Assessment of dermal exposure and histopathologic changes of different sized nano-silver in healthy adult rabbits

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Abstract. The purpose of this study is to evaluate the dermal toxicity (Irritation/Corrosion) of three sizes of nanosilver particles (10, 20 and 30 nm) during 3 min, 1 and 4 hours according to the OECD/OCDE guideline. Histopathological effects in secondary organs from liver, kidney, heart, spleen and brain 14 day post dermal administration are also reported. 10 and 20 nm Ag nanoparticles treated group showed well defined dermal erythema and oedema. Histopathological findings of 10 and 20 nm (4 hours exposure) on 14-day post dermal administration showed hyperkeratosis, acanthosis, hair-filled follicles and papillomatosis in an irregular epidermis, fibrosis, hyperemia, erythema, intracellular oedema and hyalinisation of collagen in dermis of skin. Liver revealed midzonal and periacinar necrosis, portal mononuclear infiltration, liver fatty change, liver congestion and hyperemic central vein. Splenic red pulp congestion and white pulp hyperreactivity, splenic trabeculae and sinusoidal congestion and hyaline change were found in spleen. Fatty degeneration in some cardiovascular cells and subendocardial hemorrhage without inflammation was perceived. Picnotic appearance of pyramidal neurons in the brain cortex, gliosis and mild perineuronal oedema ischemic cell change and hyperemic meninges was observed in brain. Our research concluded that dermal exposure to lesser sizes of silver nanoparticles is more disastrous than greater ones.

1. Introduction
Nanotoxicology is a new branch in toxicological research which has emerged with the aim to assess risk of novel nanotechnology products. Nanotechnology seems to have a lucrative prospect for the future. However, as the outcome of unique properties of nanoparticles, and because of increasing potential for exposure to manufactured nanoparticles, there is an increasing public concern about possible side effects of exposure to nanoparticles on human, environment and ecosystem [1-4]. Silver is a white and brilliant metallic element which has been used as a precious metal to make jewelry, dental alloy, silver kitchenware, conductors, in mirrors and in catalysts. Some silver compounds and silver ions are used as antiseptics, disinfectants and microbiocides, algaeicide and fungicide [5], thus
silver was used in cloths, silver alloy catheters, topical gels and impregnated into bandages because of its wide-spectrum antimicrobial activity [6]. However, irreversible pigmentation due to silver deposition in the skin and in the eyes, argyria or argyrosis, may develop after chronic exposure to silver. Here, a question remained unanswered whether toxic effects of nano sized silver increase as a consequence of reaching nanoscale. At the recent years, nanosilver production have had remarkably growing trend due to its potent anti-microbial activity. Nanosilver coated or embedded instruments and devices such as contraceptive devices, surgical instruments, bone prostheses, wound dressings and laundry detergents are some examples of nanosilver application [7]. Dermal exposure to nanosilver has increased through nanosilver coated / impregnated textile fibers of nano-silver manufactured clothing, nanosilver-based dressings and surgical sutures [7-9]. Skin is permeable to some specific nanoparticles and very small fraction of what is applied to the skin may penetrate. It may act as a portal of entry for localized, and possibly systemic, exposure of humans to specific nanoparticles [10]. Intradermal nanoparticles could access to systemic circulation through subcutaneous lymphatics [11] and may cause argyria like symptoms following exposure to injured skin [12]. Since rising application of nano-silver, possible toxic effects of nanosilver on human, animal and environment need to be considered.

The aim of this study is to evaluate the dermal toxicity (Irritation / Corrosion) of nano- silver particles according to the guidelines of OECD (OECD, 1992). Additionally, histopathological examination on skin, liver, kidney, heart, spleen and brain were also investigated after dermal administration of silver nanoparticles.

2. Materials and methods
Nanosilver particles (10, 20 and 30 nm) were provided from Iran Nanotechnology Initiative Council. The size of the nanosilver particles was determined by transmission electron microscopy (TEM). The purity was analyzed by X-ray fluorescence technique. The analytical results show that the purity of the nano silver particles is more than 99%.

2.1. Preparation of particle suspension
Ethanol (100%) was used as solvent for 10 nm nano-silver and mono ethylene glycol (100%) was used as solvent for 20 and 30 nm nano-silver. The concentration of nano silver in the suspension was 8000 mg/L. Solutions containing nano silver particles were stirred on vortex agitator for 15 min and before each dermal exposure with single dose of 0.5 mL/animal. Trace metal grade Ag powder in ethanol/ mono ethylene glycol and AgNO3 (Merck, Germany) was used in trace size.

