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ORIGINAL ARTICLE

Augmentation of lipid peroxidation and tissue injuries in rats exposed by sub-acute inhalation to nano-silver particles containing tween-20

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ABSTRACT

Nanosilver particles (NSPs) used by customers may contain detergents/solvents. However, in most of the toxicological studies, nanosilvers are prepared in water and very little is known about the interaction of these agents with NSPs in causing adverse effects. The aim of this study was to investigate the interaction of NSPs and tween-20 in causing tissue injuries after inhalation. In this experiment, 16 rats were divided into four groups (n=4/group) as follows. Controls, exposed to water vapors; Tween-treated exposed to tween-20 alone (0.01% V/V); NSP-tween group exposed to 1000 ppm containing tween-20; Group treated with 1000 ppm of NSP alone. Whole-body exposure technique was performed in an exposure chamber for 16 hours (4 hours/day for 4 days). At the end of exposure, blood, liver and lungs were collected and processed for histopathological analysis. Serum glucose, total protein, alkaline phosphatase (ALP), aspartate transaminase (AST) and lactate dehydrogenase (LDH) were compared between experimental groups. The results showed no significant changes in serum ALT, AST, glucose, and albumin in rats exposed to either NSP or NSP suspended in tween-20. Whereas, lipid peroxidation in liver and lungs in rats exposed to NSPs prepared in tween-20 was elevated by 50 and 95% respectively compared to their controls ($p<0.05$). The interaction of tween-20 and Ag-NSP was further confirmed by observing tissue damage (histology) in tissues. In conclusion, data show that exposure to NSPs prepared in water is associated with negligible toxicity in rats. However, the toxicity of NSPs is potentiated if prepared in tween-20. It appears that Tween-20 can increase the absorption of NSPs and enhance lipid peroxidation in cellular and subcellular membranes leading to liver damage.

KEY WORDS: nanosilver; toxicity; interaction; tissue damage; oxidative stress

Introduction

Silver nanoparticles or nanosilver (Ag-NPs or NSPs) have attracted much interest due to their unique physical, chemical and biological properties compared to their macro-scaled counterparts (Sharma *et al.,* 2009). High electrical and thermal conductivity, surface-enhanced Raman scattering, chemical stability, catalytic activity and nonlinear optical behavior are major physico-chemical properties of NSPs (Chaudari & Paria, 2011).

Silver nanoparticles are considered to be a potentially useful tool for controlling various pathogens. NSPs exhibit a broad spectrum of bactericidal, fungicidal and anti-viral activity (Lara *et al.,* 2010; Sheehy *et al.,* 2015). Hence, nanosilver is widely used in disinfectors for cleaning the surfaces and appliance in hospitals. Moreover, NSPs are added to different consumer products such as plastics, soaps, pastes, food and textiles (Fabrega *et al.,* 2011; Garcia-Barrus *et al.,* 2011; Dallas *et al.,* 2011). NSPs are used in colloid (coating and spray), liquid or solid forms (Tran & Lee, 2013).

Nanosilver particles are also used in the textile industry and in membranes used in water purification

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systems. In many of these applications, the technological idea is to store silver ions and incorporate a time-release mechanism. This usually involves some form of moisture layer that the silver ions are transported through to create a long-term protective barrier against bacterial/fungal pathogens (Chung *et al.,* 2013; McShan *et al.,* 2014; Maneewallanapinyo *et al.,* 2011). In spite of nanosilver applications, there are concerns about the release of NSPs into environmental media as they may generate adverse human health and ecological effects (Chung *et al.,* 2013).

In vitro and *in vivo* reports from different laboratories show that NSPs can exert their toxicity by different mechanisms. The mechanisms described for NSPs are their interaction with cell membrane molecules causing membrane damage, interaction with cellular macromolecules, inhibition of enzymes and induction of oxidative stress and production of reactive oxygen species (ROS) (McShan *et al.,* 2014).

