology research is to establish better models to assess the long-term effects of Ag NPs in mammalian systems, thereby enabling the design of *in vivo* studies with definable end points. The recently developed imaging modalities described above are ideally suited for monitoring the *in vivo* transport and fate of Ag NPs and may be able to contribute toward new insights into toxicological mechanisms.

In closing, we hope that future discussions on the health and risk benefits of Ag NPs will be driven by sound scientific evidence, produced from carefully designed studies using the appropriate tools. Such studies are vital to ensure that the eventual regulation of Ag-enhanced products will be determined by facts rather than by alarm or ignorance.

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Toxic Effects of Silver Nanoparticles on Liver and Some Hematological Parameters in Male and Female Mice (*Mus musculus*)

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Abstract This research was carried out to evaluate toxic effects of nanosilver (Ag-NPs) on liver function and some blood parameters of male and female mice *Mus musculus*. A group of 54 BALB/c mice was randomly divided into three groups (each with two replications): Ag-NP (2) and control (1), each with nine mice. The experiment lasted for 14 days. In the treatment groups, two different doses of 20 and 50 ppm of Ag-NP solution were administered orally, while in the untreated (control) group, no Ag-NP solution but distilled water was used. At the end of the experiment, the serum was obtained by centrifugation of the whole blood at 3000 rpm for 15 min. The biochemical levels including alanine aminotransferase (ALT), aspartate amino transferase (AST), and blood cells were assayed by an automatic biochemical analyzer. Also, liver biopsy was performed and samples were stained using hematoxylin and eosin (H&E) staining. The values of red blood cells (RBC), hemoglobin (Hb), and hematocrit (Hct) did not vary significantly in the control and Ag-NP-treated animals. There were significant changes in the treatment and control groups in the levels of liver enzymes so that at both doses, there were significantly elevated levels of ALT and AST in mice treated with Ag-NPs compared with the control ($p<0.05$). Sexuality was not significantly involved in the results. Oral exposure to Ag-NPs produced changes in blood chemistry and hepatotoxicity as indicated by increased serum activity levels of both AST and ALT and histological damages to the liver with no significant changes between male and female mice.

Keywords Silver nanoparticles · Silver toxicity · Liver · Liver enzymes · ALT · AST

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Introduction

Despite the wide application of nanoparticles, there is a serious lack of information concerning their impact on human health and the environment [1]. Silver nanoparticles (Ag-NPs) are groups of silver atoms ranging in size, in at least one dimension (typically spherical diameter), from 1 to 100 nm. These nanoparticles have been considered as antibacterial made by human and could be used as an additive instead of antibiotics due to their antibacterial properties and their adaptability to biological systems [2–4].

In fact, nanomaterials are at the leading edge of the rapidly developing field of nanotechnology and their unique size-dependent properties make these materials superior and indispensable in many areas of human activity [5]. Recently, silver and silver nanoparticles (Ag-NPs) are widely being applied to consumer products and medical uses [6]. Ag-NPs are translocated into blood circulation and accumulated in some organs to cause hepatotoxicity when administered through oral, inhalation, or subcutaneously [7]. Liver appears to be a major accumulation site of circulatory silver nanoparticles [8]. In fact, silver nanoparticles have been shown to damage liver cells [9]. The toxic effect or heavy metal poisoning is defined as "any functional or morphologic change in the body

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produced by an ingested, injected, inhaled, or absorbed drug, chemical, or biological agent" [9].

Kim et al. [7] studied relation of histopathological responses and parameters of blood serum in poisonousness of nanoparticles and found significant changes in level of AST and ALT enzymes of blood serum. Pathological studies also prove damages to liver tissue, especially to hepatic lobules [7].

The first step for diagnosis of hepatic damage is simple blood test and then multiple biochemical tests [10]. Changes in enzyme activity of plasma are used as index of tissue damage, environmental stress, or disease position. An incresae in enzyme activity depends on the concentration of enzyme in cells, rate of leakage during damage, and pure natural path of enzyme from plasma. Changes in enzyme activity happened by increase or decrease of production of enzyme, natural path excretion, increase of the alter ability of cellular membrane, or disorder in blood flow [11].

The most important aspect to be considered for enzyme assays is determining activity of aminotransferase enzymes to indicate damage to hepatocytes or hepatic cells [12]. These enzymes usually are in hepatic cells but with damaging liver; these enzymes enter to blood flow [10–12]. Aminotransferase enzymes, i.e., ALT and AST, are used to evaluate damaging hepatic cells in rats, dogs, and nonhuman primates.

Our understanding of Ag-NPs in tissue deposition and related adverse effects is limited. In the current study, we therefore looked into Ag-NP tissue accumulation and toxicological impairments in mice exposed to Ag-NPs via oral administration. Many findings suggested that liver, among others, is the major organ for Ag-NP localization in mice. In addition, Kim et al. (2008) in Sprague-Dawley rats found that the kidneys showed a sex-dependent accumulation of silver, with a twofold higher accumulation in the female when compared with the male.

It must be noted that while the population exposed to silver nanoparticles continues to increase with ever new applications, silver nanoparticles remain a controversial research area as regards their toxicity to biological systems. In particular, the oral toxicity of silver nanoparticles is of particular concern to ensure public and consumer health. In addition, Kim et al. [7] studied oxidative stress-dependent toxicity of silver nanoparticles in human hepatoma and/or Cheraghi et al. [9] just investigated in vivo effect of silver nanoparticles. This study could be new in that it specifically addressed the toxic effects of nanosilver on liver and some blood parameters. Overall, the study aimed to investigate the toxic effects of Ag-NPs with two different doses on the liver function and some blood and electrolyte parameters in male and female mice (*Mus musculus*) when administered orally.

Materials and Methods

Mice Holding

Animal test was performed with compliance of the local ethics committee. A group of BALB/c mice of about 9 weeks (weighting 27.2±3.0 g) were purchased from Medical Faculty of Shahrekord University and then transferred to the laboratory. The animals were in a single group and maintained on commercial pellet diet, given deionized water ad libitum, and kept in plastic cages in a 20±2 °C, 50–70 % relative humidity room with a 12-h light/dark cycle. The photoperiod was provided by fluorescent tubes (Thorn, 36 W, white light), and all lighting was excluded during the scotophase. A timer was used to turn the lights on and off. After 2-week acclimation, the mice were randomly divided into three groups (each with two replications): the Ag-NP (2) and control (1) groups, each with nine mice. The animals were kept fasting overnight before treatment. The mice were examined daily for infections. Equal numbers of male and female mice were used in this study.

Preparation of Ag-NPs Silver nanoparticles (Ag-NPs) were purchased from Nano Pars Co., Iran with a purity of 95 %. The mean diameter of Ag-NPs averaged 40 nm (and ranged from 35 to 45 nm), according to the manufacturer.

Experiment Anesthesia for experimentation was achieved with an intramuscular injection of 10 ml ketamine, 0.5 ml acepromazine, 2 ml diazepam, and about 0.5 ml xylazine solution at a dose of 50 mg/kg. Therefore, once a day at the same time, a volume of 50 μl from the nanosilver solution (20 and 50 ppm) was administered orally at a given time. The untreated (control) group received distilled water without Ag-NPs. Each group of mouse was housed separately. The experiment lasted for 14 days. Samplings (n=9) were conducted on days 2, 7, and 14. The blood was obtained directly from heart using heparinized tubes. The serum was obtained by centrifugation of the whole blood at 3000 rpm for 15 min. Then, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using an ion autoanalyzer. In addition, a hematological autoanalyzer measured total red blood cell (RBC)

Table 1 The blood group differentiation in 50 ppm Ag-NP-treated (male only) and control groups

Groups	Mono	Lymph	Neut	Hb	RBC	WBC	PCV
Control	1.15±6.7	9.86±55.3	3.32±38	1.27±13.2	1±7	1.04±7.2	3.05±42.5
50 ppm	1.15±3.3	8.71±62	3.35±33.3	2.9±13	2.02±6.82	0.76±9.8	6.03±46.3

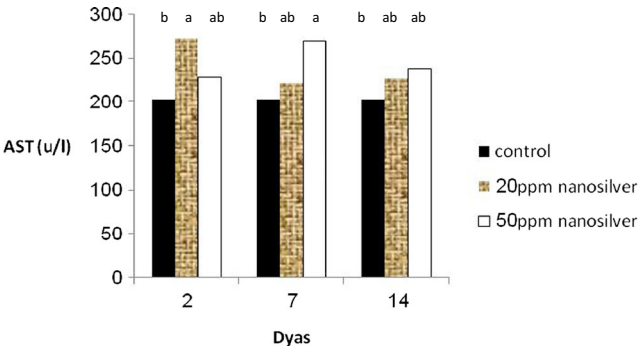

Fig. 1 Serum level activity of AST in male Ag-NP-treated and control groups during the experimental period of 14 days. Each test group was compared with control group. Means with the *same letter* are not significantly different (Duncan's test and $p<0.05$)

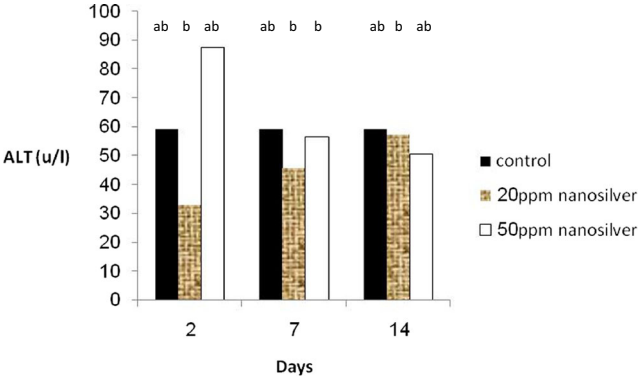

Fig. 3 Serum level activity of ALT in male Ag-NP-treated and control groups during the experimental period of 14 days. Each test group was compared with control group. Means with the *same letter* are not significantly different (Duncan's test and $p<0.05$)

count, hematocrit (Hct), hemoglobin (Hb) concentration, total white blood cell (WBC) count, and percent differential leukocytes.

Livers were taken from the mice and prefixed in 10 % (*v*/*v*) neutral buffered formalin for histopathological examination. The fixed tissues were trimmed, dehydrated, embedded in paraffin, sectioned and mounted on glass slides, stained with H&E, and examined by light microscopy.

Statistical analyses were performed using Student's paired *t* test, one-way ANOVA, and Duncan post hoc test. A *p* value of 0.05 was considered significant. The results showed the average value±standard deviation.

Results

The hematological and serum biochemical findings are shown in Table 1. The values of RBC, Hb, and Hct did not vary significantly in the control and Ag-NP-treated animals (Table 1). However, the total number of WBC in Ag-NP groups increased as compared to the control with a significant decrease in monocytes. Serum AST level in male and female mice with 20 and 50 ppm of nanosilver showed statistical increase compared to control (Figs. 1 and 2). Except for day 2 in which ALT level in male mice (50 ppm of nanosilver) elevated remarkably to control, trend of ALT level with a 50-ppm nanosilver was decreasing but it was increasing with a 20-ppm. (Figs. 3 and 4). Level of ALT in female mice again was increasing (with a 20-ppm), but after a sharp fall at day 7, it was increasing at day 14 when using 50 ppm of nanosilver.

Histological studies of liver organ are seen in Fig. 5. There were vast damages to the liver tissue in both sexes which increased with time such as the necrosis, hepatocytic inflammation, and resultant aggregation of lymphocytes in liver tissue. In fact, all treated mice exposed to silver nanoparticle had minimal to moderate lymphocyte aggregation in hepatic area. More severe changes were observed in mice exposed to 50 ppm than to 20 ppm nanosilver. Histological findings (as pie diagrams) regarding the liver damage in both sexes and with two doses of

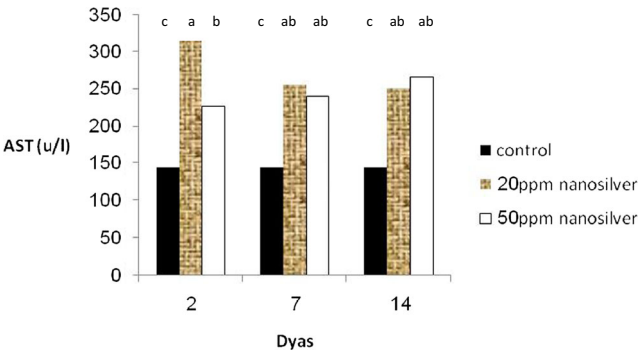

Fig. 2 Serum level activity of AST in female Ag-NP-treated and control groups during the experimental period of 14 days. Each test group was compared with control group. Means with the *same letter* are not significantly different (Duncan's test and $p<0.05$)

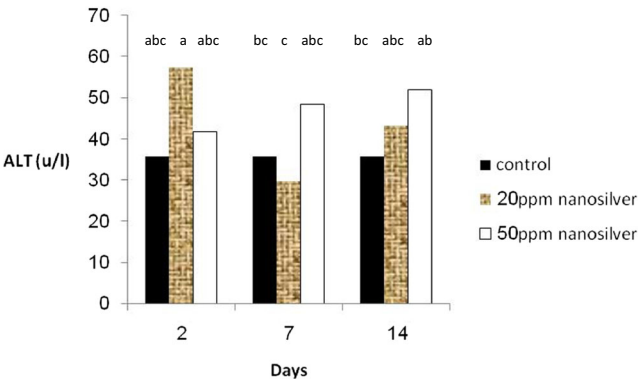

Fig 4 Serum level activity of ALT in female Ag-NP-treated and control groups during the experimental period of 14 days. Each test group was compared with control group. Means with the *same letter* are not significantly different (Duncan's test and $p<0.05$)