2.2. Animals and treatment
72 healthy adult male and female albino rabbits individually housed in steel cages in 20±2°C, 50–70% relative humidity room with a 12-h light/dark cycle. For feeding, conventional laboratory diets including 2390 kcal/kg-1 metabolic energy, 10320 kcal/kg-1 digestible energy, crude protein 19.5%, crude fiber 10%, phosphor 0.69% and calcium 0.76% with an unrestricted supply of drinking water provided.

After 2 weeks acclimation, animal were divided into 6 groups (see Table 1) for treatment with 10 nm, 20 nm, 30 nm nano particles and AgNO3, pure trace metal grade Ag powder Ag powder (CAS: 7440-22-4). The potential influence of the carrier/ vehicle on irritation/ corrosion of the skin was investigated in parallel with the nano particle samples. Each group was divided into 3 subgroups (n: 4) for 1 minute, 1 hour and 4 hours exposure to nano particles and carrier/vehicle. Approximately 24 hours before the test substances administration, fur was removed by closely clipping the dorsal area of the trunk of the animals according OECD 404 guideline.

The test substance was first applied in a single dose according OECD 404 guideline.
The test substance was first applied on a 6 cm² gauze patch and then was applied on a small area (approximately 6 cm²) of skin. Gauze patch was held in place with non-irritating tape. The patch was loosely held in contact with the skin by means of a suitable semi-occlusive dressing for the duration of the exposure period. Gauze patch was attached to the skin in such a manner that there should be a good contact and uniform distribution of the substance on the skin. Access by the animal to the patch and ingestion or inhalation of the test substance was prevented by means of Elizabeth collar. Untreated skin areas of the test animal served as the control. At the end of the exposure period residual test substance were removed using water.

| Table 1. Details of the suspensions prepared for the different treatments applied. |
|-----------------------------------|-----------------|-----------------|
| Particle            | Size | Solvant         | Time of exposure |
|---------------------|------|-----------------|-----------------|
| Nano silver         | 10 nm| ethanol         | 1 minute, 1 hour, 4 hours |
| Nano silver         | 20 nm| mono ethylene glycol | 1 minute, 1 hour, 4 hours |
| Nano silver         | 30 nm| mono ethylene glycol | 1 minute, 1 hour, 4 hours |
| AgNO₃               | Trace| ethanol         | 1 minute, 1 hour, 4 hours |
| Pure Ag powder      | Trace| ethanol         | 1 minute, 1 hour, 4 hours |
| Pure Ag powder      | Trace| mono ethylene glycol | 1 minute, 1 hour, 4 hours |

2.3. Clinical observation and histopathological examination
The test site was examined immediately after skin treatment period (1 minute, 1 hour and 4 hours) and then after 60 minutes, 24, 48 and 72 hours and until day 14 of the last administration for signs of erythema and oedema, local toxic effects and clinical signs of toxicity. Dermal reactions were graded according to the OECD instructions. On 14 day post dermal administration, all animals were sacrificed after being anaesthetized and skin, heart, liver, spleen, brain and striated muscles under the administration site were excised and histopathological tests were performed using standard laboratory procedures. The tissues were embedded in paraffin blocks, then sliced into 5 µm in thickness and placed onto glass slides. After hematoxylin–eosin (HE) staining, the slides were observed.

3. Results

3.1. Clinical manifestation, dermal symptoms observation and histopathological findings of nano-silver particles
No mortality, abnormal behavior and obvious body weight differences were found after 2 weeks of nanoparticles dermal exposure of all groups. Dermal reactions was graded and recorded according to OECD/OCDE 404 guideline.

3.1.1. Dermal toxicity symptoms of 10 nm silver particles. All 4 rabbits which were treated with 10 nm nano-silver during 3 minutes did not show any symptoms of oedema, erythema and eschar formation. However, rabbits which were treated during 1 hour showed well defined erythema during days 2-8 which decreased toward very slight erythema till to day 14 of after exposure. Slight oedema also was observed during days 2-8 after exposure. Rabbits of the subgroup which were treated during 4 hours showed definite erythema rising from moderate to severe and an increasing slight oedema with defined edges.

3.1.2. Histopathological findings of 10 nm silver particles. On 14-d post dermal administration, the pathological observation was performed on skin, liver, spleen, muscle, heart and brain of exposed rabbits to 10 nm Ag nano particles for 4 hours. Treated skin showed hyperkeratosis, hair-filled follicles and papilomatosis in an irregular epidermis and fibrosis, hyperemia, erythema, intracellular edema and hyalinisation of collagen in dermis (Figure 1). The histological examination of the liver revealed midzonal heptic necrosis, mononuclear infiltration in the portal area and liver fatty change (Figure 2). As shown in Figure 3, splenic red pulp congestion, splenic white pulp hyperreactivity,
splenic trabecule and sinusoid congestion was found in 10 nm Ag nano particle on 14-day post dermal administration. Fatty degeneration in some cardiovascular cells and subendocardial hemorrhage was induced by 10 nm Ag treatment (Figure 4). Pionotic appearance of pyramidal neurons, ischemic cell change and hyperemic meninges was observed in histopathological examination of brain (Figure 5). No sign of necrosis, inflammation, fatty degeneration or specific degenerative changes was seen in striated muscles located under the administration site.

