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Dermal Application of Silver Nano-particles on Adult Mice: A Histopathological and immunohistochemistry study of kidney and lung

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Abstract There is an increasing public concern about possible side effects of manufactured nanoparticles because of increasing potential for their exposure. Silver nanoparticles (AgNPs) are one of the most commonly used nanomaterials. So, this research aimed to highlight on the effects of AgNPs (20 nm) on kidney and lung. Fifteen adult mice were divided into two groups: control group (5 mice), and silver group (10 mice) which were exposed to 25µl AgNPs (for each mouse) for four hours dermally. After 14 days, the kidneys and lungs of all mice of the two groups were investigated histopathologically and immunohistochemically. The evaluation of immunohistochemical findings was done by the measurement of immunoreactivity score (IRS) of inducible nitric oxide synthase (iNOS). Fourteen days post dermal exposure to AgNPs revealed histopathological changes of the kidney and lung in the form of congestion and inflammatoy cellular infiltration. Renal and lung IRS of iNOS had significant high score in silver group than control group. Our research concluded that dermal exposure to low dose of small-sized silver nanoparticles was not safe because it clearly caused histopathologic abnormalities of the kidney and lung tissues, and so we need further researches for protection suggestion against this toxicity.

Keywords AgNPs, iNOS, kidney, lung

Introduction

Therapeutic application of nanoparticles in medicine has gained importance because of the reduced size and large ratio of surface-to-volume (Chakraborty et al., 2016). Nanotoxicology is a new branch in toxicological researches, and used to assess the risks of novel nanoproducts. The concern about the toxic properties of nanoparticles on human and environment was increased (Choi et al., 2009).

Silver is a white metal which can be used in making jewelry, dental alloy, conductors, and mirrors. Silver ions are used as disinfectants, antiseptics, microbiocides, and fungicide (Chopra, 2007). The strong antiseptic and antibacterial properties of silver nanoparticles (Ag-NPs) made it one of the most widely used nanomaterials (Benn et al., 2010). There are a lot of products with silver nanoparticles used in the medical field such as heart valves implants, medical face masks, wound dressings and bandages (Theivasanthi & Alagar, 2011).

Nanoparticles toxicity depends on their dose and their routes of entrance into the living system (Rastogi, 2012). The absorption of AgNPs is through the gastrointestinal tract, respiratory tract, skin and other mucous membranes (Pronk et al., 2009). The distribution of AgNPs was mainly in the liver and spleen (Elkhawass et al., 2015). These particles have the ability to generate reactive oxygen and nitrogen species and induce oxidative damage in various cells (Inkielewicz-Stepniak et al., 2014).

Inducible nitric oxide synthase (iNOS) is a calcium-independent inducible isoform of NOS which can be induced by various cytokines (Radi and Murad, 2009). The high-output iNOS are induced in an oxidative environment and so high levels of nitric oxide (NO) (*Mungrue* et al., 2002*)*. NO is a signaling molecule generated by nitric oxide synthase (NOS) that plays an important role in homeostasis (Zielinska et al., 2016). However, abnormal NO generation or metabolism increase the oxidizing stress and thus cellular damage (Heinrich et al., 2013).

The knowledge about nanosilver toxicology has been derived mainly from studies on administration of nanosilver inhalational or by mouth (Korani et al., 2011). So the current study aimed to assess the toxicity potentials of AgNPs by dermal application, and to evaluate its effect on the kidney and lung histopathologicaly and imunohistochemically.

Materials and Methods

Materials & Kits

1- Silver nanosphere (20 nm) solution, its concentration was 20 µg/ml water. Sigma Aldrich Co.

2- Epitope specific antibody to iNOS was purchased from Thermo Fisher Scientific Anatomical Pathology (CA, USA).

Ethical consideration of the study

The animals were acclimatized prior to starting dosing for a period of one week. All aspects of animal care and treatment were carried out according to the local guide lines of the Ethical Committee of Faculty of Medicine- Minia University. All approved conditions used for animal housing and handling were considered. The experimental protocol used followed the regulations for administration and painless sacrifice of the experimental animals. Mice were sacrificed by decapitation after light ether anesthesia and dissected at the end of the experiment.

