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EFFECTS OF SILVER NANOPARTICLES IN LIVER FUNCTION ENZYMES AND OXIDATIVE STRESS LEVELS

Gulboy A. Nasir[1*], Hasan F. Samir[2] and Alaa K. Mohammed[3]

[1]Section of Basic Science, College of Agriculture, University of Baghdad, Iraq.

[2]Biotechnology Dep., College of Science, University of Baghdad, Iraq.

[3]Biochemical Eng. Dept, Al- Khwarizmi Engineering College, University of Baghdad, Iraq.

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***Correspondence for Author**
Gulboy A. Nasir
Section of Basic Science,
College of Agriculture,
University of Baghdad,
Iraq.

ABSTRACT

In this study we used silver nanoparticles which have been prepared and characterized in previous study. The sliver nanoparticles were 40.5 nm in size and like spherical in shape. Three different concentrations of silver nanoparticles were injected intraperetoneally in Swiss mice (0.25, 0.5 and 1.0 mg/kg of body weight) for 7 and 14 days. The level of oxidative stress (GPx, GRx, SOD, CAT, MDA) and enzymes of liver function (AST, ALT, ALP) in serum were tested. Adverse impacts on liver function especially in activities of AST and ALP were observed in a high dose-treated group, (1.0 mg/kg).

KEYWORD: AgNPs toxicity, oxidative stress, liver enzymes.

1. INTRODUCTION

Silver nanoparticles (AgNPs) have been widely used in diverse fields due to their superior properties. Currently the biosynthesis of AgNPs is in the limelight of modern nanotechnology because of its green properties.[1] "Metal nanoparticles in various size ranges play an increasingly important role in many different fields of science, technology and medicine ranging from applications as catalyst, as antibacterial agents in medicine or plasmonic active structures in optical sensing and imaging.[2] Recently it has been identified that also plant extracts have the capability to reduce silver and gold salts and to create silver and gold nanoparticles.[3] Several reports have indicated oxidative stress as a mechanism playing an important role in cytotoxic effects of nanosilver.[4,5] AgNPs were shown to induce generation of reactive oxygen species and changes in enzyme activities that are associated with antioxidant defense systems such as glutathione peroxidase (GPx), reduced glutathione

(GSH), superoxide dismutase (SOD)and catalase[6] Changes involving metallothionein, heat shock protein 70 (Hsp70), glutathione S transferase (GST), and p53 have also been documented after nanosilver exposure.[7]

AgNP-induced oxidative stress and resulting high level of ROS is the cause of DNA lesions, such as DNA breaks, oxidative adducts, oxidative single base damage causing point mutations. Apart from the direct damage, ROS is also the cause of lipid peroxidation and its products (e.g. nonenal) react with DNA, adding to the genotoxic effect. All these lesions – if left unrepaired by the cellular repair systems – are potentially carcinogenic.[8]

Moreover, AgNPs cause inflammation and toxicity.[9] "This is of clinical significance due to some pathological conditions like inflammation is associated with increased oxidative stress and this may in turn causes alteration the sensitivity of cells and tissues to probably cytotoxic AgNPs increasing their market value.[10] "Several reports have indicated oxidative stress as a mechanism playing an important role in cytotoxic effects of nanosilver.[4,5] AgNPs can enter the cell by diffusion or endocytosis resulting mitochondrial dysfunction, leading to damage of proteins and nucleic acids, eventually inhibiting cell proliferation".[11] "In addition, AgNPs may have an entrance to systemic circulation through broken skin when using the products contain AgNPs such as bandages or wound dressings.[12] "AgNPs can translocate to the blood circulation and spread throughout the main organs, especially in the liver, spleen, kidney, lung and brain"[13], "and induce blood–brain barrier (BBB) destruction and a astrocyte swelling and cause neuronal degeneration.[14]

2. MATERIALS AND METHODS

2.1 Animals

fifty six male of *musmusculs* (Balb/C) mice (weighted 23-35 g, ages 8-10 weeks) were used by dividing them to seven groups, each group contained eight mice in independent cage, and the mice were fed a standard pellets diet with enough water daily as a follow.