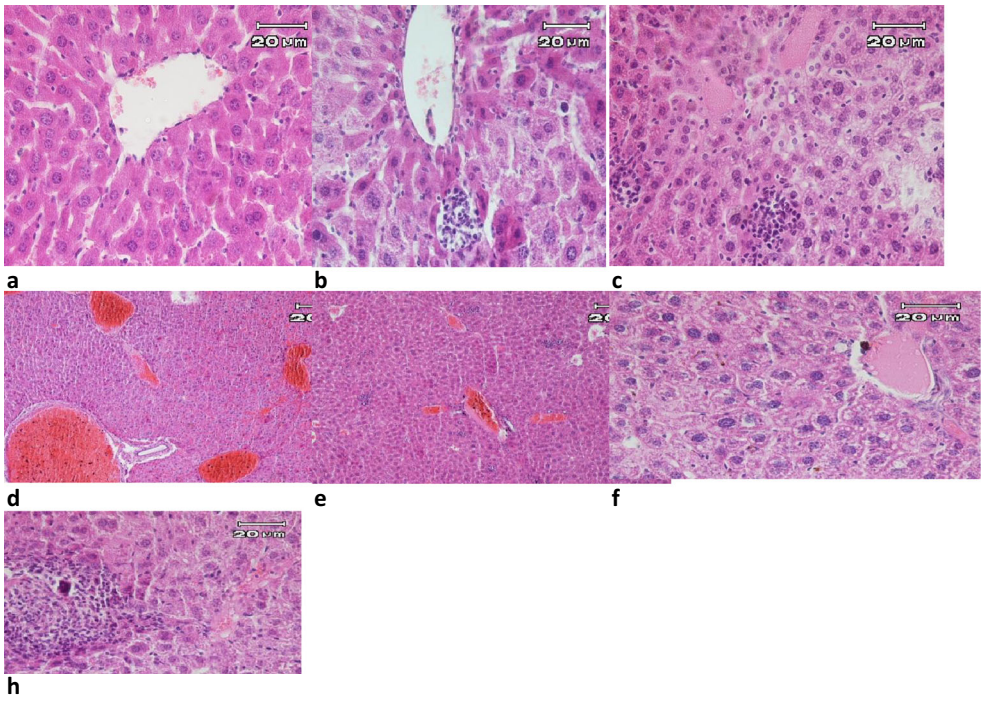

Fig. 5 Histological changes in mice liver after oral administration of Ag-NPs. **a** Normal tissue. **b–h** Treatment groups. **b** Day 2, the hepatic cells with a generalized cytoplasmic granulation. **c**, Day 2, aquatic granular degeneration of hepatic cells. **d** Day 7, portal vein congestion with high blood cells. **e** Day 7, inflammation of hepatic sinusoids. **f** Day 14, cytoplasmic vacuolation of hepatocytes with necrosis. **h** Day 14, inflammation, necrosis, and degeneration of hepatic cells

Ag-NPs (20 and 50 ppm) are shown in Figs. 6, 7, 8, and 9. The histopathological examinations of the male and female mice livers revealed that while there was a dose-dependent deposition of silver nanoparticles, the effect of the silver nanoparticles on male was more prominent than female.

Discussion

Nowadays, nanotechnology had rapid progress with the most effect on all parts of human, animal, and environmental and industrial life. The use of nanoparticles (NPs) in industrial and biomedical applications has increased significantly in recent years, yet their toxic effects have not been studied extensively [1, 3, 5, 17]. The results of the current study showed that silver nanoparticles was predominantly localized in liver in both sexes of mice, and this accumulation of nanoparticles in livers caused remarkable hepatic toxicity.

Many studies have demonstrated that exposure of silver nanoparticles may lead to clear accumulation in various organs including liver, as well as the kidneys, testes, lungs, and brain [4–8, 18]. For instance, accumulation of silver nanoparticles in the liver has been shown to induce hepatotoxicity in animal studies [18]. Studies have indicated that nanosilver has a strong toxicological effect in the range concentrations of 10–50 ppm (e.g., [19, 20]).Nanosilver enters the body through the skin, respiratory system and gastrointestinal tract. The most important way to contact it, especially in the gastrointestinal tract, is in colloidal form [13]. No consensus on the cytotoxicity of nanosilver has been reported; however, there is always reduced cell viability following exposure. As liver organ is able to actively remove compounds from the blood and transform them to chemical forms that can easily be excreted [9], so, silver nanoparticles might have impacted on the liver, as a major organ of detoxification. The hepatocytic inflammation in liver tissue of the current study is consistent with Lee et al. [18] study on rat liver following nanosilver administration, so

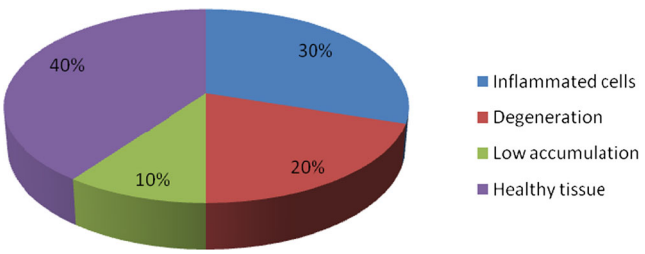

Fig. 6 Histopathological findings in the liver of Ag-NP-treated male (20 ppm), showing the percentage of liver damage in mice treated with nanosilver particles

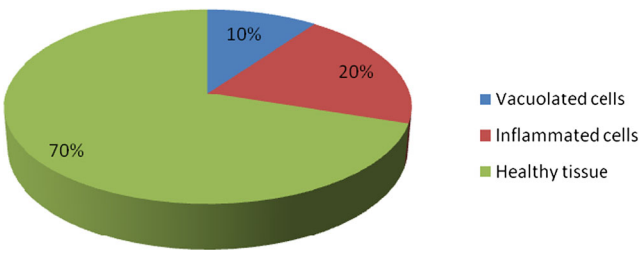

Fig. 7 Histopathological findings in the liver of Ag-NP-treated female (20 ppm), showing the percentage of liver damage in mice treated with nanosilver particles

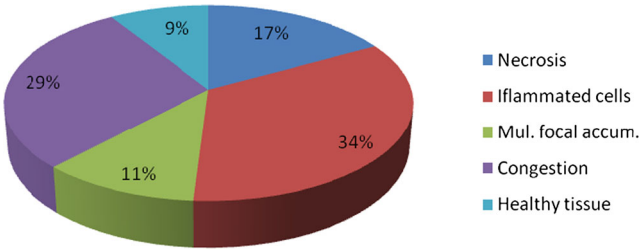

Fig. 8 Histopathological findings in the liver of Ag-NP-treated male (50 ppm), showing the percentage of liver damage in mice treated with nanosilver particles

that hepatocytes exhibited mild infiltration of inflammatory cells in portal vein area. Due to the accumulation of nanosilver in macrophages (Kupffer cells), they then concluded that Kupffer cells were involved in the process of inflammation following nanosilver exposure.

Hepatic function is evaluated by measuring AST and ALT. In the other words, liver damage induced by nanosilver particles of the present investigation physiologically affected AST (in particular) and also ALT in male and female. Increase serum AST and ALT levels indicated that liver tissues were damaged. These were confirmed by histological microscopy and by some other studies. For instance, in a histological analysis reported by Gatti et al. [14], inorganic particles, heterogeneous in nature but homogeneous in size, were identified in the liver. The results of our investigation are consistent with other studies (e.g., Cheraghi et al. [9] with nanosilver on these enzymes showing elevation of hepatic enzymes so that AST level in serum was elevated in male and female mice as compared to the control. On the other hand, the observed increase in ALT in the current study may be due to the free radicals released from the nanosilver particles when attacking hepatocytes and releasing ALT stored in them and entering into the blood serum [9–12]. Similarly, the increased level of WBC in the present study may follow phagocytosis of silver nanosilver [8, 9, 12]. The immune response of rats to an external factor has been the increase of the number of white blood cells for phagocytosis of nanosilver particles [9], whereas mice effects of nanosilver particles have been evaluated at different doses on serum. In this study, the level of ratio of WBC components changed which is in accordance with other studies. In fact, silver nanoparticles can lead changes in lymphocytes/granulocytes ratios so that the lymphocyte/granulocyte ratio may change sharply [15, 16]. Finally, while this study achieved a dose-dependent effect of the silver nanoparticles, the gender-related difference between the male and female mice livers has not been previously reported. Likewise, Kim et al. [7] found the gender-related distribution of silver nanoparticles in the rat kidneys (but not in the liver).

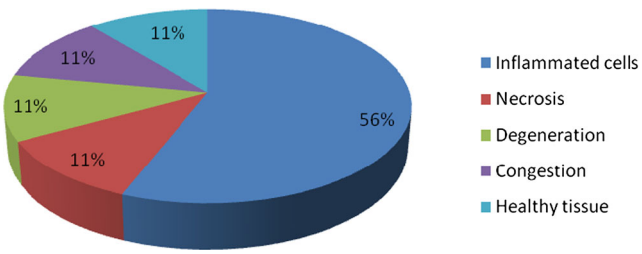

Fig. 9 Histopathological findings in the liver of Ag-NP-treated female (50 ppm), showing the percentage of liver damage in mice treated with nanosilver particles

Conclusions

From the study, it was concluded that the oral exposure to silver nanoparticles produced changes in blood chemistry and hepatotoxicity as indicated by increased serum activity levels of both AST and ALT and histological damages to the liver with no significant changes between male and female mice.

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Conflict of interests The authors declare that they have no competing interests.

Authors' contributions MSH carried out the preliminary studies, established the methods, and drafted the manuscript. He performed experiments and drafted the manuscript. RJS and SA collected data, performed experiments, and provided substantial input in data interpretation and analysis. All authors gave final approval to the version to be published.

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بررسی سمیت دوزهای مختلف نانو ذرات نقره بر بافت ریه به صورت خوراکی در موش صحرایی نر

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چکیده

مقدمه: نانوتکنولوژی به فناوری نانو اطلاق می‌شود که ابعادی در حدود ۱ تا ۱۰۰ نانومتر دارند. در تحقیقات نشان داده شده است که ترکیب، شکل و اندازه‌های مختلف نانو نقره به آن ویژگی‌هایی می‌دهد که در مقایسه با مواد شیمیایی با ترکیب مشابه اما درشت‌تر(میکرونقره)، اثرات سمی متفاوتی دارد. افزایش تقسیمات سلولی، استرس اکسیداتیو وآپوپتوز یا مرگ سلولی با اثرات سمی نانو نقره در ارتباط است. بنابراین هدف این مطالعه بررسی اثرات سمی نانو نقره بر بافت ریه می‌باشد.

روش بررسی: در این مطالعه تجربی ۵۰ سر موش صحرایی نر بالغ از نژاد ویستار(پنج گروه ده تایی) در گروه‌های تجربی و کنترل مورد بررسی قرار گرفتند. نانو ذره نقره ۷۰ نانومتر با مقادیر ۰/۲۵، ۰/۵، ۱ و ۲ میلی‌گرم بر کیلوگرم به صورت خوراکی به مدت ۳۰ روز داده شد. برای بررسی تغییرات پاتولوژیکی پس از ۳۰ روز بافت ریه موش‌های هر گروه را جدا و برای آزمایشات هیستوپاتولوژی در فرمالین نگهداری و پس از قالب و برش‌گیری، نمونه‌ها به روش هماتوکسیلین-ائوزین رنگ‌آمیزی شدند. سپس با میکروسکوپ نوری اینورت مشاهده و بررسی گردید.

نتایج: با توجه به نتایج گروه‌های با مصرف دوز بالا(N4 ,N5) نسبت به دیگر گروه‌ها(N2 ,N3) و گروه شاهد اثرات پاتولوژیکی(خونریزی، آپوپتوزو نکروز) بیشتری را نشان دادند. به طور کلی می‌توان گفت که اثر نانو ذره نقره بر بافت ریه قابل ملاحظه بود و تغییرهای حاصله نشانگر آسیب‌های سلولی و بافتی می‌باشد.

نتیجه‌گیری: سمیت نانو ذرات نقره که به صورت خوراکی به مدت ۳۰ روز در گروه‌های تجربی مذکور مورد بررسی قرار گرفت، اثرات مضری بر بافت ریه گذاشت که توسط آزمایش‌های پاتولوژیکی تعیین گردید.

واژه‌های کلیدی: نقره، نانو ذرات، ریه، سمیت، رت

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مقدمه

فناوری نانو، واژه‌ای است کلی که به تمام فناوری‌های پیشرفته در عرصه کار با مقیاس نانو اطلاق می‌شود. منظور از مقیاس نانو، ابعادی در حدود ۱ تا ۱۰۰ نانومتر می‌باشد. گسترش فناوری نانو در سطح جهانی و استفاده روز افزون از تولیدات حاصل از این فناوری، با توجه به کاربردهای فراوان نانو مواد در کاهش عفونت میکروبی پوست و زخم‌های سوختگی، همچنین برای جلوگیری از تجمع باکتری برسطح ابزار مختلف مثل پروتزها، مورد استفاده قرار گرفتند(۱). نانو ذرات ممکن است از مسیرهای متفاوت وارد بدن شوند و این موضوع تعیین خطرات مربوط به هر ماده را با دشواری روبرو می‌کند. اغلب تحقیقات برای ارزیابی تأثیرات سمی نانو ذرات، در سیستم تنفسی گزارش شده است، اما مسیر ورودی معدی روده‌ای هم به تحقیقات بیشتر نیاز دارد، زیرا نانو ذرات می‌توانند مستقیماً از طریق آب، غذا، مواد آرایشی، داروها، وسایل انتقال دارو و غیره وارد سیستم گوارش شوند(۲). افزایش ذراتی با اندازه‌های مختلف در سیستم گوارش می‌تواند منجر به تأثیرات سمی گوناگون گردد(۳).