Experimental protocol

Fifteen mice were randomly divided into two groups: Control group (5 mice), and silver nanoprticles group (10 mice). An area of 0.90 cm × 0.90 cm of the back zone near the vertebral column (the least hair covering) of each animal was shaved for clearing the exposed skin. At these shaved areas, a volume of 25µl of AgNPs solution (20 µg/ml water) taken by micropipette was applied to the silver group and the same amount of distilled water was applied to the control group. These areas were covered with bandages to not lose any of the applied doses when the mice bite or lick themselves. The silver free sterile bandages were applied and fixed with cloth glue for 4 hours, then the bandages were removed (Yarmohammadi et al., 2014).

After two weeks all mice of the two groups were scarified and dissected for removal of their kidneys and lungs which embedded in paraffin. Serial sections from the paraffin-embedded tissues were cut at 5µm-thick, where half numbers of sections was stained with haematoxylin & eosin (H&E) for routine histopathological examination and the other half sections were used for immunohistochemical staining.

N.B. Dermal LD50 of low-sized AgNPs was >2000 mg/kg in rats according to Organization for Economic Cooperation and Development Test Guidelines 402 (Kim et al., 2013). Mouse dose equals half rat dose (Shin et al., 2010), and so dermal LD50 of low-sized AgNPs was >4000 mg/kg in mice. The current used dose was nearly 1/200000 of dermal LD50.

Assessment of immuno-expression of iNOS protein

Half number of paraffin sections was immunostained for anti-iNOS according to manufacture's guidelines, which finally counterstained with hematoxylin (El-Tahawy et al., 2017).

In each section, three high-power fields (×400) were selected for assessment of immunoreactivity score (IRS). IRS was defined as the product of staining intensity (SI) and the percentage of positive cells (PP). Staining intensity was graded as 0 (negative), 1 (weak), 2 (modarate), and 3 (strong); percentage of positive cells was scored as 0 (negative), 1 (< 10%), 2 (11-50%), 3 (51-80%), and 4 (> 80%). IRS values was from 0-12 as follow: 0 as negative, 1-3 as weak, values 4, 6 as moderate positive, and multiplication values 8, 9, 12 as strongly positive (Metindir et al., 2008).

Statistical analysis

Values were expressed as means ± standard deviation (SD). The data were analyzed by using SPSS. The significance of differences between groups was calculated by using student t test and chi-square test. $P < 0.05$ was considered statistically significant.

Results

I- histopathological examination of the renal & lung tissues

Renal sections examined from control mice showed normal kidney structures **(fig.1)**. Administration of AgNPs resulted in congestion and inflammatory cellular infiltration. There was necrosis in some glomerular cells and bowman capsules **(fig. 2).**

Sections from lung tissues of control animals showed no abnormality histopathologicaly **(fig. 3)**, while lung sections of silver group revealed massive inflammatory cellular infiltration and marked congestion **(fig. 4).**The histopathological findings of the kidney and lung noticed in the 2^{nd} group had significant abnormality than the control group **(table1)**.

II- Immuno-expression of iNOS protein in renal & lung tissues

Expression of iNOS in most control kidney sections had weak intensity and percentage of positive cells (pp) score 2 **(fig. 5A)**. Immunohistochemical assessment of renal tissues of silver group showed a significant increase in iNOS protein expression as follow: intensity became moderate **(fig. 5B)** to intense **(fig. 5C)**, and pp revealed score 2-3. So IRS was significantly very high in silver group than control group **(table 1)**.

iNOS expression in lung tissues of the control group displayed weak to moderate intensity and PP score 2 **(fig. 6A)**. Immunohistochemical analysis of lung tissues after AgNPs exposure revealed a significant increase in iNOS protein expression as follow: intensity became moderate **(fig. 6B)** in most sections, and pp was score 3. IRS, which was calculated and expressed in **table 1**, showed significant increase in silver group.

Table (1): Pearson chi-square test of light microscopic findings of the kidney and lung

Changes \ Group	Group(1)	Group(2)	Chi-Square
Renal congestion & inflammation	20%	80%	X^2=5 P=0.025*
Necrosis in glomerular cells or bowman capsules	0%	30%	X^2=1.88 P=0.17
Lung congestion & inflammation	0%	70%	X^2=6.56 P=0.01*

**p<0.05: Significant*

Table (2): Student "t" test statistical analysis of IRS in control and silver groups.

