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ORIGINAL RESEARCH PAPER

Evaluation the effect of silver nanoparticles on oxidative stress biomarkers in blood serum and liver and kidney tissues

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ABSTRACT

Objective(s): Silver nanoparticles (Ag-NPs) are one of the most widely used nanomaterials recently. Despite the wide application of nanomaterials, there is limited information concerning their impact on human health and the environment. This study aimed to find the effects of Ag-NPs (40 nm) on blood serum, liver and kidney tissues of homing pigeons (Columbia livia).

Materials and Methods: Columba livia, in vivo model used in ecotoxicity experiments were gavaged 3 times daily with 75 and 150 ppm of Ag-NPs within 14 days. A group of 30 Pigeon were randomly divided into three groups: Ag-NPs exposed and control groups (n=10). Data analysis was counducted by performing one-way variance (ANOVA) in SPSS.v.16.0

Results: The results of this study illustrated that in the enzyme activity of Glutathione S –transferase (GST), Aspartate amino transferase (AST), Alanine amino transferase (ALT) and lactate dehydrogenas (LDH) there is a significant difference between treatment groups with Ag-NPs and the control group. Also, lipid peroxidation (LPO) analysis and catalase activity CAT) suggest Ag-NPs cause the main damage to the liver tissue. On the other hand: Ag-NPs have toxic and harmful effects in both concentrations (75 and 150 ppm), and cause LPO induction, oxidative stress and increase of biomarkers of liver necrosis in under treatment pigeons.

Conclusion: The results of this study show that the organism's exposure to Ag-NPs cause toxicity that is dose-dependant. in this study, the highest damage was observed in the liver. However, this issue will have to be considered more extensively in further studies.

Keywords: *Aspartate amino transferase (AST), Alanine amino transferase (ALT), Oxidative stress biomarkers, Pigeon, Silver nanoparticles (Ag-NPs)*

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INTRODUCTION

Nanotechnology is an expanding science, which its products will have a dramatic effect on the lives of humans. Metal nanoparticles possess unique properties due to their size, shape, surface structure, aggregation characteristics and chemical composition that separates them from their corresponding soluble metals.

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The distinct characteristics of nanoparticles (e.g. preparation method, type of capping agent, size, and shape) may reverse their effects on living organisms, leading some scientists to suggest that the toxicity of these new materials needs to be investigated case by case. Many studies conducted in recent years indicated negative and harmful effects of silver nanoparticles. Nanosilver (silver nanoparticle, Ag NP) materials have a wide range of application in

circumstances such as: biomedical settings, medical health care and antimicrobial sterilization [1-2].

Nanosilver products, which have well-known antimicrobial properties, have been used extensively in the field of medical setting include wound coverage, and medical devices [3].

The development of new bacterial resistance against antibiotics is a significant problem in the realm of health. Since silver (Ag) is well known for its antimicrobial, anti-fungal and anti-protozoan activity, it can be used to overcome these problems [4]. Antibacterial effect is the most prominent characteristic of Ag NP.

Typical applications are coatings on cloth or other textiles, food cosmetics and health care products. Some studies suggest that silver nanoparticles not only have antibacterial actions, but also can have cytotoxic properties and may induce over production of cellular reactive oxygen species (ROS) [5].

Many studies have shown that elevated cellular oxidative stress (OS) after exposure to nanoparticles plays an important role in cellular signaling, inflammatory, genotoxic and proliferative responses [6]. Oxidative stress is a normal cellular process involved in many aspects of cellular signaling, though excessive oxidative stress can be harmful [7].

At higher and more prolonged oxidative stress, cellular perturbation and disarray would result indecreased mitochondrial membrane potential, leading to cell death.

The fundamental factor that governs initiation of cellular oxidative stress by transition metal oxides is still unknown. Once metal oxides enter the circulatory system and cells, the oxygen accepter molecules might release free radicals [8]. Because nanoparticles are able to pass through biological membranes, they can affect the physiology of any cell in an animal body.

It has been shown that exposure to heavy metals such as Lead and Cadmium can cause LPO and formation of reactive oxygen species [8-9].

Also these heavy metals adversely affect general health and reproduction of birds. Pigeons are appropriate animal models in ecotoxicology studies. Furthermore, to the best of our knowledge the toxic effects of silver ions and nanoparticleson birds have not yet been investigated.

The increasing commercial application of this material has showed potential side effects on humans and environmental health. So the present study, as a primary research in this field, aimed to evaluate the potential toxic effects of silver nanoparticleson the liver, kidney and serum parameters of *Columba livia* as a bird model.