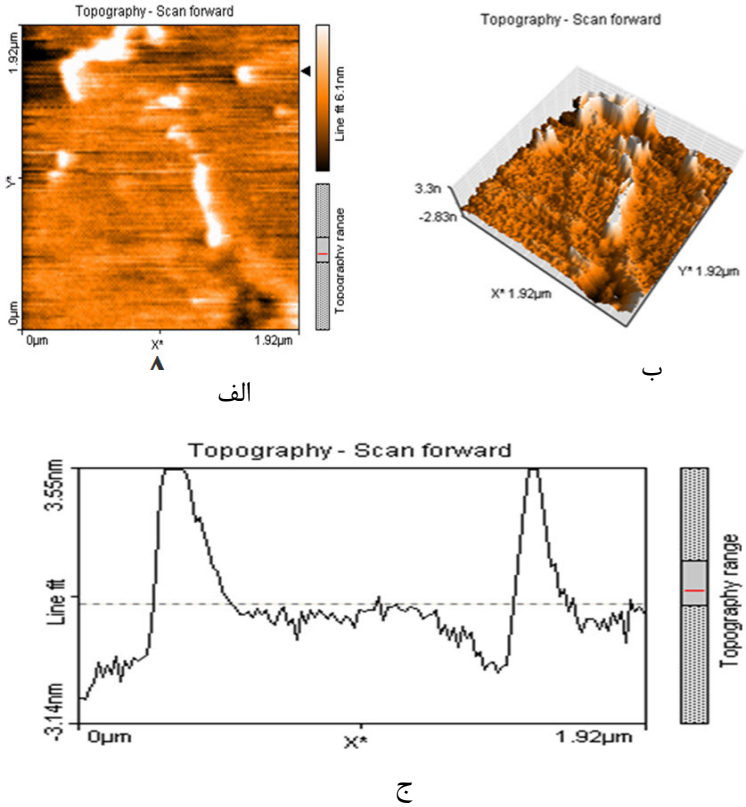
بعضی گزارشات در زمینه‌های پزشکی و بیولوژیکی ثابت کرده‌اند که بسیاری از وسایل پزشکی نقره‌دار، یون‌های نقره آزاد می‌کنند که وارد خون و در کبد، کلیه،ریه و مغز انباشته شده و باعث سمی شدن آنها و در نهایت منجر به مرگ می‌شود(۴) بنابراین نانو ذرات نقره ممکن است اثرات سمی داشته باشند که مکانیسم سمیت آنها روشن نیست(۵) و نگرانی‌های زیادی را در ارتباط با استفاده در طبیعت برای سلامتی انسان‌ها به وجود آورده است(۱). در نانو مواد اندازه هر ذره بسیار با اهمیت است که نقش کلیدی در تعیین ویژگی‌های نهایی نانو ذره دارد. اندازه ذره می‌تواند ویژگی‌های فیزیکی- شیمیایی نانو ماده را تغییر دهد و احتمال جذب و تعامل با بافت‌های بیولوژیکی را افزایش دهد(۶،۵). در حقیقت اندازه ذره در مقایسه با دیگر ویژگی‌های نانو مواد بیشتر مورد بررسی قرار گرفته است(۷). امروزه نتیجه بررسی‌ها نشان می‌دهد که فعالیت بیولوژیکی نانو مواد با کاهش اندازه ذره افزایش می‌یابد(۸،۷). قابل توجه‌ترین روش قرار گرفتن در معرض نانو ذرات، روش تنفسی است، در حالی که مصرف خوراکی و پوست، عمدتاً از دیگر مسیرهای ورود به بدن می‌باشد. تنفس عمیق نانو ذرات، ممکن است سبب فرار این ذرات از فاگوسیت‌ها و غشاهای سلولی ریه شده و به دیگر بخش‌های بدن انتشار یافته و باعث اثرات سیستماتیک می‌گردد(۶). ریه، اندام هدف برای ذرات آلاینده هوا است و ارتباط بین افزایش آلودگی هوا و اثرات مضر برای سلامتی در کودکان، مبتلایان آسم و سالخوردگان ثابت شده است(۹). جذب نانوذرات اکسید سریوم(۵۰-۲۵ نانومتر و ۵۰۰-۲۵۰ نانومتر) در فیبروبلاست‌های ریوی انسانی در محیط آزمایشگاهی مورد بررسی قرارگرفت(۱۰۰نانوگرم بر گرم تا ۱۰۰ میکروگرم بر گرم). Limbach و همکاران جذب نانو ذرات را توسط فیبروبلاست‌ها مشاهده کردند(۱۰). در آزمایش تنفس موش‌ها، افزایش قابل توجهی را در علایم یا شاخص‌های التهابی در طول ورود ذرات ۲۰ نانومتری اکسید تیتانیوم در مقایسه با همان جرم از ذرات ۲۵۰ نانومتری، مشاهده کردند. این نتایج نشان می‌دهند که اگر ذرات در مقیاس نانو قرارگیرند از لحاظ بیولوژیکی فعال می‌شوند(۲). Zhang و همکاران، همچنین Ostiguy و همکاران گزارش دادند که شکل‌های نانوذره ۵۰ نانومتر اکسید تیتانیوم، اکسید آلومینیوم و کربن سیاه، شاخص‌های التهاب تنفسی را افزایش می‌دهند(۱۱،۱۲). در مجله سرطان ریه، متذکر می‌شود که ذرات ناپایداری مثل کربن سیاه و اکسید تیتانیوم، سبب بروز فیبروز، ضایعات نئوپلازی و تومورهای ریوی در موش‌ها می‌شوند. مقدار لازم این ذرات برای ایجاد این تأثیرات، در نانو ذرات بسیار کمتر می‌باشد. منطقی است فرض کنیم که مکانیسم‌های مولکولی مختلفی ممکن است در ایجاد بیماری‌های قلبی- عروقی و ریوی نقش داشته باشند. تحقیقات آزمایشگاهی با قاطعیت ثابت کرده‌اند که قرار گرفتن در معرض نانو ذرات، باعث التهاب ریه می‌شود که به اندازه ذره، ویژگی‌های شیمیایی و ویژگی‌های سطح بستگی دارد(۱۳). در این تحقیق سمیت نانو ذرات نقره در دوزهای مختلف در بافت ریه موش‌های صحرایی نر پس از ۳۰ روز مصرف خوراکی مورد ارزیابی قرار گرفت.

روش بررسی

به منظور انجام این مطالعه تجربی، از ۵۰ سر رت(موش

صحرایی) نر بالغ(سن ۸ هفته و وزن ۲۵۰-۲۰۰گرم) از نژاد ویستار استفاده شد که از دانشگاه علوم پزشکی شهید صدوقی یزد خریداری و به ۵ گروه ۱۰ تایی N1, N2, N3, N4, N5 تقسیم شدند. به گروه کنترل N1 سرم فیزیولوژی داده شد و ۴ گروه تجربی دیگر N2-N5 در شرایط کنترل شده درجه حرارت ۲۲±۱°c، رطوبت حدود۱۰±۶۰٪، نور ۱۲ ساعت روشنایی و ۱۲ ساعت تاریکی با دسترسی آسان به آب و غذای کامل طبق ضوابط قانون نگهداری از حیوانات آزمایشگاهی نگهداری شدند.

نانو ذرات نقره(۷۰ نانومتر) سنتز شده(شکل۱) از پژوهشکده علوم و فناوری نانو دانشگاه پیام نور استان یزد تهیه و مساحت سطح نانو بوسیله میکروسکوب AFM این مرکز جهت استفاده در این پژوهش آنالیز شد و سپس هر ۲۴ ساعت ۱ میلی‌لیتر از سوسپانسیون نانو ذرات نقره با دوزهای متفاوت(۱، ۲، ۰/۵، ۰/۲۵ میلی گرم بر میکروگرم) بوسیله گاواژ به مدت ۳۰ روز به موش‌ها خورانده شد(۴).



شکل ۱: تصویر نانو ذره نقره ۷۰ مورد استفاده در این آزمایش توسط میکروسکوپ AFM
الف: تصویر دو بعدی- ب: تصویر سه بعدی- ج: نمودار ارتفاع و سطح نانو ذره

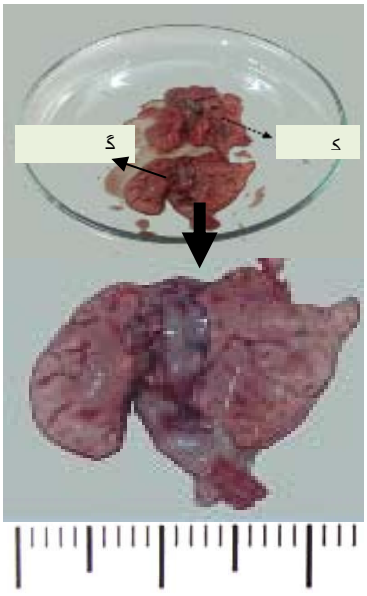
پس از ۳۰ روز موش‌ها توسط اتر بیهوش و کالبد شکافی شدند. ریه موش‌ها با دقت و ظرافت از بدن جدا و با سرم فیزیولوژی شسته سپس از نظر مورفولوژی مورد بررسی قرار گرفته شد. سپس اندام مورد نظر در داخل فرمالین ۱۰٪ ثابت و برای آزمایشات هیستوپاتولوژی نگهداری شد. بافت‌های نمونه پس از پاساژ دادن توسط دستگاه Tissue Prosecing مرکز تحقیقات و ناباروری استان یزد در قالب‌های لوکهارت به وسیله پارافین قالب‌گیری شده و سپس توسط دستگاه میکروتوم برش تهیه نموده و به روش رنگ آمیزی هماتوکسیلین- ائوزین و با استفاده از میکروسکوپ اینورت مشاهده و عکس‌هایی گرفته شد. سپس برای شناسایی ویژگی‌های بافت مورد نظر به پاتولوژیست واگذار گردید.

نتایج

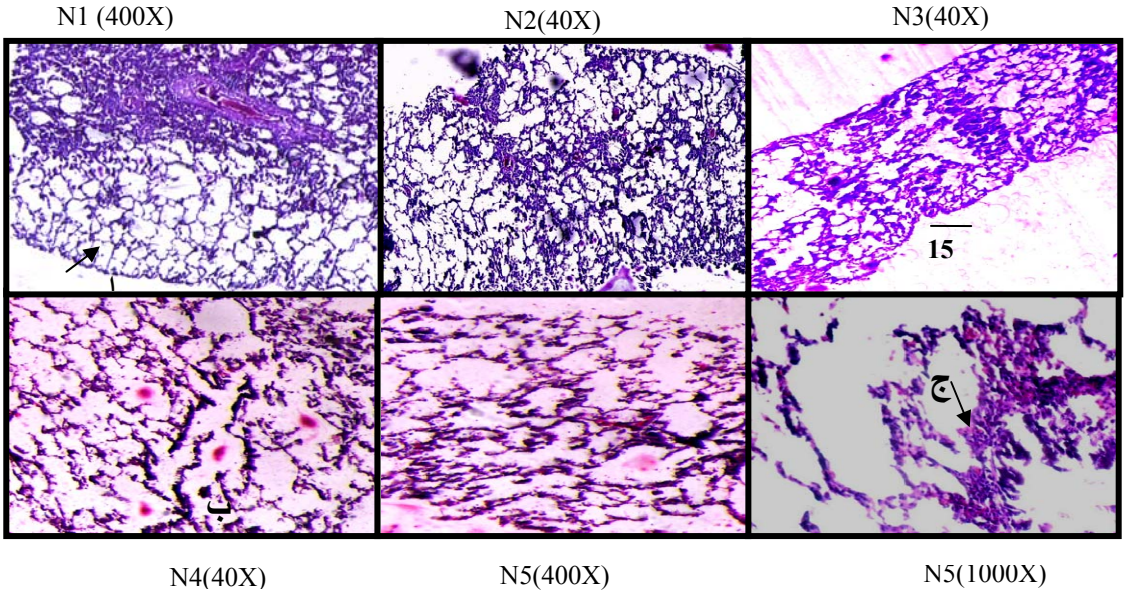
بر اساس بررسی‌های انجام شده برروی بافت ریه موش صحرایی

نر که به مدت ۳۰ روز بصورت خوراکی که در معرض نانو ذره نفره ۷۰ نانومتر بودند نشان داد که در گروه‌های مختلف اثرات پاتولوژیکی متفاوتی داشته است. در نمونه‌های بافت ریه‌ی گروه‌های N2 و N3 در مقایسه با گروه N1(گروه کنترل) تغییرات هیستوپاتولوژیکی مشاهده نگردید، در حالی که در گروه‌های N4 و N5 تغییرات قابل توجهی شامل خونریزی وجود پروتئن و نکروز بافتی مشاهده شدند(شکل ۳-ج و د).

تغییرات مورفولوژیکی در گروه‌های تجربی(N4 و N5) نسبت به گروه کنترل(N1) بافت ریه بی رنگ و حجیم‌تر بود که از نشانه‌های آمفیزم ریه است(شکل۲-ب). بررسی پاتولوژیکی آمفیزم ریه که خونریزی و رسوب پروتئین(در فضای بین بافتی) و افزایش پنوموسیت‌ها در آلوئول‌های گروه‌های N4 و N5 که دوزهای بالا ۱ و ۲ میلی‌گرم بر کیلوگرم دریافت کرده بودند به مراتب بیشتر دیده شد(شکل۳). وجود این نوع ضایعات هیستوپاتولوژیک در بافت مورد بررسی در این تحقیق می‌تواند نشان دهنده وجود یک عامل توکسیک در بدن باشد.



