İstanbul Üniversitesi
Veteriner Fakültesi Dergisi

Journal of the Faculty of
Veterinary Medicine Istanbul University

*İstanbul Üniv. Vet. Fak. Derg. / J. Fac. Vet. Med. Istanbul Univ., 41 (2), 205-211, 2015*
*doi: 10.16988/iuvfd.2015.93581*

***Araştırma Makalesi*** ***Research Article***

# Evaluation of Protective Effect of Penicillamine on Silver Nanoparticles-Induced Oxidative Stress in BALB/c Mice

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***Geliş Tarihi / Received:***
8 October 2014

***Kabul Tarihi / Accepted:***
1 December 2014

***Anahtar Kelimeler:***
Gümüş nanopartikülleri, oksidatif stres, penisilamin, histopatoloji, biyokimyasal parametreler, BALB/c fareler

***Key Words:***
Silver nanoparticles, oxidative stress, penicillamine, histopathology, biochemical parameters, BALB/c mice

**Abstract**

Despite the negative effects of silver nanoparticles, their application is growing rapidly, so this study was designed to investigate the adverse effects of nanosilver administration on histopathological changes and biochemical parameters as well as therapeutic efficacy of penicillamine. Four groups of six BALB/c mice were treated intraperitoneally with various dose of nanosilver and the LD50 was calculated. To investigate therapeutic efficacy of penicillamine, forty-two mice were assigned into equal seven groups. The animals of each group were treated intraperitoneally with LD50 of nanosilver and 50 or 200 mg/kg penicillamine concurrently, or 4 hours after nanosilver injection. Histopathological and serum biochemical analyses including total protein, albumin, total globulin, cholesterol, triglyceride, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total antioxidant capacity (TAC) and Malondialdehyde (MDA) were carried out. The LD50 was 1500 PPM. Results showed an increase in the levels of AST and ALT enzymes and the MDA in the mice receiving nanosilver while TAC concentration decreased. These alterations were statistically significant between the mice receiving nanosilver and those receiving nanosilver and 200 mg/kg penicillamine. Also, histopathological examination of the tissue sections of the the mice receiving 1500 PPM nanosilver showed hepatic and renal damage. Whereas, the biochemical parameters and tissue lesions improved in mice receiving 200 mg/kg penicillamine in comparison with the nanosilver exposed mice and it was shown that the extent of oxidative stress was decreased.

**Özet**

**BALB/c Farelerinde Oluşan Oksidatif Stres Üzerine Gümüş Nanopartiküllerle İndüklenmiş Penisilaminin Koruyucu Etkisinin Değerlendirilmesi**

Gümüş nanopartiküllerinin olumsuz etkilerine rağmen uygulamaları hızla artmaktadır. Bu çalışma nanogümüş uygulamasının histopatolojik değişiklikler ve biyokimyasal parametreler üzerindeki istenmeyen etkilerini ve aynı zamanda penisilaminin terapötik etkinliğini araştırmak için düzenlenmiştir. Altışarlı dört grup BALB/c faresine değişik dozlardaki nanogümüş intraperitoneal yoldan uygulandı ve $LD_{50}$ hesaplandı. Penisilaminin terapötik etkinliğini araştırmak amacıyla 42 fare eşit yedi gruba ayrıldı. Her gruptaki hayvanlara $LD_{50}$ nanogümüş ve eşzamanlı olarak ya da nanogümüş enjeksiyonundan 4 saat sonra 50 ya da 200 mg/kg penisilinamin intraperitoneal yoldan uygulandı. Toplam protein, albumin, toplam globulin, kolesterol, trigliserit, aspartat aminotransferaz (ALT), alanin aminotransferaz (ALT), alkalin fosfataz (ALP), toplam antioksidan kapasite (TAC) ve malondialdehit (MDA) de dahil olmak üzere serum biyokimyasal ve histopatolojik analizler yürütüldü. $LD_{50}$ 1500 ppm'di. Sonuçlar nanogümüş alan farelerde AST ve ALT enzimleri ve MDA düzeylerinde artış gösterirken TAC düzeylerinde azalma oluştu. Bu farklılıklar özellikle nanogümüş alan fareler ile nanogümüş ve 200 mg/kg penisilamin alan farelerde istatistiksel olarak anlamlıydı. Ayrıca, 1500 ppm nanogümüş alan farelerin doku bölümlerinin histopatolojik incelenmesinde karaciğer ve böbrek hasarı olduğu görüldü. Öte yandan, nanogümüşe maruz kalmış farelere karşılık 200 mg/kg penisilamin alan farelerde biyokimyasal parametreler ve doku lezyonlarında iyileşme oldu ve oksidatif stres boyutunun azaldığı belirlendi.

