

Apoptotic effect of silver nanoparticles in rat

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ABSTRACT

Background: Silver nanoparticles (AgNPs) was recommended as a disinfectant and can be used as drug in the treatment of some non- curable disease in lives stock. **Aim:** the toxicity of nanosilver is not well known, it is essential to examine its safety. **Material and methods:** fifty male rats were used to assess the dose – dependent manner effects of AgNPs. Adult male rats were divided randomly into five groups, in the four experimental groups, nanosilver particles were injected intrapretoneally for 30 consecutive days at doses of 0.25, 0.50, 1.0 and 2.0 mg/kg 1 B.W. Rats in control group received equal injections with deionized water. Liver sample were collected and stored in liquid nitrogen tank for estimation of some gene expressions. **Results:** AgNPs administration showed a significant increase in the gene expressions of TNF- α , P₅₃ and caspase -3 levels **conclusions:** It was concluded that injection of AgNPs in high doses for long period produced changes in gene expression and apoptotic effects were indicated

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Introduction:

Nanoparticles are engineered materials produced within the nanoscale range of 1-100 nm in one or more dimenions, pure silver has the highest electrical and thermal conductivity of all the metals and has low contact resistance [1].

Silver nanoparticles (AgNPs) have been used in a wide variety of applications, as antimicrobial agents and have been incorporated into a number of products as industrial and food products in addition to biological and medical applications [2].

Nanosilver with a particle size less than 20 nm in diameter has been reported to be effective in the treatment of certain infectious diseases [3], and is effective in retarding growth of bacteria , molds and harmful spores. It has been reported that silver is re-emerging as a viable treatment options for infections associated with burns, open wounds, and chronic ulcers [4].

Silver nanoporticles (AgNPs) can be ingested via water, food cosmetics , drugs and drug delivery devices [5]. Other investigators have demonstrated

that silver ions released from ingested products into the blood and can accumulate in body organs and have toxic effect in the liver and kidney[6]; it has been reported that AgNPs are more toxic than other metal nanoparticles, but the mechanism of their toxicity is not clear [7]and [8].

The distribution of nanoparticles into major organs including liver, kidney, lung, spleen, lymph nodes and bone marrow has been reported in mice [9] and [10].

On the other hand NPs can elicit a spectrum of tissue responses such as cell activation, generation of reactive oxygen species (ROS), inflammation and cell death [11]; these studies provided evidence that the cytotoxicity of AgNPs may be partially due to their induction of cellular oxidative stress through the generation of free radicals and ROS [11], this is of clinical significance because certain pathological conditions as inflammation is associated with elevated oxidative stress and this may in turn alter the sensitivity of cells and tissues to potentially cytotoxic AgNPs increasing their market value [12].

Liver damage has been proven to be associated with redox imbalance and oxidative stress [13]. Cytotoxicity induced by Ag-NP exposure has been demonstrated in a number of studies with a variety of factors influencing observed cyto- and geno-toxic effects [14] and [15].

Nano-Genotoxicology is another new term that represent the growing trend of research into NP-induced genotoxicity and carcinogenesis [16]. Recent reports pointed that, long-term inflammation and oxidative stress present in tissue due to NPs toxicity, can eventually induces DNA damage in cells and tissues. Continuous ROS production in

the cell can cause gene mutations/deletions leading to mutagenesis, carcinogenicity, and subsequently development of tumors and cancer. Particularly the metal based NPs like Ag NPs [15], Au NPs [17] and TiO₂ NPs [18] are important for that kind of ROS production and genetic damage. As a result of DNA damage induced by NPs, single-strand DNA breaks, double strand breaks, DNA deletions and genomic instability in the form of increase in 8-hydroxy-2-deoxyguanosine levels are formed [19]. According to [20] long-term exposure of cells to NPs displayed genome instability under comet assay analysis, altered cell cycle kinetics in flow cytometry and induced protein expression of p53, having a critical role in responding to various stresses that cause DNA damage, and DNA repair-related proteins.