شکل ۲: تغییرات مرفولوژیکی در بافت ریه رات



شکل۳: تغییرات پاتولوژیکی در بافت ریه موش صحرایی
الف: آلوئول، ب: پروتئین، ج: گلبول قرمز

بحث و نتیجه گیری

اگرچه نانوذرات نقره به صورت گسترده در محصولات مصرفی بکار می‌رود، اطلاعات سم شناسی کافی در مورد آنها در دسترس نمی‌باشد. مسیرهای ورود نانو ذرات به بدن(تنفسی، دهانی و پوستی) و انتقال آنها به دلیل اندازه کوچک نانو ذرات در مقاله‌های فراوان مورد بحث قرار گرفته است(۲،۵،۱۴). در آزمایشات انجام شده در ۳ سایز مختلف نانو ذره نقره ۲۲، ۷۱ و ۳۲۳ نانومتر که به صورت خوراکی به موش‌ها داده شد، پس از کالبدشکافی و بررسی بافت‌های گروه کنترل با گروه دریافت کننده نقره نشان دادند که نانو ذره نقره با سایز ۳۲۳ نانومتر در هیچ یک از بافت‌ها مشاهده نگردید اما دو سایز ۲۲ و ۷۱ نانومتر در بافت‌ها دیده شد که سایز کوچکتر نانو نقره ۲۲ نانومتر بیشترین جذب را در سطح سلول‌های معدی- روده‌ای داشته است(۵). بر اساس مطالعات گزارش شده آزمایشگاهی، هیچگونه تغییر قابل توجه توکسیکولوژیکی در موش‌هایی که ۲۸ روز در معرض نانوذرات بصورت استنشاقی بودند دیده نشد(۱۵). مقایسه اثرات توکسیکی ذرات کوچک با ذرات بزرگ و تجمع و انتشار ذره در بافت‌های مغز، ریه، کبد، کلیه و بیضه به نحوه مصرف از راه دهانی یا تزریق بستگی دارد(۵-۲). موش‌هایی که نانو ذرات نقره با سایزهای متفاوت از طریق تزریقی دریافت کرده بودند، نانو ذرات وارد جریان خون شده و در بافت‌ها مخصوصاً کلیه، کبد، طحال، مغز و ریه انباشته شده بودند(۵). بر اساس این تحقیق، ثابت شده است که نانو ذرات نقره سبب تخریب دیواره خونی- مغزی شده و تخریب عصبی را به وجود می‌آورند. مسیر دیگر ورود نانو ذرات، دهانی یا خوراکی است که ممکن است در مورد بسیاری از محصولات مصرفی مثل خمیر دندان‌ها، بطری‌های قابل بازیافت، پستانک کودک، لوازم آشپزخانه و اسباب بازی‌ها مهم باشد(۱۶-۲).

در این تحقیق، تلاش کردیم تا اثرات مضر نانو ذرات نقره ۷۰ نانومتربادوزهای مختلف را برروی ریه موش‌های صحرایی بصورت خوراکی، شناسایی کنیم.

سمیت نانو ذره نقره که به صورت خوراکی در دوزهای ۰،۱/۰،۵/۲۵و ۲ میلی گرم بر کیلوگرم به موش صحرایی داده شد، در مقایسه با گروه کنترل مورد بررسی قرار گرفت. نتایج حاصل از آن نشان داد که با افزایش دوز، اثر سمیت بیشتری را در حیوان ایجاد می‌کند. ریه‌های نمونه نسبت به کنترل حجیم و بی‌رنگ بود که از نشانه‌های مرفولوژیکی آمفیزم ریه است و بررسی‌های پاتولوژیکی آمفیزم ریه و آسیب‌های ایجاد شده به مقدار دوز نانو ذره بستگی دارد. ثابت شده است که سمیت نانوذرات، بستگی به عوامل زیادی دارد که شامل اندازه، شکل، ترکیب شیمیایی، حلالی، مساحت سطح و بار سطحی می‌باشند. ذرات ریز، سطح فعال وسیع‌تری نسبت به ذرات درشت‌تر دارند و برای ایجاد پاسخ‌های بیولوژیکی یا توکسیکولوژ، فعال‌تر هستند(۸).

در تحقیق‌های صورت گرفته گزارش شده است که هنگامی که موش‌ها ۲۸ روز متوالی در معرض مصرف خوراکی دوزهای ۳۰۰،۳۰ و ۱۰۰۰ میلی‌گرم بر کیلو گرم از نانوذرات نقره(۶۰ نانومتر) در کربوکسیل متیل سلولز(CMC) قرار گرفتند، اندازه آلکالین فسفاتاز(ALP) و کلسترول، بصورت قابل توجهی در گروه‌هایی که تحت تأثیر بیش از ۳۰۰ میلی گرم بر کیلوگرم نانو ذرات نقره قرار گرفته بودند افزایش یافت. تجمع یا انباشتگی در بافت‌ها وابسته به دوز بود اما واکنش‌های مربوط به سمیت ژنتیک در هیچکدام از گروه‌ها نشان داده نشد(۱۷).

این نتایج به این معناست که نانو ذرات نقره روکش‌دار میزان جذب در مجرای گاستروانتستینال را کاهش می‌دهد. نانو ذرات نقره مورد استفاده در تحقیق ما، ذراتی بدون روکش بودند که جذب جریان خون شده و سمیت بیشتری را ایجاد کردند.

زمانی که نانو ذره نقره وارد مجاری تنفسی می‌شود، ذره‌های درشت‌تر در قسمت‌های بالاتر مجاری تنفسی ته‌نشین می‌شوند و ذره‌های کوچک‌تر که قابلیت عبور بیشتری به داخل فضاهای آلوئولی دارند به قسمت‌های عمیق‌تر دستگاه تنفسی رفته، که این مسئله باعث جذب ریوی نانو ذره نقره می‌شود. با توجه به مکانیسم‌های مولکولی مربوط به تعامل نانو ذرات تنفس شده با اندازه‌گیرنده‌های غشای سلول در مقایسه با ذرات دانه درشت‌تر

باعث کاهش پاک سازی ریوی می‌شود(۱۴). در نتیجه پاسخ التهابی بافت نیز کاهش می‌یابد با این حال اندازه و زمان ماندگاری نانو ذرات در فضای آلوئولی ممکن است چالشی بزرگ در سلول‌های هدف ایجاد کند. از جمله آمفیزم که ریه‌های حجیم، همراه بزرگی شدید کیسه‌های هوایی با نازک شدن و تخریب دیواره‌های آلوئولی است که در اثر دو عدم تعادل پروتئاز- آنتی پروتئاز و اکسیدان- آنتی اکسیدان بوجود می‌آید که نتیجه این عمل افزایش استرس اکسیداتیو می‌باشد(۱۸). به طور کلی می‌توان گفت که در مسمومیت‌های نقره در بافت ریه که در این بررسی مورد مطالعه قرار گرفت تغییرهای حاصله نشانگر خونریزی و دژنراسیون شدید سلول‌ها، آسیب‌های بافتی و گسترش ضایعات ایجاد شده توسط نانوذرات، روند وابسته به دوز را آشکارا نشان می‌دهند. هرچه دوز نانوذرات بالاتر رود، تخریب بافت حیوانات آزمایشی هم بیشتر خواهد بود، اما تحقیقات بیشتر مکانیسم دقیق روند تاثیر نانوذرات بر بافت‌های مختلف را روشن خواهد ساخت.

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Toxicological Effects of Silver Nanoparticles in Rats' Lung

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Abstract

Introduction: Nanotechnology deals with structures that their dimensions are approximately between 1 to 100 nanometers. Research has shown that the composition, shape and different sizes of nanosilver make the features that possess different toxic effects compared with the chemicals with same but larger composition (microsilver). Nanosilver is associated with cell division, oxidative stress, and apoptosis or cell death. The aim of this study is to investigate the effects of nanosilver on lung tissues.

Methods: In this study, 50 adult male Wistar rats were examined in two groups of experimental and control. 70 nm silver nanoparticles with four different concentrations 0. 25, 0.5,1 and 2 mg kg-1 were orally administered for 30 days. To investigate the pathological changes after 30 days, the lung tissue of mice in each group was apart and kept in formalin for histopathological examination. After molding and cutting, template samples were stained with H &E staining method. Then slides were observed by invert microscope.

Results: According to the results, the high-dose groups(N4, N5) showed more pathological effects compared with other groups(N2, N3) and controls. In general, the effect of silver nanoparticles on lung tissue was significant and the resulting changes revealed cell and tissue damage.

Conclusion: The toxicity of silver nanoparticles, administrated orally for 30 days in the experimental groups, was investigated that showed its harmful effect on lung tissue determined by pathological examination.

Keywords: Silver, Nanoparticle, Lung, Toxicity, Rat

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Review

Silver nanoparticles as an effective disinfectant: A review

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ABSTRACT

The paradigm modifications in the metallic crystals from bulky to micro-size to nano-scale have resulted in excellent and amazing properties; which have been the remarkable interests in a wider range of applications. Particularly, Ag NPs have much attention owing to their distinctive optical, chemical, electrical and catalytic properties that can be tuned with surface nature, size, shapes, etc. and hence these crystals have been used in various fields such as catalysis, sensor, electronic components, antimicrobial agents in the health industry etc. Among them, Ag NPs based disinfectants have paid attention due to the practical applications in our daily life. Therefore the Ag NPs have been used in different sectors such as silver-based air/water filters, textile, animal husbandry, biomedical and food packaging etc. In this review, the Ag NPs as a disinfectant in different sectors have been included in detail.

1. Introduction

In spite of the contemporary improvement of the hygiene in the biomedical (hospitals), education (school/colleges), surrounding environment (air/water), and industry (food/textile/animal husbandry); it is an increasingly important public health issue globally. In particular, the infectious diseases are the major challenges to the human being because of emerging > 300 infectious diseases with a new adaptation. The microbial based infections are a key cause of the diverse infections because of which > 50% people are dying in Africa due to a variety of infections [1]. To overcome the various strategies have been used to reduce infections by using different disinfectant. The disinfectants are chemical substances applied on the surface to kill or inhibit microorganisms. It is an ideal way to disinfect various surfaces in hospitals, kitchens and in clinics. They are useful in our daily life because they particularly kill microorganisms without causing health hazards to human beings. In addition to that, they are abundant in quantity, efficient, a cheaper antimicrobial agent in short periods and unable to generate toxic compounds after their use [2]. The various chemical compounds such as alcohols, quaternary ammonium cation, aldehydes, oxidizing agents such as sodium hypochlorite, hydrogen peroxides, iodine etc. have been used as disinfectant effectively. However, these compounds are suffered from various constraints such as harmfulness, corrosive nature and bacterial resistance.

To overcome those problems, the nanomaterials have created a new field in wider sectors. The International Organization for Standardization states a nanomaterial as a material with any exterior dimension in between 1 and 100 nm; which have also been in the multifold domain due to their remarkable properties. The various nanomaterials have been employed as efficient disinfectants by optimizing their physicochemical properties. Hence, many investigators are probing to generate multifunctional nanomaterials as a potent disinfectant. The nanomaterials have a wide range of uses like water disinfectant, hospital acquired disinfectant, food preservative, and medical devices etc. Among the various materials inorganic metals such as the copper, silver and gold are used eating utensils, plates, cups, jewellery, and coins water/food container for disinfection of water/food as well as human infections. Particularly, silver ions and silver-based compounds are the well-known antimicrobial agent for the medicinal importance from the 1000 BCE and they have been used as an efficient health additive in Chinese and Indian Ayurveda medicine [3]. The choice of silver is due to its multiple functions in the medical field. As usual silver nitrate is used for antimicrobial action a long time, but nowadays nano-based silver has efficient in antimicrobial action due to its physicochemical property in which larger surface to volume ratio resulted into higher surface exposure to the microbes which leads to furnish better antimicrobial activity. In addition, the special properties such as size, shape, phases play a crucial role of bacterial inactivation or killing of bacteria. These physicochemical properties of the silver nanomaterials and its compounds have foremost applications in the environmental,

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Table 1
The physicochemical properties of silver nanoparticles as a disinfectant.

Sr. No.	Materials	Physicochemical properties of Ag NPs	Disinfectant activity	References
1.	Ag/TiO_2	Reduction in band gap leads to visible active material	Visible active antibacterial activity	[6,76]
2.	Ag@ZnO	O_2^- , ·OH	Photo-oxidative killing of bacteria	[37,61]
3.	Ag NPs	Surface functionalization	Photocatalytic, self-cleaning bacterial inactivation	[52,54,58,61]
4.	Ag polyamide	Size 40–60 nm	Sustainable release of Ag + ion for antibacterial effect	[62]
5.	PLA/ZnO:Cu/Ag bionanocomposites	Mechanical/structural, antibacterial and barrier property to UV light	Enhance shelf life of food	[73]
6.	Ag NPs	Anti-viral	Elimination of aerosolized bacteriophage MS2 virus particles	[26]
7.	Ag@Co-NPs	Magnetic and antibacterial	Water purification	[21]
8.	$AgNP@SiO_2$	Spherical morphology	Prompt and synergic antibacterial activity of air filter	[25]
9.	Ag NPs/Chitosan	Porosity, moisture retention capability, blood-clotting capability	Wound healing ability	[47]
10.	Ag/BC	Porosity	Wound healing ability	[54]

biomedical and industry sectors. Ag NPs are playing the crucial role in the air/water purifications, in biomedical fields as a therapeutic agent, textile consumer products as well as wound dressing which are shown in Table 1. Its bactericide effects are observed on *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus*, *Listeria innocua*, *Salmonella choleraesuis* bacteria due to higher toxic effect to the bacterial cells [4]. Ag NPs have imparting broad scope to enhance efficiency by optimizing its physicochemical parameters, which also leads to greater binding capability with sulphur and phosphorous functionalized biomolecules of bacteria for killing the cells [5]. Our research group exhibited a detail study of nanocrystalline Ag connected to the surface of TiO_2 electrostatically for the photoinactivation antibacterial studies in the presence of UV/Visible light [6]. Therefore, due to its multi-domain uses, Ag NPs would have the broad spectrum of the biomedical sector for innovative formulation to resist the bacterial growth. This review is majorly focus on the role of Ag NPs as a disinfectant for controlling the various infections observed in water, air, textile, poultry, hospital acquired infections, wound healing infection and food packaging infection.