## Introduction

Nanoparticles are small scale substances (<100 nm), which their exposure is increasing among humans and animals due to the increase in their applications (Stebounova et al., 2011). Different types of the industrial silver compounds such as nitrate, sulfate and oxide (Weast et al., 1988) can be released into the environment from different sources (Rosenman et al., 1972).

Nanosilver particles can be used in various sciences, especially in medicine. Antimicrobial properties of silver nanoparticles have recently been recognized and they have been extensively used in some medical applications such as silver-coated medical devices (Park et al., 1999). Although nanosilver elevates the therapeutic effects of silver, its safety has been discussed in comparison with silver compounds. The antibacterial features of silver nanoparticles are related to some biological events including linking to cells membranes, binding to thiol groups on proteins, superficial absorption, changes of membranous permeability, creating oxidative stress and inactivation of cell enzymes (Akradi et al., 2012). Hence, they can produce the poisonous properties of nanosilver and have negative effects on health and the environment. On the other hand, significant concerns have lately been presented regarding the potential risk of nanosilver because of their high exposure and probable toxicity in the environment (Klaine et al., 2008; Luoma, 2008).

Most of the compounds that enter the body are metabolized in the liver. So liver is damaged by compounds with side effects more than any other tissue (Akradi et al., 2012). One of the main concerns for the use of nanoparticles is oxidative injury (Kim et al., 2009). It has recently been reported that nanoparticles could produce free radicals. Regarding the previous studies, silver nanoparticles can damage different organs, especially liver tissue (Akradi et al., 2012; Ji et al., 2007).

Penicillamine is a pharmaceutical of the chelator class which is used in treatment of metal poisoning, especially copper and lead. Its chelating effect is related to the supply of a sulphydryl group that combines with lead or copper to produce ring compounds and excrete them (Beattie, 1977). Squitti et al. (2002) have reported that copper could play a role in the production of peroxides in Alzheimer's disease and penicillamine has been effective in reducing oxidative damage in Alzheimer's disease.

Despite the negative effects of Silver nanoparticles, their application is growing rapidly, so the present study was conducted to investigate the adverse effects of nanosilver administration on histopathological and biochemical parameters and therapeutic efficacy of penicillamine.

## Materials and Methods

### Preparation of Nanoparticles

Suspension of silver nanoparticles (Nanonasb Pars Company, Tehran, Iran) were provided with a concentration of 2000 PPM, 20 nm diameter and 98% purity. The suspension was processed by ultrasonication (3×20 s at 65 W) at 1°C yielding a homogeneous soluble. Then they were diluted with deionized water and sterilized with microfilter (0.22 micron).

### Animal Ethics

This experiment was accomplished under the approval of the state committee on animal ethics, Shiraz University, Shiraz, Iran. Also, the recommendations of European Council Directive (86/609/EC) of November 24, 1986, were used regarding the standards in the protection of animals used for experimental purposes.

### LD50 Determination

Four groups of six BALB/c mice, approximately 6 weeks old, were treated intraperitoneally with 900, 1200, 1500 and 1800PPM nanosilver. The LD50 was calculated by the up and down dosing (Choi, 1990) and this estimated dose of the nanosilver was used for experimental protocol.