- The 1st and 4th groups were challenged intraperitoneally dose of 0.25mg/kg of body weight of AgNPs (50 µl) for 7 and 14 consecutive days respectively.
- The 2nd and 5thgroups were received 0.5mg/kg of body weight of AgNPs (50 µl) intraperitoneally for 7 and 14 consecutive days respectively.
- The 3rdand 6thgroups were challenged intraperitoneally dose of 1mg/kg of body weight of AgNPs (50 µl) for 7 and 14 consecutive days respectively.

- The 7th group was left as a control group with intraperitoneally dose (50 µl) of distilled water.

At the next day of the end of the dosing period (after 7 days), the 1st, 2nd and 3rd groups of mice were sacrificed by cervical dislocation. The 4th, 5thand 6thgroups of mice were sacrificed by cervical dislocation, at the next day of the end of the dosing period (after 14 days). The blood samples of each mouse was collected in non-anticoagulant tubes, and then centrifuged for 10 min. at 3000 rpm and isolated serum was kept in -20 ºC, until measurement of biochemical aspects.

2.2 Measurement of liver functions

The liver functions test (ALP, ALT and AST) was measured using kits for Reflotron® plus (Roche-Germany), as manufactured company.

2.3 Measurement of oxidative stress levels

2.3.1 Measurement of Malondialdehyde (MDA)

The level of malondialdehyde was determined by a modified procedure described by Guidet B. and Shah S.V., (1989).[15] The principle of the test was based on the reaction of MDA with thiobarbituric acid (TBA); forming an MDA-TBA_2 product that absorbs strongly at 532 nm.

2.3.2 Measurement of Catalase Activity (CAT)

Bioassay Systems' improved assay directly measures catalase degradation of H_2O_2 using a redox dye. The change in color intensity at 570nm or fluorescence intensity (λ em/ex = 585/530nm) was directly proportional to the catalase activity in the sample.

2.3.3 Measurement of superoxide dismutase Activity (SOD)

To determine SOD activity, several direct and indirect methods have been developed. A common and convenient indirect method utilizes nitro blue tetrazolium (NBT) conversion to NBT diformazan (formazan dye) via superoxide radical. However, there are several disadvantages to the NBT method, such as poor water solubility of the formazan dye and the interaction with the reduced form of xanthine oxidase. Though cytochrome C is also commonly used for SOD activity detection, its reactivity with superoxide is too high to determine low levels of SOD activity. Cell Technology's SOD kit utilizes a highly water-soluble tetrazolium salt,WST-1(2-(4Iodophenyl)-3-(4nitrophenyl)-5-(2,4disulfophenyl)-2Htetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion as showed in figure3-4 .The rate of the reduction with O

$_2$.- is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD, as shown in Figure 3- 4, by this colorimetric method. Absorbance can be measured at 440nm Therefore, the IC 50 (50% inhibition activity of SOD or SOD-like materials) can be determined.

2.3.4 Measurement of Serum Glutathione Peroxidase (GPx_1)

Measurement of Serum GPx was done by using a sandwich enzyme immunoassay Elisa kit. The microtiter plate provided in this kit has been pre-coated with an antibody specific to GP_{X1}. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for GP_{X1}. Next, Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. Then a TMB substrate solution is added to each well. Only those wells that contain GP_{X1}, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of GP_{X1} in the samples was then determined by comparing the O.D. of the samples to the standard curve.

2.3.5 Measurement of Glutathione Reeducates (GRx)

The level of glutathione reductase was determined using Glutathione Reductase. Assay Kit Catalog # 7510-100-K, from TREVIGEN ® Instruction, amsbio. The procedure was as manufactured factory.

3. RESULTS AND DISCUSSION

3.1 Effect of AgNPs intake on activity of liver Enzymes

When mice were injected intraperitoneally with AgNPs (size≈50 nm, doses: 0.25 mg/kg, 0.5 mg/kg, and 1mg/kg) for 7 and 14 consecutive days, the serum levels of ALP, AST and ALT were most significantly increased in male mice in the group treated with 1mg/kg.b.w AgNPs than 0.5 and 0.25 mg/kg.b.w compared with control mice group and this result indicated that this effect was dose dependent as shown in Table 1, No other significant changes were observed in all three liver enzymes when the mice treated with the same three different doses of AgNPs after 14 days of treatment. These results agreed with Park *et al.*(2010) who mentioned that the injection for 28 days with the same doses which used in this study , ALP and AST were significantly increased with 1mg/kg while ALT didn't[13], and the same with

Maneewattanapinyo, *et al*.(2011), who found that there is no significant differences in ALT and AST in treated mice after 7, 14 days.[16]

Table 1: Activities of serum liver enzymes in male mice after 7 and 14 days AgNPs intake at three different dose (0.25,0.5 and 1.0 mg/kg.b.w).