Acute toxicity studies of NSPs in experimental studies suggest that no significant NSPs exposure, related to dermal tissue, changes were observed microscopically. According to this study, colloidal AgNPs given orally to mice at a dose of 5000 mg/kg BW produced neither mortality nor acute toxic signs suggesting that nanosilver is relatively safe when administered to oral, eye and skin of the animal models (Maneewallanapinyo *et al.,* 2011). Likewise, mice exposed to nanosilver by inhalation showed minimal pulmonary inflammation and cytotoxicity (Stebounova *et al.,* 2011).

The toxicological experiments carried out with nanosilver often use nanosilver solution prepared in water, whereas some of the to consumer supplied nanoparticle formulation contains Polyoxyethylene (20) sorbitan monolaurate (tween-20). Tween-20 is a surfactant that has been used primarily as a water-in-oil emulsifier, which is used mainly in cosmetic and pharmaceutical products. Tween-20 is known as a physical stabilizing agent in many topical pharmaceutical formulations such as the formulation of various drugs with lipidic nano-carriers in drug delivery systems. Tween-20 is considered a relatively low-toxic agent, but may cause inflammatory reactions in mucosa and respiratory epithelial linings. The aim of this study was to investigate the possible interaction of NSPs with tween-20 in causing toxic responses due to pulmonary exposure using an animal model.

Materials and methods

Silver nanoparticles (NSPs)

In this study, a colloid solution of silver nanoparticles of >95% purity was used for whole-body exposure of the experimental animals. Transmission electron microscopy (TEM) analysis of the colloid NSPs showed that the particles had a spherical configuration with a particle diameter of about 40±5 nm.

The NSP solution at a concentration of 1000 ppm was prepared in distilled water and wherever indicated the solution contained tween-20 (0.01% V/V).

Animals and whole-body exposure

Animals

Male adult rats of Wistar strain weighing 175–200 g were used in this study. The animals were purchased from Pasteur Institute of Iran, Tehran. The rats were maintained at a constant temperature and relative humidity of 22°C±2°C and 55%±10%, respectively, with a light/dark cycle (12 hours/12 hours) during the study. The animals were housed in plastic cages and were provided rat feed pellets and water *ad libitum* during acclimatization as well as pre-exposure and post-exposure. The animal experiments were carried out in compliance with the animal and ethics review committee of the Laboratory Animal Centre at the Tarbiat Modares University, Tehran, Iran.

Rats were randomly divided into 4 groups (n=4/group) before transferring to the inhalation chamber (Figure 1). Whole-body exposure of the rats was carried out as described below:

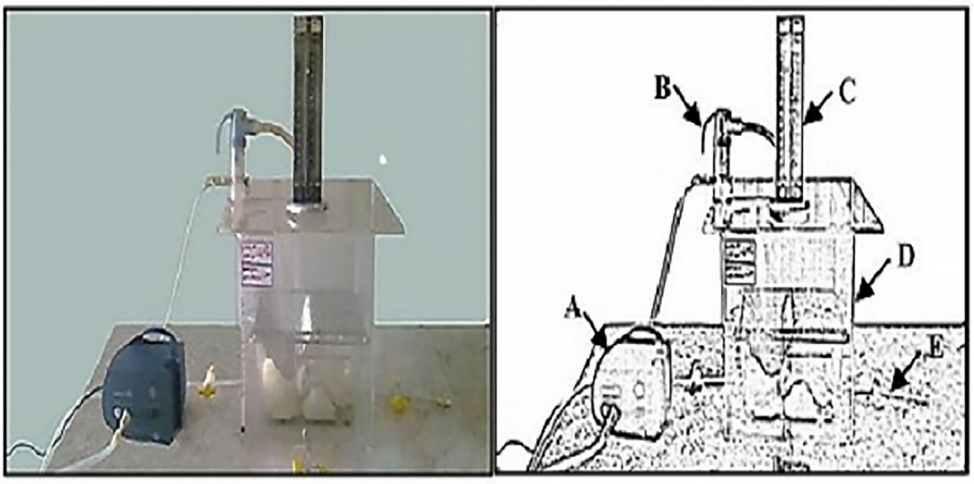

Figure 1. Schematic image of the whole-body exposure chamber designed for rats. Nebulizer (A),(B), air pressure production pump (A), special vessel of nebulizer (B), rotameter (C), special respiration tank (D), air discharge valve.