	Mean	SD	t	P
Control kidney	1.6	6.6	4.874	0.0003*
silver kidney	5.5	2.22		
Control lung	2.6	1.34	4.176	0.0011*
Silver lung	5.2	1.03		

**p<0.05: Significant*

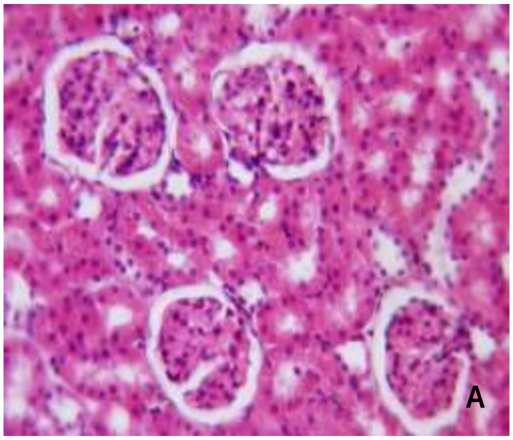

Fig (1): photomicrograph of kidney sections of the control group showing normal parenchyma (H&E X250)

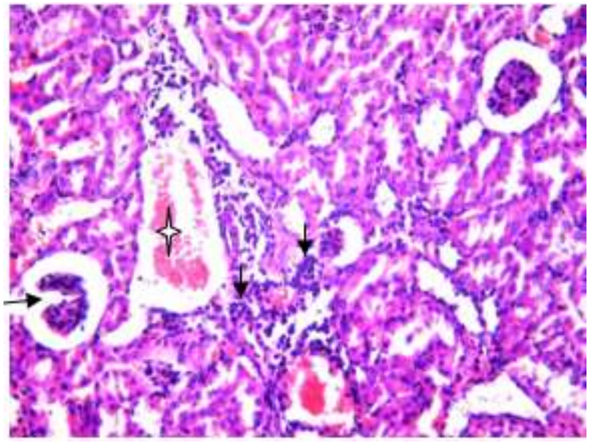

Fig (2): photomicrograph of kidney sections of silver group showed necrosis of glomerular cells and bowman capsule (long arrow), congestion (star) and inflammatory cell infiltration (short arrow) (H&E X250)

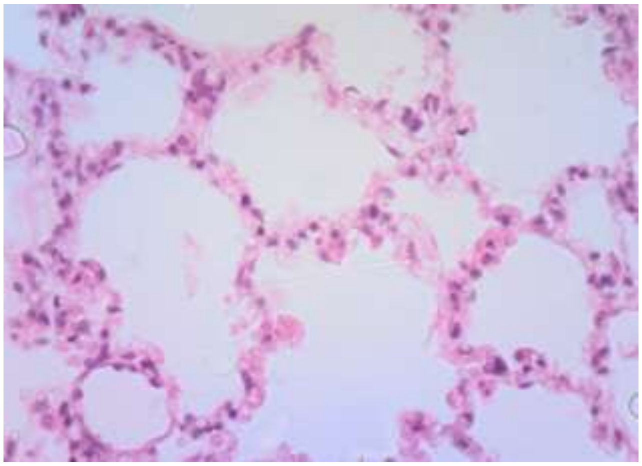

Fig (3): photomicrograph of kidney sections of control group showing normal lung architecture (H&E X250)

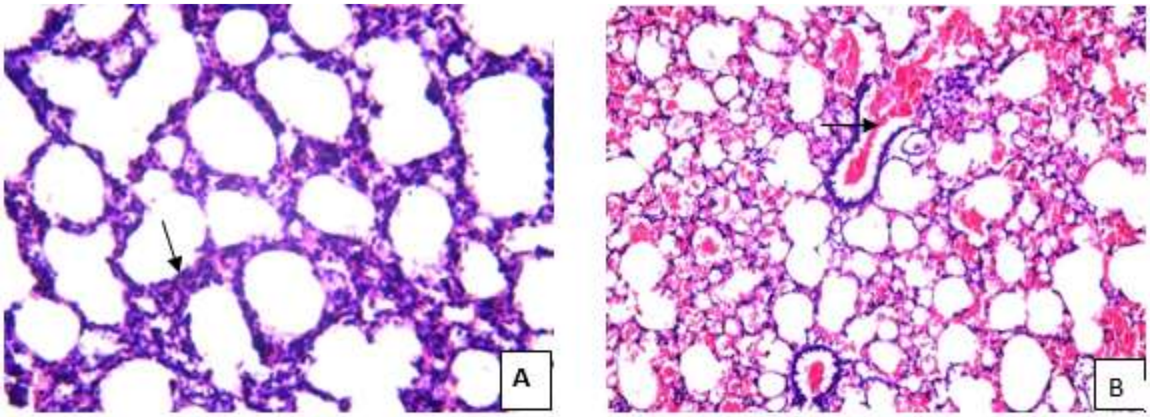

Fig (4): photomicrograph of kidney sections of the silver group showed: A) massive inflammatory cellular infiltration, B) marked congestion (H&E X250)