### Experimental Protocol

To investigate therapeutic efficacy of penicillamine, forty-two female BALB/c mice were included. All mice were housed in stainless steel cages and allowed to adapt to the conditions of the animal house for 14 days before the experiments. The animals were randomly assigned into seven equal groups of six. The seventh group served as control (G-0). The animals of each group were treated intraperitoneally with LD50 of nanosilver (G-1), LD50 of nanosilver and 50 mg/kg penicillamine (Capsule 250 mg, Haupt Pharma, Germany) concurrently (G-4) or 4 hours after nanosilver injection (G-5), LD50 of nanosilver and 200 mg/kg penicillamine concurrently (G-3) or 4 hours after nanosilver injection (G-6), just penicillamine (G-2). Blood samples were collected from the heart of the mice into vacutainers without anticoagulant, and serum was separated by centrifugation at 750 g for 15 min and stored in a freezer at -20°C until use.

### Serum Biochemical Analyses

All biochemical parameters including total protein, albumin, cholesterol, triglyceride, AST, ALT and ALP were measured by commercial kits (Pars Azmoon Co., Tehran, Iran). Total globulin was measured by the difference of total protein and albumin. All the enzyme

activities were measured at 37°C and the results have been presented in units per liter (Burtis and Ashwood, 1994). Biochemical analyses were measured using a standard autoanalyser with veterinary software (Cobas-Mira, ABX-Diagnostics, Japan).

**Measurement of MDA**

To evaluate lipid peroxidation in serum, a modified HPLC method was used based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA), to form a colored MDA–TBA adduct (Lykkesfeldt, 2001). 40 µL of sample was diluted with 100 µL of $H_2O$ and mixed with 20 µL of 2.8 mmol/L butylated-hydroxytoluene (BHT) in ethanol, 40 µL of 81 g/L sodium dodecyl sulfate and 600 µL of TBA reagent (8 g/L TBA diluted 1:1 with 200 ml/L acetic acid adjusted to pH 3.5 with NaOH). The mixture was immediately heated (60 min at 95°C) and cooled with running water; 200 µL of $H_2O$ and 1000 µL of butanol–pyridine (15:1, v/v) were then added. After vigorous mixing, the organic layer was separated by centrifugation (3 min at 16,000 g). The supernatant was analyzed on a UV-visible spectrophotometer fitted with an 80 µL flow cell. The absorbance was measured at 532 nm (the mobile phase consisted of 300 ml/L methanol in 50 mmol/L potassium dihydrogen phosphate buffer, pH 7.0). 1, 1, 3, 3-tetraethoxypropane was used as a standard, and MDA-TBA reactive substances' values were expressed as MDA mili-mole per liter (mmol/L). The HPLC system consisted of a solvent delivery pump (JASCO 980-PU, Tokyo, Japan), a reversed-phase column (Luna C18, 250 mm × 4.6 mm, Phenomenex, CA, USA), and a UV–Vis detector (Jasco, UV-975, Tokyo, Japan) operated at 532 nm.

**Measurement of total antioxidant capacity (TAC)**

Determination of total antioxidant capacity (TAC) in serum by commercial kit (Labor Diagnostika Nord (LDN) Com, Nordhorn, Germany) was based on the reaction of peroxides with peroxidase followed by a color reaction of the chromogenic substrate tetramethylbenzidine. The change in color was measured colorimetrically at 450 nm and expressed as millimoles per liter.

**Histopathological examination**

Liver, kidneys, lungs, brain and heart tissues were collected from animals. The tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 µm and stained with haematoxylin and eosin (H&E) for light microscopic examination.

**Statistical analysis**

The results were expressed as mean ± standard deviation (SD). The data were analyzed statistically by one-way ANOVA with Tukey's post-hoc test for comparison of different biochemical parameters, using SPSS software, version 11.5. $P<0.05$ was considered as significant.

## Results

As is showed in Table 1, In the groups treated with 1800, 1500 and 1200 PPM nanosilver; 6, 3 and 2 mice died respectively and they showed clinical symptoms including ruffled coat, inactivity, recumbence and anorexia before death. All the mice treated with 900 PPM survived without any clinical symptoms. The LD50 for silver nanoparticles as determined by up and down dosing was 1500 PPM.