Group 1: A group of rats (n=4) were exposed to vapors generated by distilled water for 4 consecutive days (4 h/day) in the whole-body exposure chamber.
Group 2: Consists of 4 rats exposed to tween-20 vapors for 4 consecutive days (4 h/day).
Group3: exposed to a solution of NSPs (1000 ppm) prepared in tween-20 (0.01% V/V).
Group 4: exposed to NSP suspension (1000 ppm) prepared in distilled water.

Nanosilver generation device

In this study, an evaporation-condensation method was used to generate NSP vapors. Using a nebulizer, the colloid nanoparticle was dispersed by evaporation of solvent into fine droplets, which were conducted into the exposure chamber. The generator was able to distribute the desired concentrations of NSPs into the exposure chambers (Figure 1). In this procedure, droplet distributions with a peak diameter of approximately 2.0 µm, which leads to a significant amount of non-volatile residue interference in the aerosol.

Normally the exposure was started following a short time of pre-test exposure, the pump, storage container and nebulizer were adjusted before starting the exposure (4 h/day for 4 days). During the exposure period, rats did not have access to water and food. After the exposure period, rats were transferred to their cages and kept under normal conditions for overnight before collection of blood and tissue specimens.

Blood collection

The rats in each experimental group were slightly anesthetized by injection of xylazine and ketamine mixture. Approximately 2–3 ml of blood was collected by heart puncture from each animal and transferred to a test tube. All the tubes containing the blood sample were centrifuged, serum was separated and stored in the freezer for further use.

Measurement of serum parameters: Serum levels of alkaline transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), glucose, total protein and albumin were estimated in serum samples using commercially available kits purchased from Pars Azmoon (Tehran, Iran).

Tissue homogenization and measurement of lipid peroxidation

The rats were sacrificed, liver and lung tissues were removed, a portion of each tissue was weighed (150 mg) and homogenized in 1.5 ml of phosphate buffer (100mM, pH 7). The homogenate was then centrifuged for 10 min at 300×*g*, to obtain a clear supernatant. The rate of lipid peroxidation in tissue preparation was measured using TBARS assay according to the method of Buege and Aust (1978). In this reaction, MDA and other aldehydes have been identified as products of lipid peroxidation, which react with TBA to give a pink colored product that absorbs at 532 nm. In this method, prepared tissue is heated with TBA reagent for 20 min. Then the mixture was centrifuged, the supernatant was collected and absorption was determined at 532 nm against a blank. The MDA equivalents of the samples were calculated using extinction coefficient of 1.56×10^5 M^{-1}.

Histological analysis

For histological analysis, at the end of the exposure period the rats were sacrificed, liver and lungs were excised and rinsed with phosphate buffered-saline (PBS). Specimens from each organ were fixed in 10% neutral buffered formalin and processed for preparation of paraffin-embedded blocks. The blocks were then sectioned, and stained with hematoxylin and eosin (H&E) before examination under a light microscope by the histopathologist.

Statistical analysis

Statistical analysis was performed using SPSS 18b software. The difference between the treatment groups was determined using Mann-Whitney and Student's t-test. Difference among different groups was determined using the Kruskal-Wallis test. The data are presented as mean±standard deviation (SD) and $p<0.05$ was considered significant.

Results

As shown in Figure 2, the biochemical parameters measured in serum of rats treated with tween-20 alone were within the normal range. Likewise, there was no significant difference in enzyme markers and other serum factors among the animal groups treated with tween alone or in combination with the NSPs (1000 ppm). Serum factors were also unaffected in rats following 16 days exposure to NSPs at a concentration of 1000 ppm. Exceptional exposure of rats to NSPs resulted in a significant decrease in serum total protein ($p<0.05$). Serum levels of toxicity enzyme markers such as ALT, AST and ALP were unaffected in all the treatment groups.