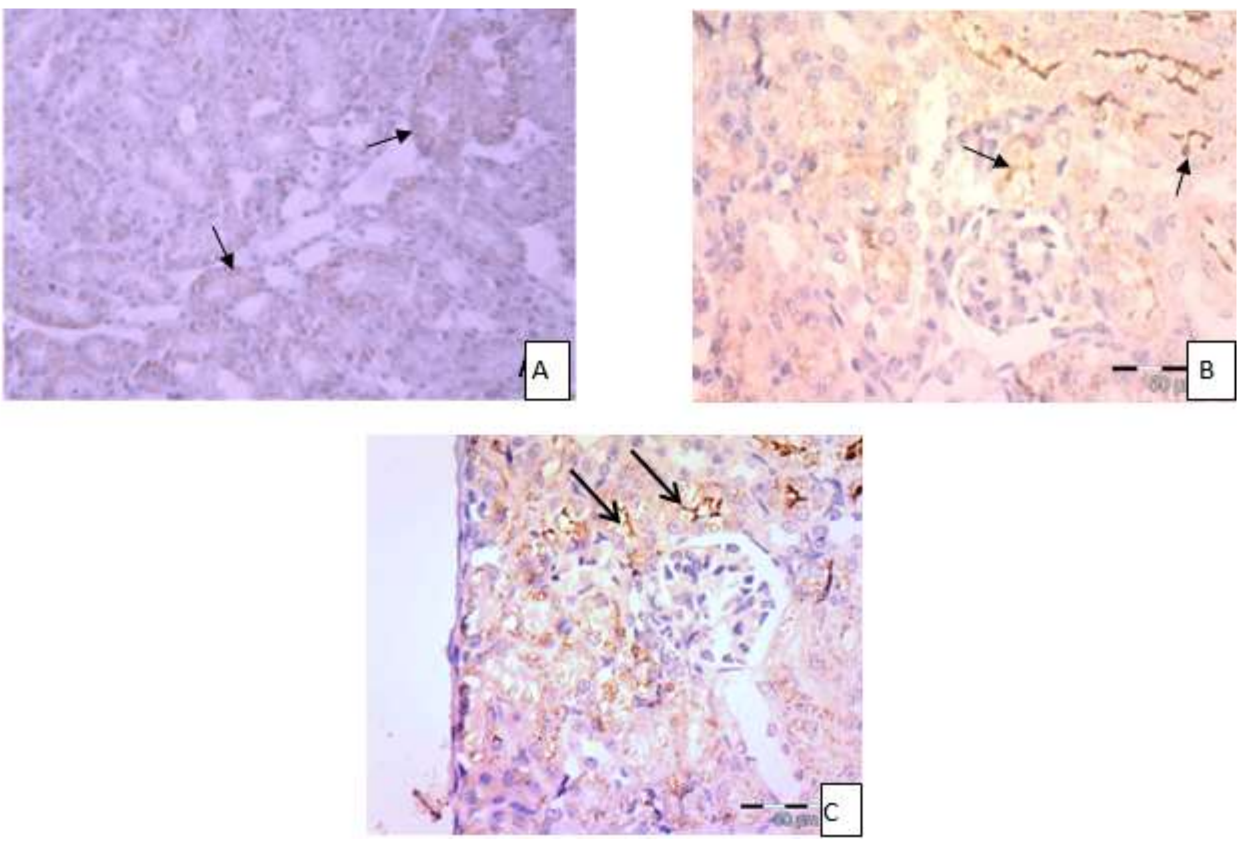

Fig. (5): Immunohistochemical expression of iNOS in the kidney tissue showed: A) control group with low intensity. B) silver group with moderate intensity. C) silver group with intense intensity (H X 400)