Schematic representation for potential applications of silver nanoparticles (Ag NPs) shown in the Fig. 1.

2. Environmental sector

2.1. Water disinfections

Providing the pure water to human beings is one of the sustainable development goals to be achieved up to 2030. Presently, the various methods are available to pure the water. Filtration is commonly used everywhere; but it can remove only the suspended or micro-particles present in water. Advanced oxidation treatment is another technique used to decompose the organic matter present in water; but it is not feasible to remove all moieties. In reverse osmosis, one can get the pure water; but having more cost as well. In addition, UV-treated filters are also available to remove the microorganisms present in water. Therefore, the present water filters should be multi-functional to remove or decompose the moieties or microbes present in water and hence these are un-economical to the common peoples. However, the bare or composites of Ag NPs would have the multi-tasking ability as these nanomaterials can degrade the moieties as well as can also kill the different organisms in a single treatment [7]. Therefore, in connection to these constraints, we have highlighted the use of composites in water disinfection fields.

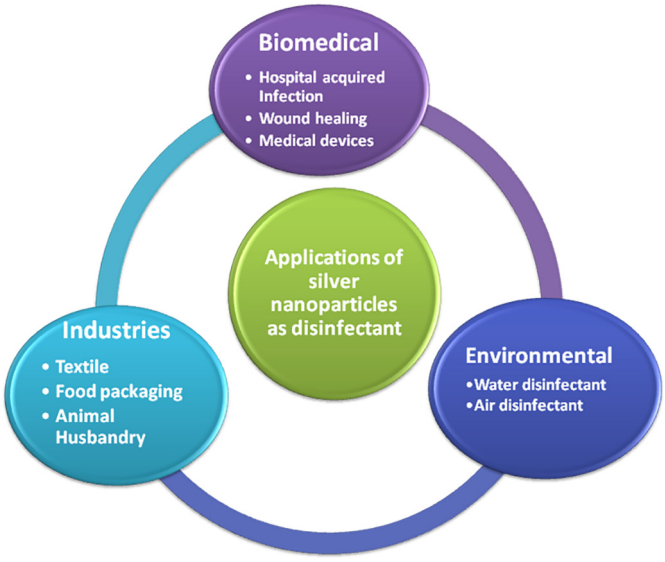

Fig. 1. Applications of silver nanoparticles as a disinfectant.

Estimated 1.87 million deaths occur worldwide due to lack of pure drinking water and sanitation problems. As per evaluated data, 780 million people did not have a safe water supply for their daily needs [8]. World Health Organization (WHO) report suggests household drinking water quality needs to be improved by various treatments at the point of use (POU) [9]. As increasing water inadequacy is an alarming rate, it is required to generate POU or advanced water treatment facilities for safe, easy, secure drinking water. In this regards, the use of nanomaterials as a disinfectant is a new approach. Drinking water purification has involved the various water treatment processes such as settlement, coagulation and filtration in addition of chemical process which covers ozonation and chlorination. Chlorination is widely used as a disinfectant method but it has some disadvantages such as bad taste and odor. In some cases, it is ineffective due to microbial resistance and generated toxic product in the water. The aim of water disinfection is to remove or inactivate microbiological contaminates from the water, without changing the physicochemical properties of water, in addition to these process, silver as a disinfectant is one of the traditional routes to kill or inactivate the bacteria [10]. Also, per the U.S. Environmental Protection Agency (EPA) and WHO, silver at a low concentration in drinking water i.e. < 0.1 mg/L is safe [11]. Silver disinfection depends on various parameters such as concentration, pH, exposure time and temperature. In addition, the other content in the water such as calcium, chlorine and sulphide also play a crucial role in the disinfection process. Calcium and sulphide in water affect the bactericidal activity of silver, but chloride has less effect on its bactericidal activity [12]. Therefore nanocrystalline silver form would have better efficacy in water disinfection. The impregnation of silver nanocrystals in ceramic water filter can act as an effective disinfectant than simple filter [13]. Water filters implanted with Ag NPs proved efficient for the removal and deactivation of microbes by using two mechanisms such as metallic disinfection and physical filtration. Actual impact of silver salt and its NPs on the *C. parvum* pathogen and its complete removal from water by filtration has been revealed [14]. Nowadays, low-pressure membrane filtrations methods have increasing usage in water treatment due to lower processing cost. Generally, low-pressure membrane equipment involve microfiltration (MF) and ultrafiltration (UF) processes. But, they have some issues related to performance. In which, biofouling is the major problem which can created bacterial film on the membrane resulted into decreasing efficiency of membrane flux, thereby reducing the life of membranes. Therefore, it is needed to add antimicrobial agent into the membrane to reduce biofouling and increase capacity of the membrane. Thus, Ag NPs play dual role such as anti-adhesive and antimicrobial agent on the surface of membrane to inhibit bacterial adsorption, attachment and growth; resulted into the prohibition of biofilm formation on the membrane [15]. The antibiofouling performance depends on the particle morphology such as microparticle (mAg), nanoparticle (npAg), nanowire (nwAg) and leaching ability of silver which has incorporated polysulfone membrane. Here, water flux is enhanced by 7 fold due to nanowire Ag NPs and overall antibiofouling property of polysulfone membrane has been enhanced [16].Thin film composite was formed with uniform distribution of Ag NPs on reverse osmosis membranes having surface roughness, hydrophilicity and zeta potential properties. It exhibited high functional property, in which 75% active bacteria anchored on the membrane hence filtered water is free from bacteria and it also reduces biofilm formation rate. Such type of ease method for potent antibacterial ability resulted into practical approach to water filter membrane [17]. The cryogels are another efficient superabsorbent material focused as alternative to ceramic filter with efficient disinfection process. Silver impregnated polysodium acrylate (PSA) cryogels are used as efficient disinfectant in POU process. This is because of its higher porosity, better mechanical and water absorption ability. Homogeneous dispersion of silver nanocrystals on pore surface of cryogels resulted into efficient disinfection with lower release rate of silver ions [13].In another method the percolation of water through silver adsorbed blotting paper sheet has play major role in the bacterial deactivation. It's deactivation performance and leaching test confirmed that, this method is good for water purification process [18]. Polyurethane foams are homogeneously coated with Ag NPs without leaching during the washing in presence of water. This foam is used in water filtration in which flow rate of 0.5 L/min was maintained and filtered water does not have any *E. coli* bacteria. It confirmed that this water filtration unit is compatible with drinking water purposes [19]. To reduce hardness of water cation exchange polymer matrices being routinely used in water treatment process with little focus on the trapping of bacteria in the membrane. Glover et al. reported the dynamic behaviour of Ag NPs on the surface under ambient conditions. The humidity dependent particle generation from the host NPs explored with successive steps are involved in this process. In the first stage Ag NPs are dissolved and oxidized on the highly humid surfaces that lead to formation of the silver ions. Afterwards, in the second stage these silver ions are diffused in the water layer from the parent particles that are different from the parent particles. Later in the third stage formation of Ag NPs are observed at the surface through the photo-reduction or mild reduction process [20]. Various stages involved in the Ag NPs generation under the ambient conditions are shown in Fig. 2.

.

For bacterial inactivation, Ag@Co NPs were embedded into polymer matrices by soft reaction condition. These nanoparticles and their functional groups are efficiently acted as antibacterial agents for water purification [21]. In the current era, self-propelled micro- and nanomotor have projected in the various fields, but in the water disinfection process micro- or nano-devices have been effectively used. The self-propelled Janus microbots incorporated Ag NPs acts as a potent bactericide for water disinfection process, anchored with magnetic iron

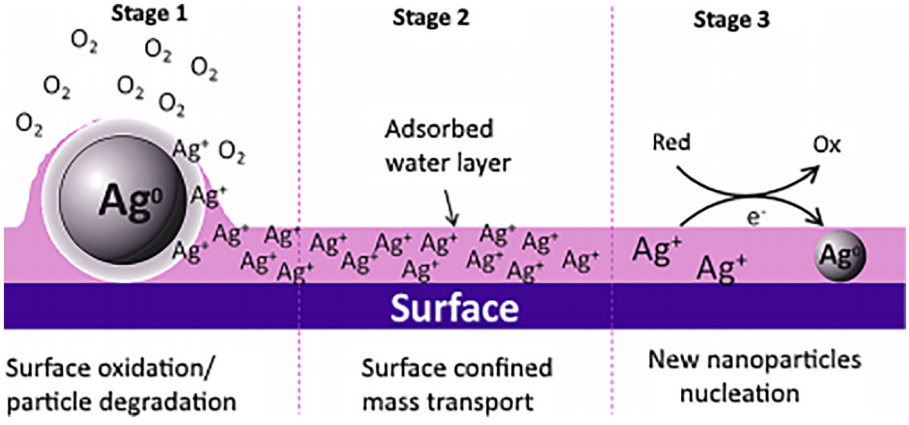

Fig. 2. Proposed steps involved in Ag nanoparticle generation from the parent nanoparticles.

particles for controlling its migration and recovery after use from the treated water. It has specific properties, such as better swimming capability and deactivation of bacteria within short time. Such innovative ways for water disinfection are phenomenally effective and encouraging for the development of micro- and nano-scale devices [22]. Therefore, incorporation of silver in materials like ceramic filter, cryogels, blotting papers, polyurethane foams and cation exchange polymer matrices to design nano- or micro-devices have generated new avenues for water purification than routine water disinfection methods.

2.2. Air disinfection

In the contemporary era, multiple human activities during the various development processes lead to have an adverse effect on the air quality. The current confront involve the clean environment free from toxic gases, particles, volatile organic compounds, airborne pathogenic bacteria and viruses. Among the various challenges of air infections, the removal of airborne microorganisms such as viruses, bacteria and fungi have received great attention as these microbes are responsible for the chronic communicable diseases. It increases vigilance about air purification in the health context. The air purification is needed to eradicate the generation of airborne particles from the various viruses, bacteria, fungi and all of biological living organism. They are majorly responsible for the various diseases such as anthrax, SARS (severe acute respiratory syndrome), asthma etc. These bioaerosols are deposited on the filter, air conditioning systems in excessive amount and multiply due to the higher amount of moisture condition [23]. Therefore, various engineering solutions are available for the removal of bioaerosols using ultraviolet germicide irradiations, photocatalytic oxidation and air ozonolysis methods. To control air quality in the engineering nanomaterials the Ag NPs are used as efficient antimicrobial activity. Silver aerosol nanoparticles are generated from atomizer has been studied as an antimicrobial agent against *B. subtilis* bioaerosols under meticulous conditions. It is observed that Ag NPs are good enough to improve the air quality using air filters [24].Young et al. reported the simple approach used to fabricate the mono-disperse Ag NPs decorated silica particles for synergic antibacterial activity with gram positive and gram negative bacteria in the air filtration unit. In addition, the solution of AgNP@SiO_2 was stable up to six months and exhibited 99.99% antibacterial efficiencies of the both bacteria. Therefore, such hybrid materials are useful as coating for the air purification devices and appliances for prospect green environment applications [25]. Silver coated silica particles are designed and coated on the air filter for the measurement of filtration efficiency and anti-viral ability in the presence of aerosolized virus particles. Subsequently, mathematical model has been used for the determination of anti-viral ability of the air filters. As per calculated data the stable inlet virus concentration 500 PFU/m^3; three inlet dust concentrations 0, 100, and 200 g/m^3; and three outside layer areal densities of 9.34 × 108, 2.8 × 109, and 4.7 × 109 particles/cm^2. These measurements show the anti-viral efficiency of the filter increased with coating areal density and diminish with dust loading. Moreover, it does not affect filtration quality of air of the air filter [26].

Fig. 3 shows the antiviral action of Ag NPs on dust particles of the air filter. Ag NPs are coated on medium air filter as an anti-viral agent and analysed its efficiency as well as anti-viral capability with aerosolized bacteriophage MS2 virus in the presence of dust particles as compared with the theoretical model [26]. Herzong et al. demonstrated the human epithelial airway barrier model at the air-liquid interface using aerosolized Ag NPs exhibited minimum cytotoxicity [27]. The utilization of nanotechnology-based products have been increased and thus the exposures of NPs to the human have also been amplified. These NPs have exposed to the consumer via inhalation, ingestion, and dermal pathways, but inhalation is the more routine way to introduce NPs into the human body. Therefore, the releases of airborne particles and silver compounds from nanotechnology-based sprays have studied in the context of model population exposure and human health effect. Thus air quality has improved by using Ag NPs incorporated in various supporting materials without affecting the air filtration efficiency [28,29]. Thus air quality has improved by using Ag NPs incorporated in various supporting materials without affecting the air filtration efficiency. Though various strategies have been employed for the air/water purification using improved devices with NPs exhibiting better performance; still there is a lot of scope for the optimization based research output with long-term impact of NPs on living organism as well as optimum concentration for increasing efficacy without toxic effect.