Figure 3 compares the effects of different exposures of animal groups on the rate of lipid peroxidation in liver and lung tissues. As shown in this figure there is a significant increase in lipid peroxidation (TBARS) in both lung (95%) and liver (50%) tissues following exposure to the NSPs containing tween-20 (0.01% V/V). Exposure to NPS prepared in tween-20 resulted in approximately 1.5 and 3.3-folds increase in lipid peroxidation in lungs and livers respectively.

Histopathological examination on tissue biopsies revealed that such changes were greater in liver tissues compared to those detected in lungs. The histopathological appearance of the liver in groups exposed by inhalation to NSPs alone or Tween-20 alone showed inflammatory signs and congestion of the portal vein and focal necrosis of hepatocytes. Similar changes with more severe effects were observed in liver tissues prepared from rats exposed to NSPs containing tween-20.

Histopathological examinations of lung tissues prepared from rats following pulmonary exposure to either NSPs or Tween-20 alone showed no obvious tissue abnormality such as inflammation, and alveolities. Likewise, there were

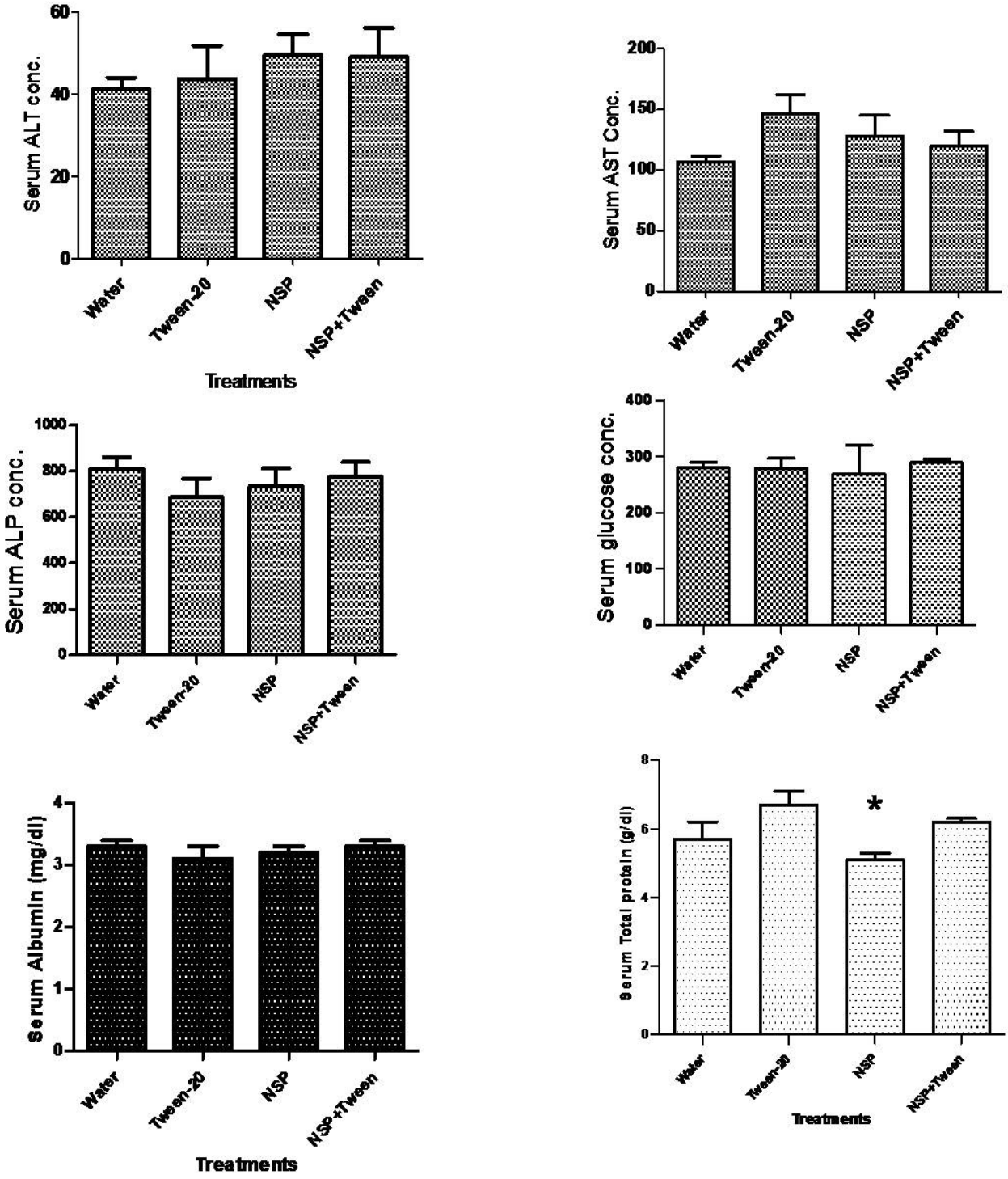