Also, by the influence and aggregation of Ag-NPs, some structural and physiological disorders in tissues such as liver and kidney would possibility be expected.

MATERIALS AND METHODS

Chemicals, animals and treatment groups

Ag NPs were purchased from Nano-shop Company (Tehran, Iran).

This nano solution has 40 nanometer size, concentration of 8000 ppm and brown color with a purity of 95%. 30 *Columba livia* were purchased from domestic birds store (Shahrkord, Iran). These *Columba livias* were acclimated, for 3 days in the cage containing food and water.

The animals were randomly divided into 3 groups (weighting 335 g±30), which received Ag-NP with concentrations of 0, 75, 150 ppm (According to 1 ml/kg) respectively.

Experimental design

Treatment with nanoparticles was undertaken within 14 days. Concentration of 75 and 150 ppm was given to *Columba livia* by gavage 3 times a day with water based on 1 ml/kg of birds weight. *Columba livia* were killed and their liver and kidney tissues sampling were performed after 14 days of treatment. Nanosilver has a strong toxic effect in the concentrations of 50-250 ppm [10]. Thus, in this study, the final concentration of solution were 75 and 150 ppm.

Sample preparation

In this section, using a syringe of physiological serum that was entered to the deepest area of tissues, additional blood was extracted with the generated flow. Afterwards tissues were crushed and ground by help of homogenizer device. Tissues were placed into cold homogenization buffer containing 5 parts: 30 m Molar KH_2PO_4 and 5 m Molar Ethylene di amine tetra cetic acid (EDTA) and 0.3 Molar sucrose and 0.3 m Molar phenyl methane sulfonyl fluoride (PMSF). Homogenizer then centrifuged for 10 minutes at low rpm and the supernatant was removed using a sampler.

Measurement of malondialdehyde (MDA) in both blood serum and tissue

The malondialdehyde (MDA) was measured based on the Buege method [11]. Contribute to a solution of 10% Trichloro acetic acid (TCA), cold, Homogenates and samples of blood serum will be diluted and mixed together for two minutes by vortex device. Then the resulting homogenate was centrifuged and the resulting supernatant was diluted with Thiobarbituric acid (TBA) by 1:1. After that samples were placed in water bath with a temperature of 90 °C.

After cooling the tube the material was transferred to a cuvette and optical density (OD) of the samples were measured at spectrophotometer 532 nm (Ultrospec 1100 pro).

Mesurement of catalase activity in both blood serum and tissue (CAT, EC 1.11.1.6)

The catalase activity was measured by Aebi method [12]. 30 ml of the supernatant with 5 ml of potassium phosphate buffer (50 mM, pH=6) has been diluted. 2 mL of fluid obtained from the previous step (supernatant diluted) was poured into the cuvette and was placed at the spectrophotometer to reach equilibrium with the environment temperature. Then 1 mL of 30 m Molar of hydrogen peroxides were added and changes in absorbance (OD) at a wavelength of 240 nm were observed within 30 seconds. The OD was recorded every five seconds. Specific activity of catalase was expressed in terms of international units mg/ protein.

Measurement of glutathione S–transferase (GST) activity in blood serum

In this study, the enzymatic assay was carried out according to the method of Benson and colleagues [13]. This enzyme is responsible for neutralizing the lipid radicals using revivaled GSH.

Also its activity level (optical absorbance) was measured using the reaction mixture including: phosphate buffer (a mixture of NaH2PO4 and Na2HPO4), 2 & 4 dinitrobenzene (CDNB) and revivaled GSH at a wavelength of 340 nm, for a minute.

Mesurement of Alanine amino transferase (ALT) and Aspartate amino transferase (AST) in blood serum

Blood sample was gathered using a syringe. Thereafter it was centrifuged at 1500 rpm for 10 minutes and finally the top layer solution was extracted and frozen at -70° Celsius for further experiments.

Analysis of serum AST, ALT were carried out by IFCC standard on commercial kit from Pars Azmon (Tehran, Iran).

Measurement of lactate dehydrogenas in blood serum (LDH, EC 1.1.1.27)

The change in the activity of this enzyme was analyzed based on the Wootton method [14]. This enzyme is cell membrane which damages biomarkers that can be measured in animal's blood serum. This enzyme enters the blood which results in damage and even cell death in tissues of many animals. Briefly, the samples of blood serum were added into the test tubes and the resulting serum was placed (settled) within half an hour at 37. Then, 1 ml of a denitrophenyl hydrazine (DNPH) solution and 2 ml of a normal (soda 0.4 normal) solution was added to the mentioned tubes. Gradually, color of brown became apparent in these tubes. At the end, after 20 minutes, the absorbance was read with a spectrophotometer at a wavelength of 440 nm. In this method, a combination of sodium pyruvate was used as the standard.