3. Biomedical sector

3.1. Hospital acquired infections (HAIs)

The current challenges in the biomedical sector are the antibiotic resistant of the organism, product development protocols and its utility in terms of toxicity, healing time period and a side effect on the human cells. In addition, the detection of infection causing non-bacterial pathogen, monitoring infection control and prevention of nosocomial infections are key confront towards the scientific community [30]. HAIs are well known as nosocomial infections that occurred in the hospital and health care facility centre. There are many factors responsible for HAIs, such as decreased immunity of patient, multistep treatment of patient resulted into increase in infections, spreading of drug resistant bacteria and less care is taken for employing bacterial infection protocols. Fig. 4 shows the various factors responsible for HAIs. A worldwide survey conducted by WHO revealed average 8.7% peoples are suffered by nosocomial infection and about 1.4 million people got affected with HAIs. Eastern Mediterranean and south-east Asia regions are more prone to such infections compared to the western world. Nosocomial infections include urinary tract infections, surgical wounds and lower respiratory tract infections [31]. Prevention of nosocomial infections requires integrated and monitoring programme in which different aspects are essential to be considered. The transfer of microorganism from patient to care taker has been reduced by personal hygiene including hand washing, hand gloves, masks, working clothes,

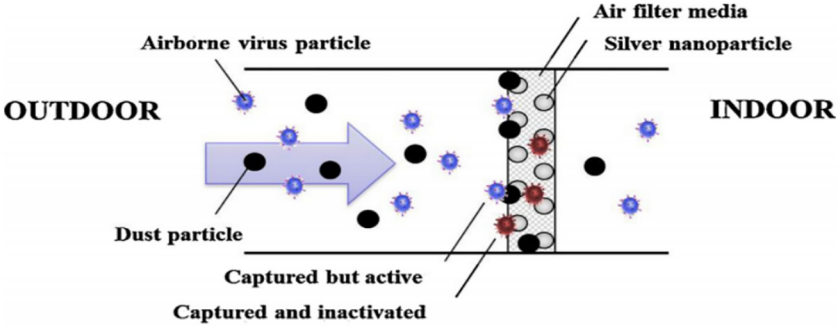

Fig. 3. Antiviral action of silver nanoparticles with dust particles.

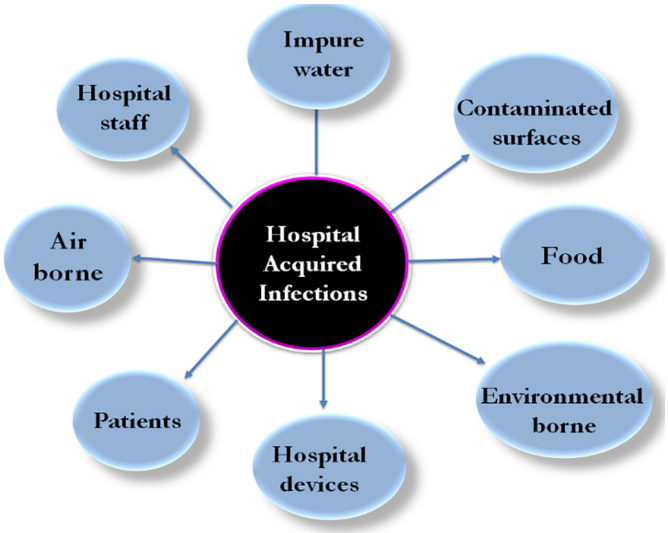

Fig. 4. Various factors responsible for hospital acquired infections.

shoes and sterilization of hospital equipment [32]. Moreover, increasing bacterial resistance has a major impact on health and enhancing economic burdens. A large number of policies have been applied for campaigning against HAIs. Particularly, the use of Ag NPs is emerging one to control the HAIs and efficient nano-weapon against multidrug resistance bacteria [33]. The metal NPs synthesized by the biological route benefits more than those prepared by the physical and chemical methods. This is because of a cheaper processing cost and eco-friendly nature of the biological method. In the biological protocol, biomass has been formed around the metal to neutralize its toxic effect. Biologically prepared Ag NPs using *Bacillus marisflavi* showed high antibacterial activity against bacteria responsible for HAIs [34]. The uncontrolled and excessive use of antibiotics and their bacterial resistance is a present threat in front of the medical community, which is also addressed by synergetic combinations of antibiotics with Ag NPs [35].

The allicin and Ag NPs combination have been studied for skin infection occurred due to Methicillin-Resistant *Staphylococcus aureus*. This study revealed that the Minimum inhibitory concentration (MIC) and Minimum bacterial concentration (MBC) values for this drug combination are lower and thus useful in skin treatment to avoid skin infections. Various medical tools used in hospitals are also potential sources of infections. These are coated with Ag NPs to avoid bacterial contamination. Ag NPs are effectively used in catheters for better antimicrobial activity and zero thrombogenicity [36]. The effect of Ag NPs due to higher surface to volume ratio and release of silver ion on the coagulation of contacting blood has been examined. Visible light assisted disinfections by using antimicrobial agent are another path to reduce infections. Antimicrobial effect of silver coated titania films was revealed under indoor light condition for photoinactivation of bacteria. A complete killing of MRSA bacteria due to synergic effect of Ag NPs as a photocatalyst and visible light has been observed [37,38]. The stents and catheters used in cardiovascular applications required to be coated with antimicrobial agents like Ag NPs to prevent thrombosis. The Ag NPs have prolonged activity, higher bactericidal and bacteriostatic property, biocompatibility and lower vivo toxicity [39]. A bone cement used in the hip and knee replacement surgery, where infection rate is lower with Ag NPs along with poly (methyl methacrylate) (PMMA) in order to reduce risk of bacterial infections. It does not display any cytotoxicity in mouse fibroblasts or human osteoblasts indicating good biocompatibility [40]. Wound dressing is another area in which nanocrystalline silver has been used as commercial product from the decade. In clinical trial, the wound healing efficiency of exiting 1% silver sulfadiazine was compared with new chitosan-nanocrystalline silver. Healing rate of chitosan-nanocrystalline silver after 13 days was higher than control by at 98.98 ± 6.09% which is compared with 1% silver sulfadiazine at 81.67 ± 6.30%. In addition, the healing period was 13.51 ± 4.56 days and 17.45 ± 6.23 days for chitosan-nanocrystalline silver dressing group and 1% silver sulfadiazine group, respectively. It has been found that antibacterial efficacy and wound healing property is significantly high for the chitosan-nanocrystalline silver [41]. The chronic infections are mainly related to biofilm formation on the surface of medical devices in which these bacteria have resistance to antibiotic agent. Such biofilms are effectively degraded by using Ag NPs. After isolation of biofilm from wounds its anti-biofilm efficacy of Ag NPs is examined. The observed lower MIC value range of 11.25–45 μg/mL and anti-biofilm efficiency of Ag NPs is higher at lower concentration of 50 μg/mL [42]. Ag NPs were incubated into polymer matrix exhibit hydrophilic properties that reduce surface attachment of microorganism, biofilm formation and proteins accumulations. Hence, the regular dispersion of activated Ag NPs on the inner and outer catheters avoids formation of biofilm and showed greater antimicrobial property into wide range of in vitro studies [43]. In addition, the human infections occur due to the *C. albicans* pathogenic microorganisms that correspond to the commensal fungi which are usually present on skin, oral cavity, vaginal and gastrointestinal tracts. Candida biofilms have been mainly studied on abiotic surfaces of medical devices but it is found that lower dose has limited efficacy. In order to improve its efficacy use of higher dose required that results in damage of various organs such as kidney, liver etc. To overcome these problems, the biogenic Ag NPs are used to control the biofilms formation on the surface of catheters at the lower dose [44]. External ventricular drainage catheters impregnated with Ag NPs are new path ways to avoid catheter-associated ventriculitis in neurocritical patients. It is validated with recent vitro study on the biomedical devices exhibited the segregation of silver ion in catheter is lower than accepted levels [45]. In conclusion, in vitro and animal studies exhibited Ag NPs have significant level of toxicity. In vivo studies showed long term exposure causes increased argyremia. Subsequently, Ag NPs have been used for wound dressing purpose for safe and broad spectrum. So, the Ag NPs or its composites are useful materials for the different purposes for the control of various infections occurs in the hospitals. In addition, proper precautions are needed to be taken to avoid their toxic effects on human.

3.2. Wound healing

Silver has been used as an effective antimicrobial agent from century to decrease bio-burden. Worldwide spreading of microbial resistance is the alarming concern in the clinical practice. The various antibiotic agents are effectively used from many years for the treatment, but excessive use resulted in antimicrobial resistance. So, to reduce microbial resistance it is needed to design new strategies making use of nanotechnology. Generally, infections are responsible for the postpone healing of closed wounds, traumatic, burn wounds and chronic skin ulcers. The silver based antimicrobial agent is emerging disinfectant promoted for the wound healing to combat infections without affecting human cells. But, its resistance, toxicity and analysis of product protocols are main factors of consideration before its use. [46]. Ag NPs (AgNPs)/chitosan composite as wound healing agent are prepared by simple two step route. The inclusion of Ag NPs into chitosan could improve the antibacterial action against drug responsive and drug-resistant harmful bacteria. The hydrophobic parts of dressing exhibit waterproof and anti-adhesion properties to avoid contamination of the surfaces. On the contrary, hydrophilic surface shows water penetrating ability and diminish bacterial growth [47]. In chronic wound care dressing, the hydrogels with wide functions are the new emerging technologies to avoid infections. Hydrogels, with a high amount of moisture and prepared with silver encapsulation exhibited that this low

concentration (0.1 and 1.0% w/v) have promising potent antibacterial performance against *S. aureus*, *P. aeruginosa*, and *E. coli* which are majorly responsible for development of antibiotic resistance [48]. Green methodologies for the synthesis of nanomaterials are point of focus due to health concern and biocompatibility. Therefore, Ag NPs have been synthesized by *Lansium domesticum* (LD) fruit peel extract for the wound healing purposes. In vivo wound healing analysis demonstrated that it intensify wound closure time and excellent histocompatibility. Thus, it has good prospectus in the disinfection applications [49]. Typically, inflammatory skin diseases, with atopic dermatitis, psoriasis, and contact dermatitis are the majorly widespread skin conditions impact on adults and children. Therefore, immunosuppressant drugs have been used for the treatments but they have lower efficacy and with bad effects. Hence, nanocrystalline silver cream is used as substitute treatments for inflammatory skin diseases and it is found that it has efficient anti-inflammatory activity in comparison with routine steroids and immune suppressant in presence of pig model of dermatitis diseases. It reduced erythema in 1 day of treatment at variation in concentration with noteworthy reduction at silver concentrations of 0.5% and 1% ($P < 0.05$) and this decreases during the study stage [50]. Direct application of such antimicrobial agents on the human skin may cause toxicity. Toxicity is the major concern to humans rather than bacteria. Therefore, in vitro study of Ag NPs for toxicity assessment exhibited that antimicrobial concentrations (1.56–6.25 g/mL) are safe for its use. Lower concentration of silver selectively attacked on bacterial cell lines without harming host cells and therefore useful as well as safe to cure burn wound infections. It established its utility as a good antimicrobial agent due to less cytotoxicity, oxidative stress and apoptosis of the bacteria [51]. There is a wide range of typical silver containing antibacterial products, such as silver nitrate, silver sulphadiazine, silver sulphadiazine/chlorhexidine, silver sulphadiazine with cerium nitrate and silver sulphadiazine impregnated lipidocolloid wound dressing. On the other hand, newly improved products such as ActicoatTM and Silverlon1 have a more systematic and prolonged release of nanocrystalline silver to the wound surfaces. The modes of action like liberation of silver have changed the path of wound dressing. This is due to the reducing threat of nosocomial infection, cost of product, and without damaging tissue efficient healing would be takes place [52]. The release of silver ions depends on the size of the particles. When the size is < 20 nm, it exhibited 100 times more release rate than bulk silver particles. It has been revealed from many studies that Ag NPs are efficient bactericidal in vivo conditions as compared to the bulk silver. The wound dressings are designed in such a way that, they can constantly release silver ion to the wound for the period up to seven days for bacterial death [53]. Ag NPs are functionalized with bacterial cellulose using green synthetic methods with formation of three dimensional webs like structure through the UV light irradiation. Bacterial cellulose is used as supporting material for wound dressing. A greater porosity of the cellulose is due to the nano-fibrous network which enhances water retention ability. The zone of inhibition of silver/bacterial cellulose is observed at 6.5 mm as compared to reference bacterial cellulose is 10 mm, shows efficient antimicrobial substrate against *E. coli* bacteria for wound healing treatment [54]. To understand antibacterial action of silver ion on infected wounds in the presence of organic materials or inorganic ions like chloride, sulphate and phosphate ions have major impact on wound healing process. Therefore, to recognize the bacterial death mechanism there is major causes such as the penetration of silver ions into peptidoglycan cell wall and interaction with plasma membrane, bacterial cytoplasmic DNA and bacterial proteins [55]. In clinical studies the wider chronic non-healing wounds of 29 patients have been quantitatively studied. The bacterial biopsies depicted same number of bacteria. But, swabs used for healing showed reduced number of bacteria on the wound surfaces and causing less pain in wound healing process [56]. In controlled clinical examinations, it is observed that Ag NPs are efficient disinfectant for the wound healing at lower dose. Despite phenomenally efficient wound healing property of silver, its production cost, clinical excellence and safety as compared with well-known antiseptic agent povidone-iodine are also considered. At the end, wound healing using Ag NPs as the potent antimicrobial agent is an advanced process with lower toxicity to the human cell line and shorter time period of wound healing. In the future, the excessive use of Ag NPs based drugs in the biomedical sector may cause of resistance to the organism so that there is scope for designing, developing and modification of the Ag NPs based drug to avoid bacterial resistance.