Figure 2. Effects of sub-acute exposure to nanosilver particles on serum biochemical factors in rats. The details about the exposure and experimental groups are as described in the methods section. The assays were performed in duplicate. Data are mean±SD of 4 samples from 4 rats. $p<0.05$ was considered.

no major histological changes in the lungs of experimental animals exposed to NSPs containing Tween-20. In contrast, under the similar conditions of whole-body exposure, rats exposed to vapors of NSPs in Tween-20 showed signs of liver damage. The disturbed architecture of hepatocyte plate around central vein in liver tissue was the main change observed in this group compared to rats exposed to NSPs alone. In addition, there was congestive the central vein and mild hepatitis around central vein without obvious necrosis in NSP-Tween-20 treated group (Figure 4).

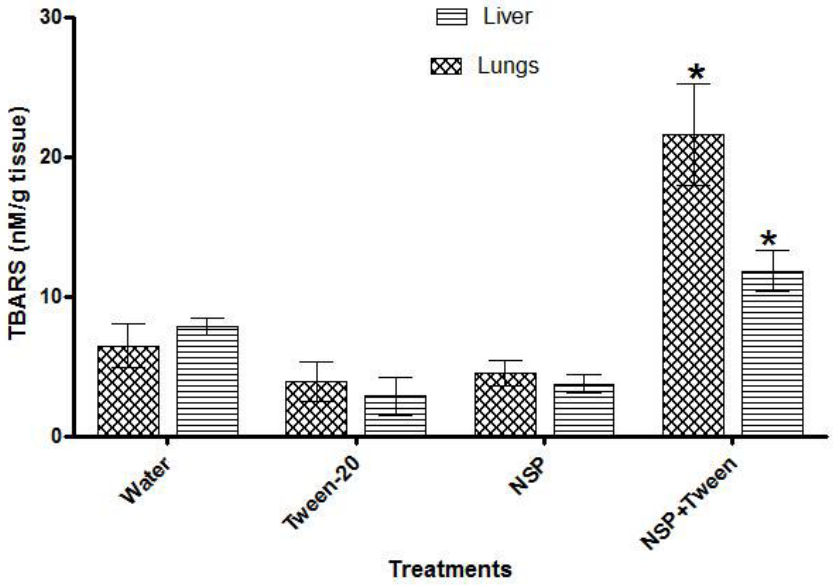

Figure 3. Comparison of lipid peroxidation products (TBARS) in rat liver and lungs following exposure to nanosilver particles. The details about the exposure and experimental groups are as described in the methods section. The assays were performed in duplicate. Data are mean±SD of 4 samples from 4 rats. The concentration of TBARS is expressed as nmol/gr tissue. * sign indicates that $p<0.05$ was considered.