Period of treatment/ Days	Dose mg/kg.b. w	Mean ±SE		
		S.AST(IU/L)	S.ALT(IU/L)	S.ALP(IU/L)
7days	0.25	91.2±11.6*	53.9±8.3*	88.6±4.6*
	0.50	97.8 ± 34.1*	60.3 ± 9.6**	95.3 ± 8.8**
	1.00	146.9± 31.9**	199.6 ± 14.7***	120.7 ± 9.3**
14 days	0.25	94.2 ± 9.8	56.2 ± 7.7	90.2 ± 3.9
	0.50	100.8 ± 32.8	65.3 ± 9.61	99.1 ± 7.3
	1.00	155.9 ± 38.7	106 ± 12.9	124.8 ± 8.8
Control		77.4±9.4	46.4±5.8	78.3±7.1

*Note: Significantly different from control group, *P < 0.05, **P < 0.01,***P<0.001. (n = 8, mean±SE)*

In the present study, physiological effects of AgNPs have been evaluated at different doses on serum ALT, AST, ALP in male mice. Hepatic damage induced by intraperitoneally injection of AgNPs in mice, has possibly caused severe irritation of oxidant system in these cells. The smaller the diameter of the nanoparticles is, the more its influence to cells and its molecular effects on the intracellular mechanisms will increase. In 1989, Machiedo *et al*. showed that free radicals induced by nanoparticles can cause destruction of red blood cells.[17]

Susan *et al*. (2009) "showed that with changes in the diameter of nanoparticles, their distribution in body tissues and their effects will become different.[18] In fact, free radicals from the AgNPs have attacked hepatocytes and released ALT stored in them and entering into the blood serum; whereas; the immune response of mice to an external factor has been the increase of the number of white blood cells for phagocytosis of AgNPs.[19] Considering the importance of role of hepatocytes in detoxification, any changes made in their structure and number can cause very large physiological changes for human body. In fact, free radicals from the nanosilver particles have attacked hepatocytes.[20] The toxic effects of AgNPs could theoretically be related to the release of free silver ions in AgNPs.

Kim *et al*., (2009) concluded that the toxicity of AgNPs exposure could not be explained only by the presence of Ag^+ in NP solution.[21] AgNPs and Ag^+ could induce oxidative stress correlating with cytotoxicity and genotoxicity.[22]

3.2 Effect of AgNPs intake on serum oxidative stress levels

The aim of this study was to determine whether AgNPs are antioxidative or prooxidants properties. Our results demonstrate that AgNPs in 0.25, 0.5 and 1.0 mg/kg increase the oxidative stress, as shown by a decreased in SOD and GPx while CAT and GRx activities were increased in animal groups which treated intraperitoneally injection after 7 days compared the control group". On the other hand after two week of treatment, the activity of enzymatic scavengers such GPx, SOD of blood samples were not significantly changed compared with these activities at 7 days period of exposure to the same doses of AgNPs. (Table 2).

Table 2: Serum oxidative stress markers levels in male mice after 7 and 14 days AgNPs intake at three different dose (0.25,0.5 and 1.0 mg/kg.b.w).

Period of treatment/ Days	Dose mg/kg. b. w	Mean±SE				
		GRx	GP_{X1}	SOD1	MDA	CAT
7days	0.25	2.1 ± 0.34	3.1± 0.56	10.3 ± 1.22	0.95 ± 0.27	2.7± 0.25*
	0.5	2.8± 0.56*	2.6± 0.49*	8.2± 1.56**	1.52± 0.21*	3.6± 0.46*
	1.0	3.2± 0.39*	2.1± 0.34*	6.9± 1.75***	1.93± 0.19**	4.8± 0.67**
14 days	0.25	2.2 ± 0.56	2.9± 0.43	9.8 ± 1.36	1.11 ± 0.22	2.9 ± 0.63
	0.5	2.8 ± 0.71	2.4± 0.61	8.0 ± 1.78	1.78 ± 0.28	3.8 ± 0.77
	1.0	3.4 ± 0.88	1.9± 0.23	6.1 ± 1.23	2.11 ± 0.27	5.1 ± 0.89
Control		1.9 ± 0.23	3.4± 0.68	12.5 ± 1.88	0.62 ± 0.15	2.2 ± 0.12