Studies showed the genotoxicity of 5 nm Ag NPs using two standard genotoxicity assays, and reported that AgNPs did not induce mutations in five different *S. typhimurium* strains (TA102, TA100, TA1537, TA98 and TA1535) [21]. However, it displayed concentration-dependent geno-toxicity in the human lymphoblast TK6 cell micronucleus assay.

Despite the growing applications for products containing Ag-NPs, there is little information about potential toxicity and side effects [22]. In vitro evidence supports the suggestion that Ag-NPs induce strong cytotoxicity and proinflammatory effects [23].

Production of reactive oxygen species (ROS) and the release of cytokines are considered to be the mechanisms by which metal nanomaterials induce toxicity [24]. ROS are continually generated and eliminated in biological systems by

endogenous or exogenous antioxidants [25] & [26], but excessive production of ROS can lead to apoptosis and cause oxidative damage [27]. Apoptosis is initiated by the sequential activation of caspases, which are a group of cysteine proteases exist in cells as inactive proenzymes [28]. Caspase -3 is a key effector caspase involved in the apoptotic cascade within cells, cleaving different cellular substrates and inducing apoptotic cell death [29].

The aim of this work was the study the effect of several doses and prolonged exposure to silver nanoparticles in rats.

MATERIAL AND METHODS

3.1. MATERIAL

3.1.1. Chemical

Silver dispersion-nanoparticles, 20 nm particles size, 0.04 mg/ml in aqueous buffer, contain sodium citrate as stabilizer was obtained from sigma – Aldrich Co.

3.1.2. Experimental animals:

Fifty (50) male adult albino rats (200-250g), obtained from National Research Centre, Dokki, Egypt were used in this study. Animals were housed in groups of ten in cages at 25±0.5°C, under 12: 12 hrs light / dark cycle, and 12 hr free access to water and food. Animals from all groups were kept under similar environmental conditions of temperature, illumination ventilation, and received the determined diet during the course of the experiment. Food and water were kept in special open containers fixed in the wall of the cages. Cleaning and changing food and water was done for all animals twice daily. The experimental protocols were approved by the Faculty of Vet. Medicine, Suez Canal University, Ismailia, Egypt. There are no restrictions in Egypt for the use of experimental animals for

Laboratory studies. Animals of this study were cared for in accordance with the principles outlined in the guide for the care and use of laboratory animal issued by the animal care Committee of National Veterinary Research and Quarantine service (NVRQS).

3.1.3. Experimental design:

The experimental albino rats were divided into five groups, each group comprising of ten rats, after adaptation in the animal houses in Faculty of Vet. Medicine Suez Canal University for one week. The groups named as N1, 2, 3, 4 and 5. Group N1, was called control group and was given deionized water IP and the other four groups, N2 to 5 were called experimental or treated groups. Silver nanoparticles were administered intraperitoneal for 30 days at doses of 0.25, 0.5, 1.0 and 2.0 mg / kg. / B.W. of rat according to protocol of [30].

3.2. METHODS

3.2.1. Sampling:

The rats were dissected; liver organs were separated carefully and washed with saline solution. After that, part of liver organs were stored in liquid nitrogen tank for genes expressions study.

Molecular determinations

- **Determination of P53 and Caspase-3 enzyme and TNF- α gene expression** using a semi-quantitative RT-PCR [31].

1. Primers sequences and preparation

Primer sequences of rat TNF- α , P53, caspase-3 and β -actin were obtained from the published sequences of [24] and for GRD from [32]. And listed below in table A.

Primers Preparation according to the manufacture instructions; Primers were prepared as follow:

1. Lyophilized primer at -20C° was equilibrated at room temperature and spin down for 3 sec. using vortex.
2. Lyophilized primer was diluted (both forward and reverse with RNase free water (the volume was added to get $100\mu\text{M}$ stock) and then the tube was gently invert for 2 min at room temperature.
3. Stock primer was diluted with RNase free water (PH 8.0) to get $5\mu\text{M}$ and kept at -20C° until used.