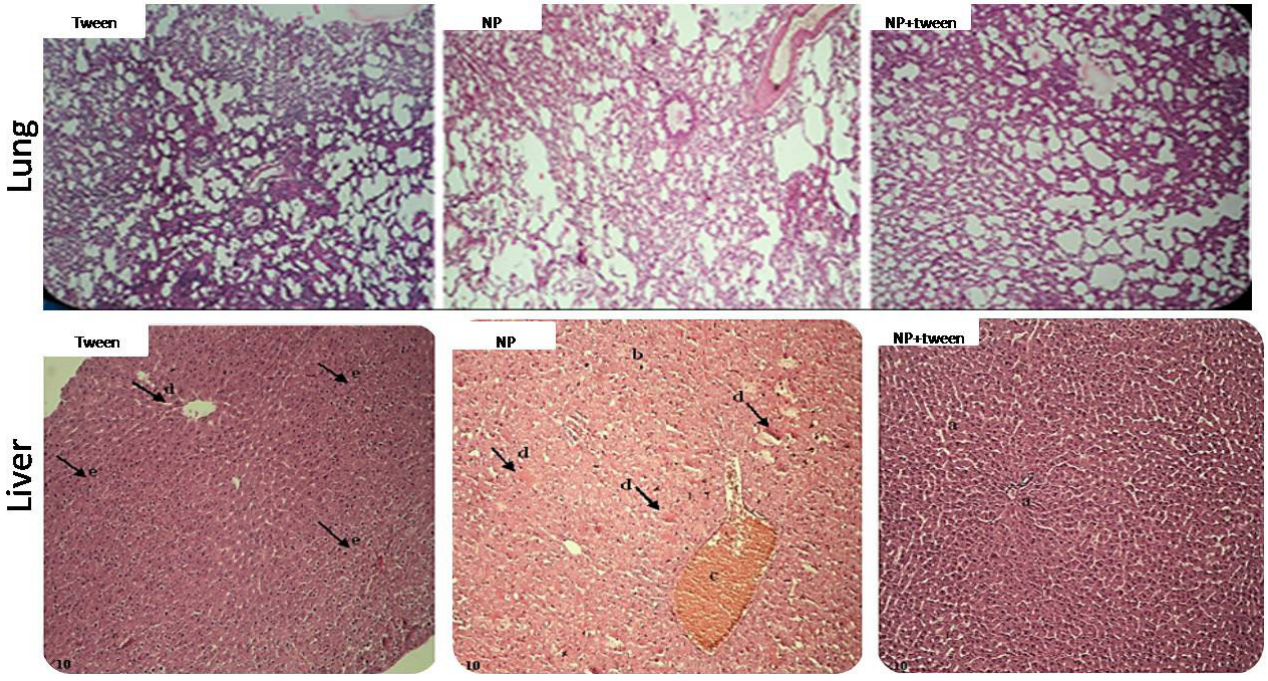

Figure 4. Photomicrographs (H&E ×40) of lung and liver tissues from experiments groups. Cross-sections of lung tissue in male rats under the treatment with tween-20, silver nanoparticles, or NSPs containing 0.01% tween-20. The duration of exposure was four consecutive days (4 h/day). H & E staining, 40X magnifications. Figure labels indicate that animals were exposed to vapors of tween-20 alone (Tween), NSPs prepared in water (NP),or NSPs prepared in Tween-20 (NP+Tween).

Discussion

Nano-sized silver particles are extensively used in different industries and the population exposed to compounds containing nanosilver continues to increase. It is generally believed that NSPs are safe and pose minimum toxic effects to humans.

Different factors such as the particle size, surface area, formulation of nanosilver and the route of administration are important determinants for the toxicity of nanosilver particles. In most of the toxicological studies carried out on nanosilver, the particles used were prepared in water.

However, little is known about the interaction of solvents and detergents added to some of the formulations on reactivity and toxicity of these particles. Nanosilver formulations supplied for some purpose may contain tween-20 as detergent, which is believed to improve the action of nanosilver.

The interaction of nanosilver particles with the human body following exposure by different routes is debated. According to Sung *et al.,* the lung function changes in rats following prolonged (90 days) inhalation exposure to nanosilver particles. These changes could be associated with inflammatory response (Sung *et al.,* 2008) .

In the present study, the toxicity of nanosilver particles containing 0.01% Tween-20 was investigated in an animal model of inhalation exposure. Exposure of rats to NSPs, NPs alone, Tween-20 alone or in combination revealed that tween-20 contributes to NSPs-related increase in lipid peroxidation in liver and lung tissues (Figure 3).