**Table 1.** Experimental design.
**Tablo 1.** Deney grupları.

| Group | n | Inoculation |
|---|---|---|
| **G-1** | 6 | 1500 PPM NS |
| **G-5** | 6 | 1500 PPM NS/after 4h 50 mg/kg PC |
| **G-4** | 6 | 1500 PPM NS/concurrent 50 mg/kg PC |
| **G-6** | 6 | 1500 PPM NS/after 4h 200 mg/kg PC |
| **G-3** | 6 | 1500 PPM NS/concurrent 200mg/kg PC |
| **G-2** | 6 | PC |
| **G-0** | 6 | - |

NS: nanosilver; PC: penicillamine

The mean ± SD of serum biochemical values are presented in Table 2. Evaluation of biochemical parameters in different groups revealed an increase in the levels of AST and ALT enzymes in G1-G6 groups when compared with G-0 group and these changes were statistically significant between the mice receiving nanosilver (G-1) and those receiving nanosilver and 200 mg/kg penicillamine (G-3 and 6). Despite the observed increase in the enzymes of mice receiving nanosilver and 50 mg/kg penicillamine (G-4 and 5), no significant differences were observed with the G-1 mice. In addition, no significant differences were seen between mice receiving 200 mg/kg penicillamine concurrently, or 4 hours after nanosilver injection.

Statistical results showed that the MDA increased while TAC concentration decreased. These alterations were significant between the nanosilver exposed mice (G-1) and those receiving penicillamine and also between those receiving nanosilver and 50 (G-4 and 5) and 200 (G-3 and 6) mg/kg penicillamine. These parameters were statistically significant ($P<0.05$).

By contrast, we found no significant differences between the different groups in ALP, total protein, albumin, total globulin, cholesterol and triglyceride concentrations.

Histopathological examination of the tissue sections from the mice of G-1 group that were treated with silver

nanoparticles, showed moderate degeneration of hepatocytes with mononuclear cells infiltration (Figure 1a). Interstitial nephritis and cell swelling were evident in the tissue sections (Figure 2a). Interstitial pneumonia, peribronchiolitis, development of bronchiolar associated lymphatic tissue (BALT) with macrophages and lymphocytes infiltration in the lumen of the alveoli was also seen in the tissue sections of these mice. No significant lesion was observed in the brain and heart.

Lesions were also seen in the tissue sections of the mice receiving penicillamine, but the severity of these lesions decreased especially in the mice receiving 200 mg/kg penicillamine in comparison with those receiving nanosilver (Figures 1b and 2b).

No lesions were found in the tissue sections of the brain, heart, lungs, liver and kidneys of the control animals.

## Discussion

Nanosilver particles are dislocated in the blood circulation and disseminated throughout the main organs (Tang et al., 2009). Since detoxification of most toxins in the body occurs in the liver, it can result in hepatic enzyme changes. It has been reported that silver nanoparticles have caused hepatotoxicity or renal toxicity by oral, inhalation or subcutaneous administration (Kim et al., 2009; Tang et al., 2009). In the present study, the significant increase in AST and ALT after nanosilver exposure was in accordance with hepatic damage in just the nanosilver exposed mice. Free radicals produced by silver nanoparticles led to hepatocyte damage and released hepatic enzymes. The hepatic and renal histopathological findings in the present study are consistent with previous studies (Choi et al., 2010; Park et al., 2010).

Previously, it was shown that nanoparticles like manganese and copper produce free radicals and oxidative stress (Hussain et al., 2006). Deposition of nanoparticles such as titanium dioxide, zinc oxide, cerium oxide and silver nanoparticle inside the cellular organelles or on the cellular surface and induction of oxidative stress signaling cascades have shown to ultimately lead to oxidative stress to the cell (Buzea et al., 2007). The increased free radicals and the inadequate response of antioxidant systems can lead to cellular damage. Silver nanoparticles result in cell death via oxidative stress-related mechanisms which cause DNA damage (Hsin et al., 2008). These particles have also presented cytotoxicity on the HeLa cells through apoptotic process (Miura and Shinohara, 2009). In fact, the nanosilver-induced apoptosis is associated with the generation of reactive oxygen species (ROS) and Jun N-terminal kinases (JNK) activation (Hsin et al., 2008).