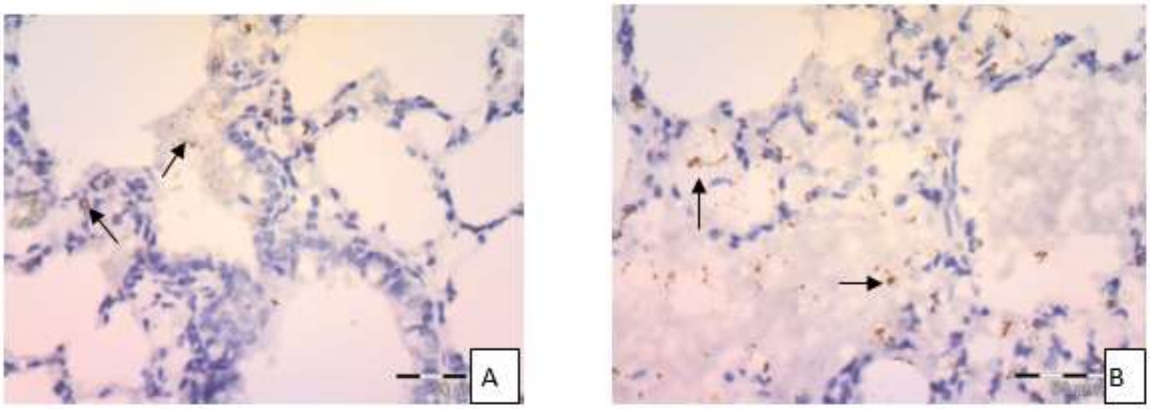

Fig. (6): Immunohistochemical expression of iNOS in the lung tissue showed: A) control group with low intensity. B) silver group with moderate intensity. (H X 400)

Discussion

The larger spectrum of Silver nanoparticles (AgNPs) applications in biomedicine and related fields are due to the great amount of their flexible properties. Recently, multiple tests have been done to give information about AgNPs toxic effects on living tissues and organisms (Marin et al., 2015).

The present study clearly showed that Ag-NPs used for wound healing in adult mice have produced the histopathological abnormalities in the kidney and lung, and these results are in line with Cha et al. (2008). Low dose of silver results in hepatic and renal toxicity, while high dose may cause death (Tang & Xi, 2008).

In our study, many histological changes in the kidney of the treated animal were observed in the form of glomerular necrosis, congestion, and inflammatory cellular infiltration. These results are in acceptance with Sardari et al. (2012), who reported tubular damage and glomerular necrosis in the kidney with the high doses of silver NPs.

Wen et al. (2017), found that intravenous AgNPs 24 hours before scarification resulted in diffuse hyaline degeneration in renal tubular epithelial cells. But Chakraborty et al. (2016), observed no abnormality in the kidney histopathologicaly, and that contrast with the present results may be due to the difference in the dose and route (1–10 mg/kg, SC).

Necrosis was the type of cell death in the current study, and this finding is against Cha et al. (2008), who found that AgNPs toxicity was in the form of apoptosis. In response to oxidative stress, necrosis not apoptosis is the main type of cell death (Hanus et al., 2013)

In the present study lungs of the rats exposed to silver NPs showed marked inflammatory cellular infiltration, and many areas of marked congestion. A recent study (Holland et al., 2016) showed vascular injury after 7 days of repeated Ag NPs (20 nm) pulmonary exposure. AgNPs induced features characteristic of asthma as pulmonary eosinophilia and neutrophilic inflammation with bronchial hyper responsiveness (Seiffert et al., 2015)

On the other hand, Wen et al. (2017), couldn't observe the acute lung toxicity induced by NPs in their short study period (24 hours). They reported that long administration period of NPs could lead to chronic lung toxic effects as these NPs could distribute into the pulmonary circulation and accumulated in the lungs. Chen et al. (2013), explained that the transformation of Ag+ ions to insoluble silver sulfide (Ag2S) decreases the lung toxicity of silver nanomaterials.

Cytotoxicity of AgNPs is mainly due to the release of Ag+ ions, which interact through different damaging mechanisms. One of these mechanisms is the interaction with cell membranes leading to lower membrane integrity and increased permeability; and another mechanism is through binding with thiol groups in proteins causing improper protein function (De Matteis *et al., 2015).* The toxic effects result from binding of AgNPs with the organ tissues are cell activation, reactive oxygen species (ROS) production, inflammation and finally cell death. High production of ROS leads to increase amount of H_2O_2 in the cellular environment that can damage DNA and oxidize cellular proteins (Patlolla et al., 2015).