Protein assay

The total protein concentration was quantified by the Markwell method [15]. using bovine serum albumin as the standards.

Statistical analysis

All data are expressed as mean ± standard deviation (S.D.). Comparison of data between treated groups was performed by one-way ANOVA method. All statistical analysis were performed with SPSS (edition of 16) statistical software and a *P*-value less than 0.05 ($P < 0.05$) was considered significant.

RESULTS

The membrane lipid peroxidatioin activity

The results indicate that silver nanoparticles, at the concentration-dependent manner were increased MDA (malondialdehyde) and peroxidation of lipids in the liver of *Columba livia*.

The liver tissue MDA proportion to control group in liver tissue is (168.6 $p > 0.05$) and (404.9 $p < 0.01$) percentage. Similarly, in kidney tissue MDA levels produced from 0.7±5 in the control group 1.78±0.45

($p < 0.05$) and 4.02±0.57 ($p < 0.01$), respectively. In the groups treated with 75 and 150 ppm of Ag-NPs reached. The serum samples were determined by applying Ag-NPs enhances LPO in the blood. In this regard, the results showed that the production of MDA in the treated group than in the control group increase as 279.5 ($p < 0.05$) and 516.9 ($p < 0.01$) respectively. Statistical analysis one-way ANOVA showed that there is significant difference ($p < 0.01$) between treated groups with nanosilver. The results of the membrane lipid peroxidatioin activity in *Columba livia* are presented in Table 1.

Catalase activity

The results of Catalase activity in *Columba livia* are presented in Table 2.

In this study it was found that the activity of catalase in the liver of *Columba livia* treated with different concentrations of Ag-NPs 75 and 150 ppm, respectively, was increased as 43.65 ($p < 0.05$) and 88.24 ($p < 0.01$) compared with the control group. Increase in catalase activity was also observed in the kidneys, so that treated group with the rate of 75 ppm of Ag-NPs than of the control group as 83.02 percent ($p < 0.05$) and treated group with a concentration of 150 ppm of Ag-NPs than of the control group as 99.67 percent ($p > 0.05$) was increased, respectively. As observed by dataset, measurement of catalase activity increased in the blood serum of *Columba livia* (AgNP 75 and 150 ppm). So that, by concentration of 75ppm, the activity of catalase in the control group is reach to 6.02±0.35 ($p < 0.05$) of 5.3±0.41.

Also in the group treated with150 ppm concentration of Ag-NPs catalase activity increased than the control group as 88.68 percent ($p < 0.01$). Statistical analysis one-way ANOVA showed that there is no asignificant difference ($p < 0.05$) between groups treated with Ag-NPs.

Glutathione S –transferase (GST) activity

The results of glutathione S –transferase (GST) activity in *Columba livia* are presented in Table 3. results demonstrated that GST activity were increased in the liver treated with 75 ppm of Ag-NPs in the range of values 325±63 ($p < 0.01$) compared to 278±94 of control group. In the treated group with 150 ppm concentration of Ag-NPs, GST activity has decreased in comparison to those of control group of -3.84 ($p < 0.05$). On the other, in the kidney of treated pigeons with Ag-NPs (75 and 150 ppm), changes of GST activity were calculated +7.48 ($p < 0.01$) and -2.38 ($p > 0.05$)

Table 1. Results experiments with different concentration of silver nanoparticles in the membrane lipid peroxidation activity (n moles MDA /mg prot/ Changes of lipid peroxidation) in liver, kidney and blood serum tissue of *Columba livia*. Data are presented as mean ± standard deviation.

	0 (control)	75 ppm Ag NPs	150 ppm Ag NPs
Liver	1.24±0.4	3.25±0.85	6.11±0.61**
kidney	0.7±0.05	1.78±0.45	4.02±0.57**
blood	0.83±0.02	3.15±0.61	5.12±0.65**

($p < 0.01$**) significant difference between the treated groups with Ag-NPs (one-way ANOVA).

Table 2. Results of changes in catalase activity (IU mg protein;unit of catalase activity) in the experiments with different concentrations of silver nanoparticles in liver, kidney and blood serum of *Columba livia*. Data are presented as mean ± standard deviation.

	0 (control)	75 ppm Ag NPs	150 ppm Ag NPs
liver	8.5±2.1	12.21±4.1	16±3.2*
kidney	6.01±1.51	11±2.1	12±2.02
Blood serum	5.3±0.41	6.02±0.35	10±0.45*

($p < 0.05$ *) significant difference between the treated groups with Ag-NPs (one-way ANOVA).