Figure 1. Hyperkeratosis, hair-filled follicles, papillomatosis in the epidermis and fibrosis, hyperemia, erythema, intracellular edema and hyalinisation of collagen in dermis of rabbits exposed to 10 nm Ag nano particle on 14-d post dermal administration (magnification = 100).

Figure 2. Oedema and degeneration, fatty change in hepatocytes of the rabbit e exposed to 10 nm Ag nano particle on 14-d post dermal administration (magnification = 40).

Figure 3. Splenic red pulp congestion, splenic red pulp hyperreactivity, splenic trabeculae and sinusoid congestion was found in 10 nm Ag nano particle on 14-day post dermal administration.
3.1.3. Dermal toxicity symptoms of 20 nm silver particles. All treated animals with 20 nm nano-silver during 3 minutes did not show any symptoms of oedema, erythema and eschar formation. However, treated animals during 1 hour showed barely perceptible erythema during days 3-4 and well defined erythema till end of experiment.

4 hours treated animals showed definite progressive symptoms rising from erythema to eschar formation preventing grading in appearance and beef redness in color. Very slight oedema also was seen during 2-14 days.

3.1.4. Histopathological findings of 20 nm silver particles. On day 14 post 4 hours dermal administration, treated skin showed hyperkeratosis, acanthosis hair-filled follicles and papillomatosis in an irregular epidermis and fibrosis, hyperemia, erythema, intracellular edema and hyalinisation of collagen in dermis (Figure 6). The histological examination of the liver revealed midzonal and periacinar hepatic necrosis. Liver fatty change, liver hyperemia/congestion and hyperemic central vein were detected with no sign of inflammation (Figure 7). As shown in Figure 8, splenic red pulp congestion, splenic white pulp hyperreactivity, splenic trabeculae, sinusoidal congestion and hyaline change was found in spleen 14-day post 4 hours dermal exposure. Fatty degeneration in some cardiovascular cells and subendocardial hemorrhage without inflammation was perceived. Picnotic (necrotic) appearance of pyramidal neurons in the brain cortex, gliosis and mild perineuronal oedema was observed in histopathological examination of brain. No encephalitis or inflammation was seen (Figure 9). No sign of necrosis, inflammation, fatty degeneration or specific degenerative changes was seen in striated muscles located under the administration site.
Figure 6. Histopathological photograph of dermis exposed to 20 nm Ag nano particle on 14-d post dermal administration (magnification = 40).

Figure 7. Histopathological photograph of liver exposed to 20 nm Ag nano particle on 14-d post dermal administration (magnification = 100).

Figure 8. Histopathological photograph of spleen exposed to 20 nm Ag nano particle on 14-d post dermal administration.

Figure 9. Histopathological photograph of brain exposed to 20 nm Ag nano particle on 14-d post dermal administration.
3.1.5. Dermal toxicity symptoms of 30 nm silver particles. No sign of oedema, erythema and eschar was observed in the treated rabbits with 30 nm silver particles during 3 minutes, 1 hour and 4 hours, even 14 days after dermal treatment.

3.1.6. Histopathological findings of 30 nm silver particles. Dermal epidermis and dermis did not show any sign of pathological lesions, inflammation (dermatitis), necrosis or hyperplasia.

3.2. Dermal toxicity symptoms and histopathological findings of Ag powder, Ag NO\(_3\) 4 hours treated rabbits with Ag NO\(_3\) demonstrated mild erythema on exposure area. They did not show any sign of pathological lesions. However, rabbits treated with pure trace grade Ag powder dispersed in ethanol and mono ethylene glycol did not show any sign of oedema, erythema and eschar formation. There were no abnormal pathology lesions in the skin tissue.

4. Conclusion

Silver is a relatively rare and poorly soluble element that has been used as an antibacterial agent. Silver compounds are absorbed via the oral, inhalation and both intact and damaged skin (1% of topically applied) routes of exposure [13-14] After absorption, excess silver is stored in the reticulendothelial cells of the skin, liver, spleen, bone marrow, renal glomerulus, lymph nodes, mucous membranes, basement membranes [15-18], central nervous system (CNS) [19], blood vessels, choroid plexus, mesenteric glands and thyroid, adrenals, lungs, dura mater, bones, cartilage, muscles and gastrointestinal tract [19,20]. Argyria, gray or blue-gray discoloration of the skin and mucous membranes, has been occurred in chronic case of silver ingestion. Also, silver-containing granules, particularly in basement membranes and elastic fibers have been observed during histopathologic examination of the skin [13]. The blue color of argyria is not only due to the deposition of metallic silver, but also due to an increased deposition of melanin because of silver melanocyte-stimulating property [21].