The oxidative stress induction and enhancement of white blood cells have been observed in the intraperitoneally injected rats by nanosilver particles (Machiedo et al., 1989; Naghsh et al., 2005). Oxidative stress can cause lipid peroxidation and DNA damage. MDA is one of the major products of lipid peroxidation (Pilz et al., 2000; Suttnar et al., 1997). Determination of this marker has been widely used as the most common approach for the assessment of lipoperoxidation (Suttnar et al., 2001). Reduced antioxidant capacity results in the accumulation of free radicals. In the present study, the content of MDA increased and the TAC concentration decreased, suggesting that the balance between the oxidative and antioxidant system was broken during the exposure of silver nanoparticles. Metals such as Cu and Fe induce MDA and 4-hydroxynonenal as DNA damaging end-products which act as inflammatory mediators (Manke et al., 2013). Exposure to such nanoparticles was reported to induce tissue damage resulting from lipid peroxidation (Napierska et al., 2012; Shukla et al., 2011; Turski et al., 2009).

Considering the high exposure and toxicity risk of silver nanoparticles, it appears a substance is needed to reduce their adverse effects. After treatment with penicillamine, the biochemical parameters and tissue lesions improved in mice receiving 200 mg/kg penicillamine in comparison with the nanosilver exposed mice and the extent of oxidative stress was decreased. Soroka et al. (1992) showed that the antioxidative action of penicillamine is because of both its chelating properties and direct anti-radical influence. Hence, It seems that penicillamine treatment of mice produced increased renal excretion of nanosilver particles.

## Conclusion

Histopathological examination of the tissue sections from the mice treated with silver nanoparticles showed hepatic and renal damage. After treatment with penicillamine, the biochemical parameters and tissue lesions improved and it was shown that the extent of oxidative stress decreased.

## Acknowledgments

The authors would like to thank the Research Council of Shiraz University and School of Veterinary Medicine, Shiraz University for financial and technical support of this study (Grant No. 71-GR-VT-5).

**Table 2.** Mean ± SD of serum biochemical values in BALB/c mice.

**Tablo 2.** BALB/c farelerindeki serum biyokimyasal değerlerinin ortalaması ve standart sapması.

| | ALT (U/L) | AST (U/L) | ALP (U/L) | Total protein (g/L) | Triglyceride (mmol/L) | Albumin (g/L) | Total Globulin (g/L) | Cholesterol (mmol/L) | TAC (mmol /L) | MDA (nmol/ml) |
|---|---|---|---|---|---|---|---|---|---|---|
| **G-1** | 72.00±5.35$^{a}$ | 76.00±4.24$^{a}$ | 220.50±29.01$^{a}$ | 7.55±0.36$^{a}$ | 209.50±22.36$^{a}$ | 4.12±0.09$^{a}$ | 3.42±0.38$^{a}$ | 44.25±4.50$^{a}$ | 1.67±0.06$^{c}$ | 3.57±0.22$^{a}$ |
| **G-5** | 68.16±7.90$^{a}$ | 72.33±8.11$^{a}$ | 213.16±26.11$^{a}$ | 7.50±0.30$^{a}$ | 197.66±18.10$^{a}$ | 4.15±0.10$^{a}$ | 3.35±0.33$^{a}$ | 38.50±5.46$^{ab}$ | 1.73±0.06$^{c}$ | 3.10±0.12$^{b}$ |
| **G-4** | 66.89±6.43$^{a}$ | 70.66±6.21$^{a}$ | 211.83±26.83$^{a}$ | 7.46±0.25$^{a}$ | 195.16±17.24$^{a}$ | 4.20±0.12$^{a}$ | 3.26±0.23$^{a}$ | 40.33±5.35$^{ab}$ | 1.87±0.05$^{bc}$ | 3.10±0.08$^{b}$ |
| **G-6** | 45.66±2.80$^{b}$ | 50.00±3.74$^{b}$ | 200.16±41.72$^{a}$ | 7.45±0.21$^{a}$ | 189.66±17.30$^{a}$ | 4.21±0.14$^{a}$ | 3.23±0.29$^{a}$ | 32.50±7.94$^{ab}$ | 1.99±0.22$^{abc}$ | 2.88±0.11$^{bc}$ |
| **G-3** | 41.66±3.32$^{bc}$ | 45.33±3.14$^{b}$ | 190.66±37.59$^{a}$ | 7.41±0.21$^{a}$ | 180.33±16.63$^{a}$ | 4.21±0.13$^{a}$ | 3.20±0.16$^{a}$ | 30.66±8.09$^{ab}$ | 2.16±0.25$^{ab}$ | 2.71±0.11$^{cd}$ |
| **G-2** | 37.25±4.03$^{bc}$ | 41.25±3.40$^{b}$ | 191.75±28.72$^{a}$ | 7.35±0.12$^{a}$ | 180.50±22.47$^{a}$ | 4.22±0.17$^{a}$ | 3.12±0.15$^{a}$ | 27.75±8.77$^{b}$ | 2.36±0.36$^{a}$ | 2.52±0.33$^{cd}$ |
| **G-0** | 35.50±3.27$^{c}$ | 41.33±4.50$^{b}$ | 185.00±32.71$^{a}$ | 7.45±0.25$^{a}$ | 175.50±16.41$^{a}$ | 4.20±0.14$^{a}$ | 3.20±0.21$^{a}$ | 26.83±8.13$^{b}$ | 2.36±0.29$^{a}$ | 2.48±0.28$^{d}$ |

ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; TAC: total antioxidant capacity; MDA: Malondialdehyde
Different letters indicate statistically significant differences (P<0.05).

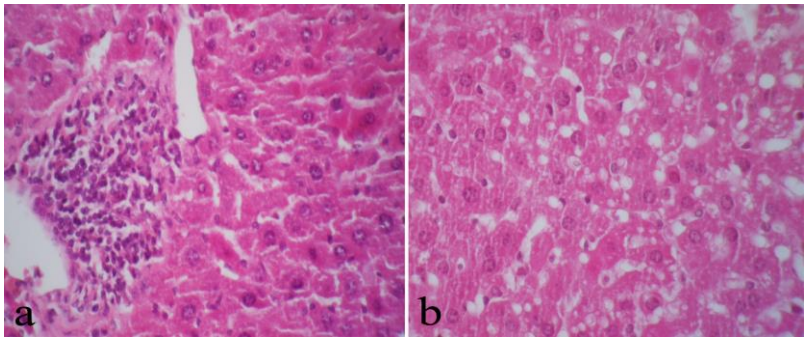


**Figure 1.** Liver sections. a) A mouse receiving nanosilver showed degeneration of hepatocytes with mononuclear cells infiltration, b) a mouse receiving 200 mg/kg penicillamine represented vacuolar degeneration in hepatocytes, H and E, x180.

**Şekil 1.** Karaciğer bölümleri. A) Nanogümüş alan bir farede mononükleer hücre infitrasyonu ile birlikte hepatosit dejenerasyonu görüldü, b) 200 mg/kg penisilamin alan bir farede hepatositlerde vaküolar dejenerasyon görüldü, H ve E, x180.

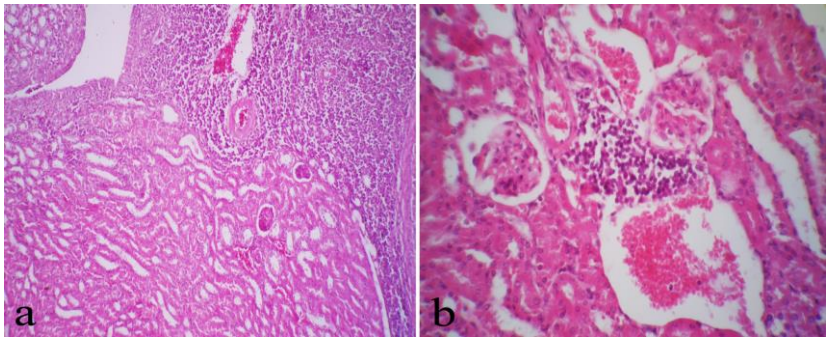


**Figure 2.** Kidney sections. a) Interstitial nephritis was evident in the tissue section of the mouse receiving nanosilver, b) the severity of these lesions decreased in the mouse receiving 200 mg/kg penicillamine, H and E, x180.

**Şekil 2.** Böbrek bölümleri. A) Nanogümüş alan farenin doku bölümünde interstisyal nefrit belirgindi, b) Bu lezyonların ciddiyeti 200 mg/kg penisilamin alan farede azaldı, H ve E, x180.

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