Table 3. Results of changes in GST activity (n moles conjugate/ mg protein/min; unit change of glutathioneS – transferase) in the experiments with different concentrations of silver nanoparticles in liver, kidney and blood serum of *Columba livia.* Data are presented as mean ± standard deviation.

	0 (cotorol)	75 ppm Ag NPs	150 ppm Ag NPs
Liver	287±94	325±63	276±52**
kidney	321±67	343±46	318±67**
Blood serum	157±37	185±41	145±77**

($p < 0.01$ **) significant difference between the treated groups with Ag-NPs (one-way ANOVA).

Table 4. Results of changes in ALT, AST and LDH activity (U/l; unit change of ALT, AST and LDH) in the experiments with different concentrations of silver nanoparticles blood serum of *Columba livia*. Data are presented as mean ± standard deviation.

	0 (cotorol)	75 ppm Ag NPs	150 ppm Ag NPs
ALT	7.41±2.02	10.02±3.15	16±4.02**
AST	47.03±6.7	65±7.3	82±14.06**
LDH	151±31.2	171±35.02	191±41.02**

($p < 0.01$ **) significant difference between the treated groups with Ag-NPs (one-way ANOVA).

compared to the control group respectively. In the blood serum was observed the same changes, So that in treated group with concentration (75 ppm) of Ag-NPs, GST activity has increased than the control group as 17.83 percent ($p < 0.01$) and the GST activity has decreased in concentration (150 ppm) of Ag-NPs than the control group as -7.64 ($p > 0.05$). Statistical analysis performed by one-way ANOVA test showed that there is no a significant difference ($p < 0.01$) between treated groups with silver nanoparticles**.**

Serum biomarkers of liver necrosis (ALT and AST) activity

The results of serum biomarkers of liver necrosis (ALT and AST) in *Columba livia* are presented in Table 4. in treated group with (75ppm) of silver nanoparticles than of the control group as 35.22 percent ($p < 0.05$).

Also treated group with (150 ppm) of silver nanoparticles has increased than of the control group as 115.93 percent ($p < 0.01$). The results showed that by concentration of 75ppm, the activity of AST in the control group is reach to 65±7.02 ($p < 0.01$) of 47.02±6.7. On the other hand, AST activity has reached to 74.39 percent ($p < 0.01$).

In the treated group with 150 ppm concentration of silver nanoparticles. Statistical analysis was performed using one-way ANOVA test showed that there is no asignificant difference ($p < 0.01$) between treated groups with silver nanoparticles.

Lactate dehydrogenase (LDH) activity

The results of the activity lactate dehydrogenase (LDH) in *Columba livia* are presented in Table4. According to obtained result of this study, silver nanoparticles (75 and 150 ppm), were increased 13.25 percent ($p < 0.01$) and 26.49 percent ($p < 0.01$) respectively, in the LDH activity in comparison to the control group.

Statistical analysis performed using one-way ANOVA test showed that there is no a significant difference ($p < 0.01$) between treated groups with silver nanoparticles.

DISCUSSION

Experiments carried out in present study indicated that the Toxic effects of nanoparticles were dose dependent, so that the highest concentrations of Ag-NPs (150 ppm) had the greatest damage in the liver and kidney.

Finally the results of the present study demonstrated that Ag-NPs are capable of causing subacute toxicity in mentioned tissues however, the toxicity differed significantly according to the particle size and concentration.

Tang and colleagues [16]. reported that after transferring to the circulatory system, Ag-NPs are targeting organs such as the kidneys, liver, spleen, brain and lung. Many studies have been conducted in recent years indicate negative and harmful effects of Ag-NPs, particularly nanoparticles in many biological systems. Possible mechanisms of Ag NP toxicity include induction of ROS, oxidative stress, DNA damage and apoptosis [17].

In order to stimulate the production of free radicals by the Ag-NPs during the work have been done on these nanoparticles with the size of 25 nm determined that human neuroblastoma cells treated with Ag-NPs are extremely vulnerable and the concentration of oxygen free radicals in the culture medium of these cells was increased significantly [18].

Also, in another study conducted in (2010) determined that Nanoparticle uptake by the gills of rainbow trout resulted in gene expression that are associated with oxidative stress [19].

Also, Statistical analysis was performed using one-way ANOVA showed that there is significant difference between treated groups with nanosilver. Consistent with these findings, Ag-NPs can provide suitable conditions for the production of free radicals and Lipid radicals in biological membranes and also increases the activity of antioxidant enzymes such as CAT and GST in order to deal with these conditions.