4. Industrial sector

4.1. Textiles

Nowadays, there is growing interest of the use of silver based nanomaterials as antimicrobial agents in the textile sector as well. Sterile fabrics are one of the common goal defined by the scientists so there would be the bacterial free fabrics subject to the various different conditions. The different usage of nanomaterials for textile fabrics has been continuously demanded due to increasing customer costumes for different purposes. To functionalize fabrics the nano-moieties can play the major role with their specific properties. The various properties of fabrics such as stimulate stain repellent, wrinkle free, antistatic, strength enhancement; water repellent and antimicrobial are very significant to enhance fabric durability, luxuries and flexibility. Textile industries have changing and adopting new technologies not only in fabric processing but also in the use of antimicrobial agent to avoid bacterial contamination. Currently, peoples are also aware about bacterial infections occurred due to textile products. The spreading of microorganisms from fabric surfaces to the human skin is the major health concern. Therefore, fabrics can be treated to avoid bacterial infections [57]. In current scenario, textile fabrics have different properties such as antimicrobial activity, UV protection and self-cleaning. The contemporary methods in textiles have adopted nanotechnology in order to accomplish antimicrobial property. But, in this direction selection of proper antimicrobial agent is a challenging task. The choice of Ag NPs is preferable as compared with traditional antimicrobial agents such as metal salts, quaternary ammonium compounds and triclosan. This is superior due to various factors such as bacterial resistance, stability, cheaper and environmental benign [58,59]. Therefore it is more useful than the ionic silver as they cannot generate stain on the fabrics, maintaining fabric breathability and handling. The antimicrobial agent in bare or composite form on the fabric cannot leads to the decolourizations or of destruction of mechanical strength of fibre [60]. Cotton fabric exhibits self-cleaning properties under visible light by anchoring its surface with Ag@ZnO nanostructured materials. In addition, the preparation of lightweight and wearable clothes with antimicrobial activity the polyamine-mediated bioinspired approach is simple for functionalization of antimicrobial agents on the surfaces. Nanostructured materials are formed by using polyamines which is coated on the surface of fabrics for better antimicrobial activity under the sunlight. It also exhibited proficient photocatalytic, self-cleaning and stain removal property of the fabric [61]. Plasma pre-treated polyamine coated with Ag NPs are studied for antimicrobial activity and aging effect. The bacterial inhibition is observed after 30 days in the presence of Ag NPs with size < 100 nm. Furthermore, a longer period bacterial inhibition is observed at lower concentration with 40–60 nm size Ag NPs [62]. Plasma treated polyester fabric developed for increasing binding property of Ag NPs and pre-treatment initiated both ionic and covalent interactions to create oxygen species on the fibres, resulting deposition of smaller size Ag NPs which promote antimicrobial property [63].The functional clothing has good scope in the market because it has designed to get better performance to the user in the extreme conditions. The various functional clothing such as protective, medical or sport clothing are available in the market. In functional clothing studies have been focused on antimicrobial effects in

laboratory scale to the real life conditions [64]. Now a day's engineered nanoparticles (ENPs) are the new technology, in which functionalized silver and titania based consumer products are used as antimicrobial and photoactive agents. However, the various external exposure pathways such as the contact between fabric and skin as well as ingestion and inhalation transfer to children by oral routes are responsible for toxicity to human cell lines. Goetz et al. studied the dermal exposure to nano-object and their aggregated and agglomerates of the loaded ENPs on the commercial textile fabrics to artificial sweat using simulated wear-and-tear through physical process. These ENPs uptake rates on skin have less exposure therefore they are exhibited less toxicity than oral pathways used in dietary supplements [65]. Silver coating by various techniques on the textile fibres having longer period of release of silver ion and uniform distribution of silver on fabric is important parameter for antimicrobial fabrics. In the realistic approach leaching of silver from fabrics used for children is studied in the presence of different liquids such as water, milk, sweat and urine. Subsequently, leaching of silver in presence of sweat and urine found to be higher than the tap water. This study reveals the less connectivity of silver particles to the fabrics; which would be further optimized for decreasing the leaching rate of Ag NPs from the fabrics so that these particles would not be harmful to the human beings [66]. Fig. 5 shows the antimicrobial efficacy of Ag NPs in the textiles. Microbial efficacy of Ag NPs was determined on the textile fibres during various life cycle stages. Multiple cycles washing exhibited range of silver release but does not affect antimicrobial efficacy of Ag NPs [67].

In supposition, the overall antimicrobial efficacy of Ag NPs depends on its concentration, surface area, size and release rate. The higher surface area and concentration of silver help to increase bacterial contact by binding with −SH group of a protein. This resulted into reduction of bacterial functions and ultimately its inhibition. It also hamper respiratory system, basal metabolism of electron transfer in the cell membrane causing bacterial death [68].Nonetheless, because of its high antimicrobial activity and stability on the fabrics next generations prefer to use various types of costumes which are free from bacterial contamination and having self-cleaning ability of the fabrics. Accordingly, the textile industries have been changing from their old fashion technology to the new nano-based technology for advanced textile products.

4.2. Food packaging

Food safety is the major concern in front of the food industry and government worldwide. A fresh, clean, hygienic and long life food without chemicals or the chemicals that are less harmful to human being globally. Market trends have also been changing to lower processed, readily prepared and ready-to-eat “fresh” food goods. On the other hand, foodborne microbial bursts, worldwide food trade and transportation of processed food to consumers are motivating for searching new ways to inhibit microbial contamination of food along with key challenges like quality, freshness and safety. The Centre for Disease Control and Prevention (CDC) of USA, evaluated that 90% of the infections are occurred due to different types of bacteria. As per CDC report almost 48 million peoples are infected out of that 128,000 are hospitalized and 3000 died in United States due to foodborne diseases. These are not only bacterial infection but also toxins released from the various microorganisms during metabolic processes [69]. Therefore, antimicrobial packaging technologies are needed to enhance shelf life of food, inhibit bacterial contamination and to prevent or delay the spoilage. Ag NPs are promising bactericidal materials in food packaging against broad range of microorganism such as bacteria, yeasts, fungi and viruses. Most of the inorganic metals and metal oxides used as antimicrobial agents are more tolerable to the drastic food processing conditions as compared to the organic materials having a less stability. Ag NPs are potent biocides against various pathogens. But, migration of silver from the packaging surfaces to the food stuffs is the potential health concern risks [70]. The European Food Safety Authority (EFSA) recommended upper limits of silver migration from packaging should not go beyond 0.05 mg/L in water and 0.05 mg/kg in food. It exhibits that determination of silver migration framework are essential to ensure potent antimicrobial activity [71]. Food packaging are classified into two categories; firstly improved packaging in which nanomaterials are embedded into the polymer to enhance its gas barrier properties and secondly active packaging in which nanomaterials interact directly with food and prevent contamination of food from microbes. In the film formation process Ag NPs were coated, absorbed, or directly incorporated by the simple chemical route [72,73]. Active packaging is the emerging technology focused on the protection of food product from microbial contamination and deteriorations. It involves

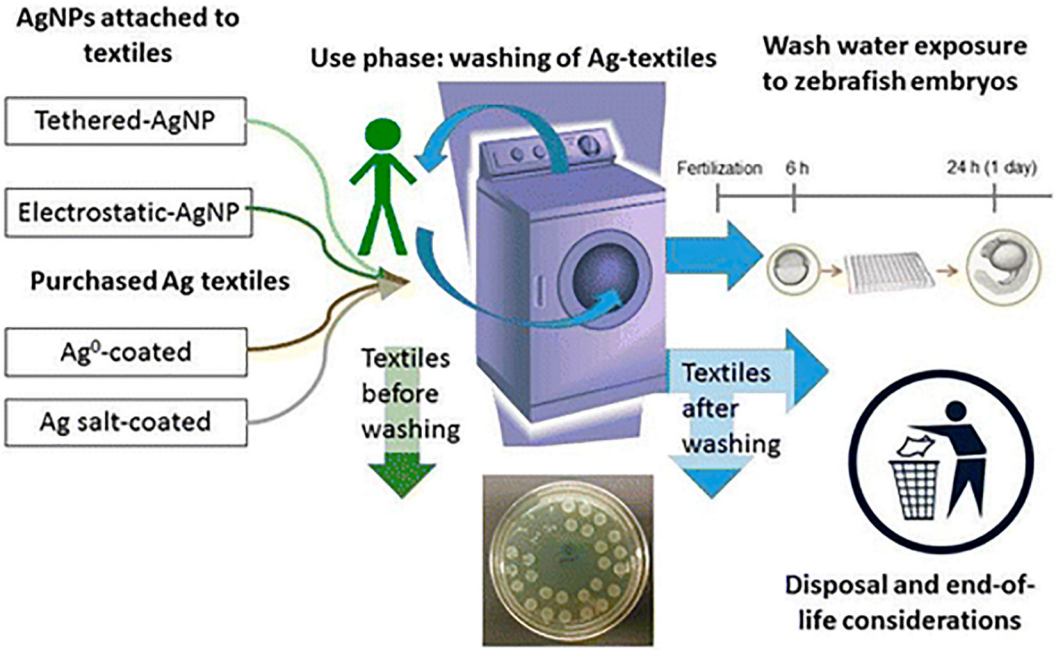

Fig. 5. Antimicrobial actions of silver nanoparticles in the textiles.

the three sub-categories, such as absorbing systems, releasing systems and other specific temperature, UV light and microwave based systems [74]. Though the Ag NPs increase shelf life of food, there is need to evaluate hazards and risks of migration from packaging to food for the customer safeness. The better food quality and shelf life have been achieved by active packaging which leads to the reduction of microbial infection from field, cold storage and consumer places. The low cost and environmental friendly packets embedded with Ag NPs for vegetable storage have been investigated. After periodic determination it was observed that shelf life of vegetables increased without reducing nutritional values [75]. Cozmuta et al. reported Ag/TiO_2 nanocomposites in high density polyethylene (HDP-P) film in packaging which increases shelf life and microbiological safety of bread in comparison with the routine packages [76]. Polyethylene based packages with Ag–TiO_2–Fe composite kept orange juice with same colour, consistency, flavour and taste as like the freshly prepared juice, even after 10 days of storage. It was also found that, silver and iron have a better antimicrobial property on the yeast and molds than TiO_2 [77]. Food packaging has various factors such as size, concentration detection limit, size resolution were determined for effective packaging. The biokinetic behaviour such as size, shape, capping agent, charge and solubility and aggregation state of the active packaging are have impact on the shelf life of food. Investigators were used SP-ICPMS and AF4-ICP-MS techniques for studying the properties of NPs for better understanding the effect of NPs on the active food packaging [78]. Ramos et al. reported the migration study of Ag NPs, from plastic baby bottle and food container revealed less agglomeration and oxidation of Ag NPs, it is depends on the nature of polymer and its storage conditions. SP-ICPMS techniques were used for the determination of ionic silver and Ag NPs in extremely diluted samples. Hence this method is better for getting precise information of NPs size and concentration in complex extracts at lower quantity with short duration of time, that avoids agglomeration and oxidation of Ag NPs [79]. Silver and copper NPs were impregnated into guar gum nanocomposites and effect of on thermo-mechanical, optical, spectral, oxygen barrier and antimicrobial properties on film have been studied. It exhibited good properties of film for active food packaging applications. While commercialization of such films require to study the effect of NPs on food and its impact on the human health [80]. Commercially available containers are used to determine migration of Ag NPs under acetic acid as stimulant at 40 °C under 10 day's observations. Migration values were found to be higher under heating in the microwave oven compared to conventional oven. These values were also depending on size and aggregation of Ag NPs [81]. PVC nanocomposite films incorporated with Ag NPs decrease the thermal with retained mechanical properties and prolonged the shelf life as well as decrease the lipid oxidation of packed chicken breast fillets [82]. Thus, active packaging with Ag NPs is breakthrough technology for food safety and its processing. Vast commercialization of this technology offers clean and fresh food in ready to eat format without losing its nutritional qualities. Fig. 6 shows the correlations between raw materials, food handlers and biofilms in the food packaging [83]. Thus, the nanoscale silver is one of the best materials for disinfection purpose with its potent antimicrobial activity. Presently, it has been profoundly used in daily life, in environment for water and air disinfection, in industries such as textile and animal husbandry, in medicine. With such widespread use of Ag NPs as disinfectant; it is required to give special emphasis on the risk of its toxicity for the welfare of human health.

4.3. Animal husbandry

Animal husbandry is the one sector of agriculture related to animals as the source of meat, fibre, milk, eggs, or other foodstuffs. During the routine activity of the animals, there is a possibility of infections due to various pathogenic microorganisms. So there is a need to expand healthy animals with fresh and better food products. Among the disinfectants, Ag NPs is also used as a surface disinfectant, water disinfectant and therapeutic material in animal husbandry including poultry, livestock and aquatic industry. The various diseases caused by bacteria, viruses, fungi and other mono-cellular microorganisms were effectively controlled by using Ag NPs. It inhibits reproduction and growth of those bacteria and fungi responsible for the infection, bad odor, itchiness and sores. Ag NPs are found to be highly efficient, fast acting, deodorizing, nontoxic, non-stimulating, non-allergic, tolerance free, hydrophilic and thus very effective for bacterial resistance. Therefore, Ag NPs are used as a disinfectant in animal husbandry to disinfect and prevent disease [84]. All the products of poultry farming viz eggs, chicken etc. are mainly based on biological material, which is the potential source of infections and thus the poultry diseases. Various microorganisms and their endotoxins are responsible for the infectious diseases and spread in the environment through bioaerosols called organic dust. This organic dust is reaching about three kilometers away from the place of its formation and causes serious respiratory tract infections [85,86]. A lot of efforts have been made to conquer such infections by eco-friendly ways without having a negative impact on human health. Many chemical compounds such as organic acids, hydrogen peroxide, sodium bicarbonates, sodium orthophosphate etc. have been used for the destruction of microorganisms. But, all those have one or more limitations like less solubility, a possibility of direct application on the product, high cost, toxicity [86].