In contrary to the previous researches and the current study, Gonzalez-Carter et al. (2017), reported that AgNPs had anti-inflammatory effects as evident in reduction of pro-inflammatory factors such as tumour necrosis factor (TNF)-α, ROS and nitric oxide (NO). AgNPs lowered microglial inflammation through up-regulation of H2S-synthesizing enzymes, and formation of Ag2S complexes around AgNPs that may represent an Ag+-sequestering and detoxifying mechanism (Vrcek *et al.,* 2016).

Immunohistochemical study of renal and lung tissues of silver group showed a significant increase in inducible nitric oxide synthase (iNOS) protein expression (score 2-3) and its intensity became moderate to intense. INOS expression attributed to increase production of NO (Amin et al., 2016). NO in the kidney has been implicated in the glomerulonephritis pathogenesis (Furusu et al., 1998). Xu et al. (2015), suggested that the inflammatory signal pathways may be important in AgNP induced toxicity. One of the direct consequences of the inflammatory process is the expression of iNOS (Suschek et al., 2004). INOS produces NO that acts as a regulatory and pro-inflammatory mediator in inflammation (Hämäläinen et al., 2008).

AgNPs with average size 18.3±2.6 nm (dose 30 or 60 μg/ml) elevated the levels of NO with concomitant upregulation of iNOS mRNA and protein (Zielinska et al., 2016). In contrast with these findings, Amin et al. (2016), showed that AgNPs with average size 30.92nm (dose 1/10 LD50) has no effect on serum NO when compared the silver group with the controls.

Conclusion

The target organs for silver nanoparticles were the kidney and lung in the male mice after dermal application. Exposure to very low dose (25μl of 20 μg/ml) of silver nanoparticles solution (20 nm) in mice which is nearly 1/200000 of LD50 is not safe and may result in kidney and lung affection. AgNPs induced inflammation as detected histopathologicaly and ensured by presence of higher expression of iNOS.

Recommendations

1- Broadcasting this effect must be done for both physicians and people, and at the same time further researches must be done to suggest protection against this toxicity.

2- The toxicity profile of different routes of different sized AgNPs should be determined in future studies.

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الملخص العربى

الاستعمال الجلدى للجسيمات النانوية للفضة على الجرذان البالغة: دراسة هستوباثولوجية و كيمونسيجية مناعية للكلى والرئة

إيمان إسماعيل حسن١ و فاطمة الزهراء فؤاد عبدالباقي علام ٢

هناك قلق عام متزايد حول الآثار الجانبية المحتملة للجسيمات النانوية المصنعة بسبب زيادة إمكانية التعرض لها. الجسيمات النانوية للفضة هي واحدة من المواد النانوية الأكثر استخداما. لذلك، يهدف هذا البحث إلى تسليط الضوء على آثار الجسيمات النانوية للفضة (٢٠ نانومتر) على الكلى والرئة.

فقد تم تقسيم خمسة عشر جرذان بالغين إلى مجموعتين: المجموعة الضابطة (٥ جرذان)، ومجموعة الفضة (١٠ جرذان) التي تعرضت ل ٢٥ ميكرولتر من الجسيمات النانوية للفضة (لكل جرذ) لمدة أربع ساعات عن طريق الجلد. ثم بعد ١٤ يوما تم فحص التغيرات الهستوباثولوجية للكلى والرئتين فى جميع الجرذان من المجموعتين. كما تم تقييم التغيرات الكيمونسيجية المناعية للكلى والرئتين من خلال قياس قيم التفاعل المناعى لبروتين سينسيز أكسيد النيتريك المحرض.

وقد كشف فحص الكلى والرئتين بعد ١٤ يوما من تعرض الجلد للجسيمات النانوية للفضة عن وجود تغيرات مرضية في صورة احتقان والتهاب خلوي.اما قيم التفاعل المناعى لبروتين سينسيز أكسيد النيتريك المحرض فى الكلى والرئة فقد كانت قيمها عالية في مجموعة الفضة بالنسبة للمجموعة الضابطة.

نستخلص من النتائج السابقة أن التعرض الجلدي لجرعة منخفضة من الجسيمات النانوية الفضية الصغيرة الحجم ليس امنا بدليل انه تسبب فى تغيرات مرضية لانسجة الكلى والرئتين بشكل واضح ، ولذا فإننا بحاجة إلى مزيد من الأبحاث لاقتراح الحماية الممكنة ضد هذه السمية.

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