Our results research demonstrate a concentration-dependent toxicity for Ag-NPs elevated MDA and LPO.

The results indicate that Ag-NPs induce lipid peroxidation in a concentration-dependent manner in the understudy pigeons bodies. In the present study we are found that increasing the concentration of Ag-NPs significantly increased CAT enzyme activity. Catalase as one of the antioxidant enzymes play essential role in the inactivation of ROS after oxidative stress in cells and tissues.

However, antioxidant enzymes increase their activity to combat oxidative stress which result in LPO. The activity of this enzyme system is directly related to deactivation and eventual elimination of oxygen free radicals, especially the type of oxygen which operate in the cells.

So far, little scientific reports have been found in scientific journals that related to effect of Ag-NPs on the activity of antioxidant enzymes on the birds body. Results from recent study appear are the first work in this field especially on birds.

The fruit fly (Drosophila melaogaster) has been used as a model organism to evaluate the toxic potential of Ag NP. This study showed that Ag NP induced oxidative stress, heat shock protein 70, DNA damage markers and apoptosis in the larvae and also a significant increase in CAT and SOD activities were observed [20].

The results obtained in this study are related to enhancement of antioxidant enzymes activity after silver nanoparticles exposure.

In the present study, it was found that the concentration of 150 ppm of Ag-NPs, not only didn't increase the GST but also it reduced the antioxidant enzyme activities in under treatment of pigeons bodies, although this reduction was not significant. Since the antioxidant enzymes have a protein natur, they can be considered as one of the goals of free radicals inside and outside of the cells (during oxidative stress).

Our results showed that with a significant increase of lipid peroxidation and the final products due it kind of function inhibition occurred for GST, in this direction, studies showed that whenever the amount of environment free radicals become more than normal, kind of function inhibition occurs to antioxidant defence enzymes. During a research conducted in 2005, on the rat hepatocyte cells, determined that Ag-NPs cause considerable decrease in amount of glutathione antioxidants.

This reduction causes mitochondria membrane potential reduction and ROS increase. These findings suggest that by the high chance Ag-NPs toxicity refers to hepatocyte cells oxidative stress [21].

Since the revivaled glutathione uses GST (GSH) to neutralize the free radicals, if any change occurs in the cells GSH, it will effect on GST activation. In 2005, two separate research groups have shown that the use of Ag-NPs increases oxygen free radicals, reduces the GST rate and mitochondrial activity in mammalian stem cells. Reducing the GST rate and reduced mitochondrial activity in mammalian stem cells [22].

So, considering the GSH reduction in Ag-NPs treatments, GST activation reduction is possible, even without the direct inhibition by nonoparticles and we can present it as a suggested mechanism in this research. In (2011), with the effect of different concentrations of Ag-NPs on rats it was shown that a significant increase in ALT, AST and bilirubin are observed following this treatment [23].

The nanoparticles, with their direct and indirect effects, cause serious damages in the cellular structure of the pigeons liver that leads biomarkers such as ALT and SLT into the blood stream through the liver cells. LDH is one of the cellular death biomarkers that can be measured in many cases such as liver disease, anemia, heart and muscle damage [24]. Studies have shown that some nanoparticles have ability to inhibit LDH activity in the cells [25-26].

The present research, with the effect of nanoparticle concentration-dependent manner, the level of biomarkers of hepatic necrosis such as ALT, AST and cell death biomarkers that is LDH, increases in the blood serum of treated pigeons. considering that in the present research determined that a severe oxidative stress has induced in the tissues of pigeons bodies and specifically hepatic tissue, so it can be concluded that by damage creation in membrane of hepatic cells, lipid proxidation due to oxidative stress caused leakage of these enzymes and their concentration increase in the blood serum of treated pigeons with the Ag-NPs.

CONCLUSION

Ag NP have emerged as an important class of nanomaterials for a wide range of industrial and medical applications that have potential risks to human health. Ag NP induced the expression level of genes involved in cell cycle progression and apoptosis. Possible mechanisms of Ag NP toxicity include induction of ROS, oxidative stress, DNA damage and apoptosis.

We used *Columba livia*, as a model bird to evaluate the toxic potential of Ag NP.

These findings appear to suggest that Ag NPs induced cell death via LPO and oxidative stress. Also, in the present study a significant increase was observed in the levels of CAT, GST, ALT, AST and LDH.

Finally, the plasma concentration of liver biomarkers and biomarkers of oxidative stress can be stated that exposure of Ag-NPs will have a negative impact on liver, kidney and blood serum. Further studies will be needed to evaluate physiological concentrations by different doses of nanoparticles on different tissues.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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