Tween-20 (Polysorbate 20) is a non-toxic detergent, which is used in pharmaceutical applications to stabilize emulsions and suspensions. Although it is a relatively non-toxic agent, the impact of its interaction with nanoparticles and their toxicological characteristics are not well known. Inhalation of tween-20 may be irritating to the mucous membranes and upper respiratory tract, which can aggravate diffusion and toxic effects of nanosilver particles. It is worth mentioning that the concentration of Tween-20 used in the nanosilver customer products is much more than that prepared in our laboratory (containing 0.01% V/V).

Increased lipid peroxidation products in tissues are considered as a primary toxicological marker and an important factor leading to oxidative stress. Hence, increased lipid peroxidation due to Ag-NSPs in tween-20, implies that Tween-20 exerts additive effects on NSPs toxicity.

The impact of nanosilver particles on oxidative stress in testicular tissue further suggests the role of oxidative stress mediators in the pathogenesis of NSPs (Rezazadeh-Reyhani *et al.,* 2015). *In vivo* studies further suggest that silver nanoparticles had the ability to induce hepatic histopathological alterations, which are assumed to be mediated by oxidative stress (El-Mahdy *et al.,* 2015).

In vitro exposure of rat liver-derived cell line (BRL 3A) to different sizes of nanoparticles (Ag; 15, 100 nm), showed a significant depletion of GSH level, reduced mitochondrial membrane potential and increase in ROS levels, which suggested that cytotoxicity of Ag (15, 100 nm) in liver cells also mediated through oxidative stress (Hussain *et al.,* 2005).

It is therefore assumed that the changes in oxidative stress factors together with inflammatory reactions are potentiated when nanosilver particles are combined with tween-20. For the first time in the present study, the interaction of nanosilver particles with tween-20 inducing lipid peroxidation in the lungs and liver of rats exposed by inhalation is interesting. The evidence shows that in addition to elevation in lipid peroxidation products, Tween-20 was responsible for the augmentation of tissue injuries.

Organ-dependent toxicity of NSPs in rats after inhalation revealed that the liver is more vulnerable to Ag-NP solution containing Tween-20 compared to lungs. Comparison of histopathological data in lungs and liver may suggest that following inhalation, the major part of the nanoparticles in tween-20 is probably translocated through the circulation to the liver. The translocation of nanosilver particles depends on several factors, and the distribution and toxico-dynamics of the particles are important issues to be studied. Moreover, regardless of the route of administration, the hepatotoxicity of NSPs is well established (El-Mahdy *et al.,* 2015; Hussain *et al.,* 2005).

Although histopathological examinations showed liver injuries in rats exposed to NSP solution in Tween-20, these changes were not reflected in biochemical markers namely; ALT, AST, LDH in plasma (Figure 2). Hence during respiratory exposure of NSPs, there were no significant changes in serum markers specific for the liver damage.

A substantial increase in lipid peroxidation products in the liver and lungs of rats exposed to tween-20 vapors containing NSPs implies that probably cellular and subcellular membranes are major targets of NSPs dissolved in Tween-20. However, according to histopathological observations, the signs of hepatotoxicity were more similar to those observed in the lungs. Cellular damages to the liver due to exposure to NSPs-Tween-20 solution were mainly the congestion in central vein and mild hepatitis around the central vein. These changes were associated with disturbance in architecture of the hepatocyte plate. Some of these changes, to a lesser extent, were observed in liver of rats exposed to Tween-20 alone suggesting that tween-20 plays a role in translocation of NSPs from the lungs to liver where it can initiate hepatotoxicity.

In conclusion, the toxicity of NSPs and their mode of action after *in vivo* exposure is a debated topic in the literature. Exposure to the combination of the NSPs and Tween-20 could increase nanoparticle absorption and translocation to the liver, causing liver damage. The toxicity of NSPs varies depending on different factors. Signs of cellular inflammation reactions in liver tissue together with increased lipid peroxidation may suggest that cellular and subcellular membranes are major targets of NSPs prepared in Tween-20.

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