Silver is a natural biocide, but compared with many nonessential heavy metals, silver nanoparticles show the highest antimicrobial efficacy against bacteria, viruses, and other eukaryotic microorganisms [22]. Widespread usage of silver nanoparticles because of their antibacterial properties in varied products including textiles, wound dressings, hospital and laboratory gowns and other products which come in direct contact with the skin, dermal exposure must be carefully evaluated. Nanosilver particles may cause inflammatory, oxidative, genotoxic, and cytotoxic effects [21]. In our experiment, the acute dermal toxicity (acute dermal irritation/corrosion) of 10, 20 and 30 nm silver particles was investigated according to the standard procedure (OECD Guidelines, No. 404) for the testing of chemicals. No obvious dermal toxicity was observed after exposure to AgNO\(_3\), trace grade Ag, ethanol and mono ethylene glycol. However, our research concluded that dermal exposure to lesser sizes of silver nanoparticles is more disastrous than greater ones. However, we did not analyze the skin or secondary organs for determination of silver/silver compounds to support that the observed effects are caused by translocated Ag nanoparticles.

Silver nanoparticles with average particle size of 25 nm were shown to penetrate into the upper layers of the epidermis in excised human skin in static diffusion cells [23]. Gopee et al showed that intradermal nanoparticles could enter subcutaneous lymphatics and Tinkle et al [24] reported phagocytosis of the nanoparticles in the skin by macrophages and Langerhans cells and immune system disorders by these particles. Also silver nanoparticles could activate mast cells [25,26]. Monteiro-Riviere et al reported nanoparticles phagocytosis ability of epidermal keratinocytes and provoking inflammatory responses by nanoparticles [27]. Several studies on some types of nanosilver coated dressings showed their cytotoxicity on keratinocytes and fibroblasts which affected on their viability, proliferation and their morphology [28-29]. Nanosilver treated cells exhibited chromosome aberrations, mitotic arrest, cytoskeleton deformations, cell surface ruffling, significant cell morphology alterations, up regulation of metallothionein, heme oxygenase-1 gene [30] decreased
mitochondrial function [31]. Some clinical observations also showed delayed wound healing and inhibition of wound reepithelialization after the use of certain topical silver dressings [32].

However, some in vivo and in vitro studies confirmed biocompatibility of nanosilver-coated dressings [7]. Samberg et al evaluated toxicity of silver nanoparticle in porcine skin and keratinocytes cell culture. They did not observe gross erythema or oedema in macroscopic observations during the entire 14-day in vivo study. However, microscopic observations showed focal inflammation and localization of silver nanoparticle on the surface and in the upper stratum corneum layers of the skin. A significant increase in IL-1β, IL-6, IL-8, and TNF-α concentration was observed. Skin treated with the dosing concentration of 0.34, 3.4 and 34 μg/mL of 20 nm silver nanoparticles showed slight, moderate and severe intracellular and intercellular epidermal edema and focal epidermal and dermal inflammation (spongiosis), epidermal hyperplasia, and parakeratosis. Also, the extension of the rete pegs increased into the superficial papillary layer of the dermis. They also dose-dependent decrease in viability of nano silver treated HEK cells. Silver nanoparticles may interact with proteins and enzymes with thiol groups like glutathione, thioredoxin, SOD and thioredoxin peroxidase within cells which are substantial components of the cell’s antioxidant defense mechanism. In the absence of these substantial components ROS can initiate an inflammatory response and perturbation and destruction of the mitochondria take place. Then apoptogenic factors are released and apoptosis is occurred. Damage to cell membranes appears to be another part of nanosilver’s mechanism of cytotoxicity [7]. Evaluation of acute dermal Irritation / Corrosion of silver nano particles will provide further context of nano particle studies and will provide method for a better production and use of nano- particles. Since staying the nanoparticles onto the skin will change their shape and activity, it is necessary to check their effect in the earliest time. Since dermal contact time should be less than the proposed time in OECD 404 or other guidelines (e.g. EPA) different criteria should be considered for the checking nanoparticles acute dermal Irritation/Corrosion effects. Monitoring time either will be depend on vehicle characterization and type of nanoparticles. However, 4 hours checking time cannot be acceptable because of nanoparticles aggregation. We propose further single oral / parenteral administration of silver nanoparticles to compare dermal administration in rabbits and verification of Ag nanoparticles across skin and into secondary organs.

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