*Significantly different from control group, *P < 0.05, **P < 0.01, *** P<0.001. (n = 8, mean ±SE)*

AgNPs have been found to increasing oxidative stress.[21] "These findings support the hypothesis (nanoparticles toxicity) that antioxidant/oxidant from the temporal exposed mice in lower doses which could ultimately lead to the observed hepato protective. These results suggest that the observed increase of spontaneous alternation in the liver function could result from AgNPs induced oxidative stress. Altogether, these results suggest that AgNPs in higher doses were capable of inducing oxidative stress, which was responsible for hepatotoxicity in experimentally mice. Additionally, the investigators demonstrated increased ROS production and increased cell lethality in rat liver cells after exposure to NPs.[23] Many studies have implicated intracellular ROS in the signal transduction pathways.[24]

Recently, it was reported that "apoptosis induced by exposure to AgNPs was mediated by oxidative stress in fibroblast, muscle and colon cells".[4] Fang *et al* 2015 suggested that Ag-NP-induced cell death in rat seem to be both apoptotic and necrotic in nature[25], and that it

occurs in a dose and exposure duration dependent manner and that data provides direct evidence that elevated mitochondrial ROS plays a critical role in AgNPs induced neuro degeneration.[26]

In the present study, antioxidant enzyme activity such as catalase (CAT) was used to measure the production of ROS in various doses of AgNPs. Importantly, after 7 days exposure, the doses which caused significant decreased in SOD and GPx was 1.0mg/kg dose (Table 2). These data suggested that AgNPs could be inducing oxidative damage through a ROS-mediated process. However, it remains to be investigated whether AgNPs induced free radicals directly or indirectly through depletion of antioxidant defense mechanisms depending dose e.g. caused by interactions with antioxidant systems.[23,27] The previous studies have shown that "small dose AgNPs were more effective antioxidant than large NPs. Recently, studies reported that micro-sized particles were less toxic than their smaller counterparts".[27]

In the present study AgNPs (about 40nm) size was fixed but in different doses, high doses were more toxic as observed after treatment for both 7 and 14 days. The ability of AgNPs to undergo the development of oxidative stress and in particular the generation of reactive oxygen species (ROS) represents the model mostly used to explain the in vitro toxicity of nanoparticles.[28] Generation of ROS is also assumed to be underlying the toxicity of AgNPs.[29] "This could occur via different mechanisms: First, nanoparticles could catalyze redox reactions directly on their surface leading to ROS formation. Another mechanism that might be of particular relevance for silver is based on direct interactions with mitochondrial membrane proteins, which often contain sulfur-containing amino acids. Disturbance of mitochondrial functions can lead to increased production of ROS. Previous studies have demonstrated that the mechanism of nano- Ag toxicity involves disruption of the mitochondrial respiratory chain leading to production of ROS and interruption of ATP synthesis, which in turn causes DNA damage.[30]

Previous studies proposed that "the induction of ROS is a general mechanism of NP-mediated cytotoxicity which was supported by studies showing that in vitro exposure to AgNPs cause reduction in GSH, elevated ROS levels, malondialdehyde as lipid peroxidation marker and increased expression of ROS responsive genes".[21, 29]

The toxic effects of AgNPs could theoretically be related to the release of free silver ions in AgNPs. Kim *et al*., (2009) concluded that the toxicity of AgNPs exposure could not be explained only by the presence of Ag^{+} in NP solution.[21] AgNPs and Ag+ could induce oxidative stress correlating with cyto- and genotoxicity.[22]

CONCLUSIONS

Adverse impacts on liver function especially in activities of AST and ALP were observed in a high dose-treated group, (1.0 mg/kg).The result suggested that repeated administration of nano-sized AgNPs may cause organs toxicity in mice".

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