One of the significant examples of an organic compound used for the disinfection is formaldehyde due to its low cost and high biocidal activity. But, it is toxic as well as carcinogenic in nature. Thus, it is needed to find effective methods for destroying bacteria and fungi without being harmful to human health. Literature survey revealed that the Ag NPs with good biocidal properties, may be an outstanding alternative [87,88]. They are effective in abolishing a wide range of Gram-negative and Gram-positive bacteria. Gram-negative bacteria include the genera *Acinetobacter*, *Escherichia*, *Pseudomonas*, *Salmonella*, and *Vibrio*, while Gram-positive bacteria include genera such as *Bacillus*, *Clostridium*, *Enterococcus*, *Listeria*, *Staphylococcus*, and *Streptococcus* [89]. Studies proved that Ag NPs with a diameter of 22.5 nm increase the antibacterial activity of some antibiotics, such as penicillin G, amoxicillin, erythromycin, clindamycin, and vancomycin [90]. Sun et al. observed that Ag NPs are effective against many viruses and it also inhibit the replication HIV 1 virus. Ag NPs are also found effective for the inhibition of a large number of fungi *Aspergillum*, *Candida* and *Saccharomyces*. On the other hand, they are markedly useful against yeast isolated from infected cow udders [91]. As a disinfectant, nanosilver plays a very significant role in animal breeding where sanitation of transport chambers or the space used for the storage of animals are important factors. Ag NPs are applied for animal disinfection. In animal husbandry, strong antibacterial, fungicidal and deodorizing properties of Ag NPs are noteworthy for the disinfection and the prevention of contamination in animal breeding facilities [92]. Some workers [93] reported that a nanomaterial-supplemented diet reduces the toxic activity of aflatoxin-contaminated feeds. Sawosz et al. assessed levels of Ag NPs residues in eggshells and tissues. This study revealed that nanosilver stimulates the oxidative stress condition in chicks hatched from nanosilver disinfected eggs. Disinfection proved to be very effective in the development of embryos and makes them sensitive to even very small amounts of toxic substances [94]. The application of Ag NPs is investigated as feed additives for encouraging birds and weaned pigs' growth. In the study, it was well established that Ag NPs with size up to 100 nm showed higher antimicrobial activity than the silver salts. This is because; silver salts get deactivated by gastric acids and easily absorbed into the body through the intestinal mucosa. At the same time, Ag NPs cannot be digested through intestinal gastric juice and render less toxic effect as compared to silver salts [95]. Ag NPs as a potential dietary additive ascertained beneficial for the growth of weaned pig which might be facilitated through its antimicrobial properties by killing bacterial groups or reducing the microbial load of the small intestine of pigs [96]. In the future, there is wide scope to build up a new

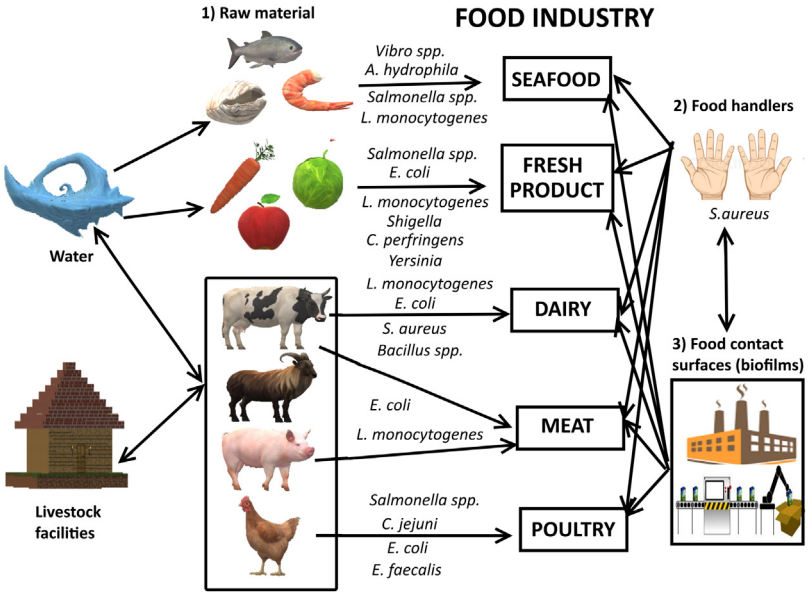

Fig. 6. Correlations between raw materials, food handlers and biofilms.

avenue in the industrial sector to avoid bacterial infections on silver based nanocomposites. In which, the pave the way to reduce cost, competent and low toxic nanomaterials for ease to use silver based fabrics, food packaging and clean, safe and fresh animal food products.

5. Antibacterial mechanism of silver nanoparticles

The antibacterial mechanisms of silver NPs are reported by the various investigators. The bacterial cell membranes contain sulphur constituting proteins and sulphur containing amino acids; inside and outside the cell membrane silver can interact with them which resulted in bacterial inactivation. In addition, silver ion released from Ag NPs interacts with phosphorus in DNA as well as with sulphur containing proteins resulted into inhibition of enzymes activities. Particle size and shape are also other parameters to determine antimicrobial activity. In the size dependent study, it can be revealed that if a size of NPs is < 20 nm, it can exhibited greater attachment of sulphur containing protein of membrane resulted into maximum permeability through membrane and finally cell death of bacteria [97].

Fig. 7 shows the detail mechanism of Ag NPs. This has to be considered together with the high surface to volume ratio typically present in nanomaterials the smaller the particles, the higher the metallic surface exposed and subsequently higher micro-biocidal effect can be expected [98,99]. The shape is the other parameters of nanocrystals that are responsible for the interaction with cell wall of bacteria. Truncated triangular shaped silver nanoplates exhibited higher antibacterial activity against *E. coli* bacteria rather than spherical and rod-shaped NPs [100]. Recently, Ag NPs < 10 nm create pores on cell wall due to these pores the cytoplasmic amount is discharged into the medium, which governs cell death without interacting the intracellular and extracellular proteins and nucleic acids of the bacteria. The interaction of Ag NPs with some cells may cause the process of programmed cell death i.e. apoptosis [101].

In summarize, the Ag NPs as an effective disinfectant in the various commercialized products such as Acticoat™ for wound dressing, Silverline® for polyurethane ventricular catheter, SilvaSorb® as hand gels, wound dressing and cavity fillers, ON-Q SilverSoaker™ as a catheter for drug delivery [39]. In addition to that it is used in various

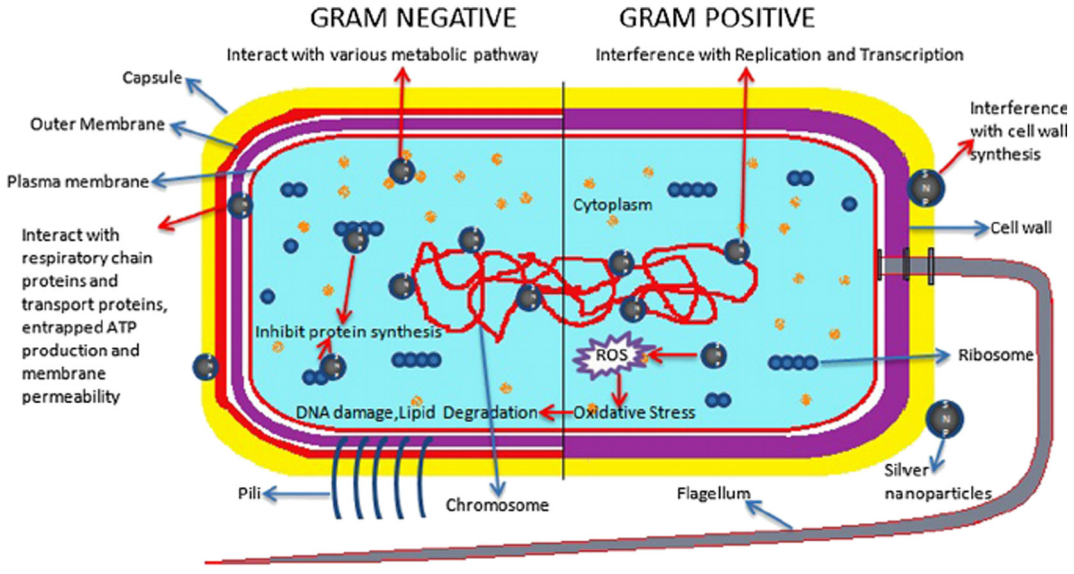

Fig. 7. Antibacterial mechanism of silver nanoparticles.

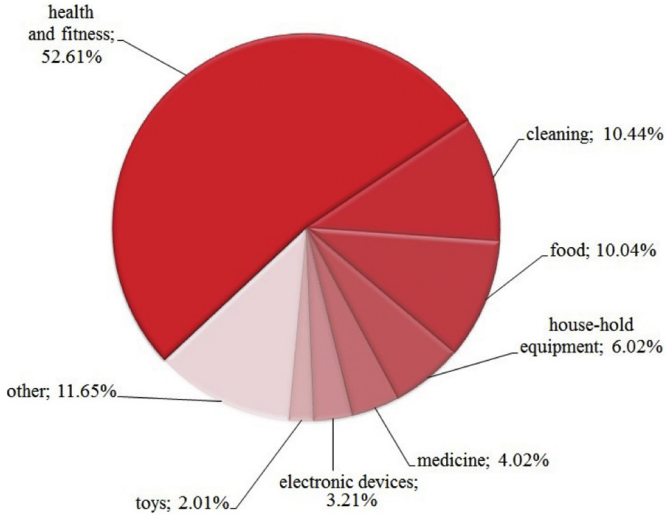

Fig. 8. Contribution of silver nanoparticles in different sectors.

products such as shirt, cloths and medical mask, toothpaste, hand wash, shampoo, toys, detergent as well as humidifiers. Nevertheless, the use of Ag NPs in a consumer product is safe or not is the current topic of debate.

6. Hazardous effects of Silver nanoparticles

Silver NPs have been used in various sectors due to fabulous and efficient antimicrobial nature; a lot of awareness has arisen among the researcher and policy makers because of the adverse effects of silver NPs on the environment as well as on the human health. Therefore it is needed to tackle its health hazards as well as to understand long-term associated risk which fulfils the knowledge gap of toxicity. As we aware of about silver NPs are efficient material used in different sectors as food materials, health and fitness, cleaning, electronics devices, household appliances, toys, medical devices are depicted in the Fig. 8 [102].

Silver NPs show signs of some vivo or virto toxicity due to their physicochemical properties. The other form of toxic effect in the environment observed due to discharge of silver NPs that are readily absorbed by the aquatic species. In addition, extensive use of silver NPs as a disinfectant may be a risk of microbial resistance that reduces its applicability. The change in bluish-gray colour of skin is reported as Argyria diseases due to the toxicity of nano-silver. Actually, toxicity of silver is low but its consequence other than Argyria was observed at a higher concentration; the available literature data exhibits the 0.9 g is threshold limit throughout life time for the Argyria diseases [103]. Furthermore, the drinking water limit is 100 μg/L for nano-silver constituents. Toxicity arises from the nano-silver or dissolved silver is a lot of debate but current research reports show the toxicity arise due to a discharge of silver in the environment in the particulate type as well as in nano-size rather than dissolved silver. The sensitivity of toxicity of silver NPs is higher for the aquatic species with the concentration of 1–5 $\mu g/L^3$ as compared to the human and mammals [104]. In the environment, the toxicity of silver in nanoscale is introduced in sequential forms as a release of nano-silver from the product, emission, distribution and effect on the aquatic life. AshaRani et al. reported Ag-np has the probable reason of toxicity to human cell line as determined by cytotoxicity, genotoxicity and antiproliferative parameters [105]. A review discusses the various aspect of the transformation of silver NPs surface property as phase transformation, aggregation and sulfidation in the environment lead to toxicity to the aquatic living organism. In addition, it revealed the toxicity of silver NPs to the aquatic, terrestrial, plant, algae, fungi, vertebrate and human cells skin (keratinocytes, lung fibroblast cells, and glioblastoma cells) [106].Gliga et al. reported the detail nanotoxicology studies of silver NPs were investigated with particles agglomeration in cell medium, cellular uptake, intracellular localization and release of silver; and revealed intracellular release of silver is accountable for the toxicity to human lung cells [107]. Even though enriching knowledge about the hazardous effect of silver NPs some issues needed to be assessed and optimized the toxicity limit, dose and concentration to the aquatic living organism and human, thereafter it can be safely and efficiently used in various functions.

7. Conclusions

At present, the use of nanomaterials in a wide range of products specifically in the medical and daily life sectors have been increased. Moreover, for the safe, peaceful and paramount life there is need to avoid various infections associated with water, textile, foods and hospital environment. To avoid microbial infections, the Ag NPs have been used from last few decades. It is used in various daily life products and medical devices which give a new approach towards the microbial resistance bacteria to avoid bacterial infections. Beside the wide range of applications of silver and silver based products in various fields as antimicrobial agents, the actual impact of its toxicity on a human has to be encountered as the major risk factor. Furthermore, researcher are also seeking to understand impact of Ag NPs for long term health effect, generating bacterial resistance that are wider scope for the future study.

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