Statistical calculation:

The obtained data were analyzed and graphically represented using the statistical package for social science (SPSS, 15.0 software 2009) for obtaining mean and standard deviation and error. The data were analyzed using one way ANOVA to determine the statistical significance of differences among groups. Duncan's test were used for making a multiple comparison among the groups for testing the inter grouping homogeneity.

Results

Results in Table (1) and Figure (1) showed that there was a significant increase in the gene expression of P53 within the increase of silver nanoparticles conc.

Results in Table (2) and Figure (2) showed that there was a significant increase in the gene expression of TNF- α within the increase of silver nanoparticles conc.

Results in Table (3) and Figure (3) showed that there was a significant increase in the gene expression of

Caspase-3 within the increase of silver nanoparticles conc.

DISCUSSION

At the present study we tried to identify the adverse effects of silver nanoparticles using rats treated with intra-peritoneal administration. In addition, we tried to investigate the effects of toxicity in the different doses of silver nanoparticles.

In this study, Tables (1-3) and Figs. (1-3), it was established that exposure to Ag-NP resulted in significant transcription of inflammatory cytokines and tumour necrosis factor (TNF)- α . Ag-Np exposure can generate intracellular reactive oxygen species (ROS) in mammalian cells, with excessive production linked to cell damage and initiation of an inflammatory response [33] and [34]. Oxidative stress in monocytes coupled with the release of inflammatory cytokines is a natural protective response, however, this process can become pathogenic when normal cell control systems are overwhelmed. Evidence has indicated a correlation between ROS production and inflammation resulting in an amplified response. Oxidative stress causes expression of cytokines and TNF- α which in turn contribute to ROS generation. This produces an amplification loop between oxidative stress and inflammation initiated by Ag-Np exposure [35]. This loop while potentially contributing to resolution may also cause continued pro-inflammatory cytokine induction past the threshold of resolution thus inducing a continued inflammatory response and toxicity [36]; [37] and [38].

In vitro studies support cell toxicity for Ag-NPs [39], which can induce oxidative stress in human hepatoma

cell [27], DNA damage in testicular cells [40], reduced cell viability in alveolar macrophages and lung epithelial cells (Soto, *et al.*, 2007), and apoptosis in Hela cells [11]. The cytotoxic effects of Ag-NPs have been reported in various cancer cell lines [41] and [42]. Most administered nanoparticulate silver has been described to be deposited in the liver, the major organ of detoxification [43].

This study revealed that administration of Ag-NPs for 30 days likely led to early apoptotic stages, we observed that administration of Ag-NPs induced upregulation of caspase -3. Caspase-3 as an effector caspase, deals with the intrinsic pathway of apoptosis [17]. Eckle, *et al.*, have shown that expression of caspase-3 represents a reliable marker of apoptosis in the rat liver [28].

Ag-NPs exposure increased apoptosis, as demonstrated by increase in P₅₃, P₂₁ and caspases 3,8 and 9. Ag-NPs caused DNA damage and reduced the interaction between P₅₃ and NF-κB [44].

Since silver nanoparticles (Ag-NPs) possess unique cytotoxic features, we examined, whether their activity could be exploited to kill tumour suppressor deficient cancer cells. This found that both sized Ag-NPs (5 and 35 nm) targeted mitochondria and induced apoptosis in wild-type P₅₃ containing V20s and P₅₃-deficient Saos-2 cells. Ag-NPs are able to kill osteosarcoma cells independently from their actual P₅₃ status and induce P₅₃ independent cancer cell apoptosis [45]. Although many studies have reported the apoptotic effects exhibited by silver nanoparticles (Ag-NPs) in various circumstances, the apoptosis mechanisms of Ag-NPs is unclear. As a result, Ag-NPs –significantly

enhanced DNA fragmentation dose – dependently and treatment with P₅₃ siRNA or pifithrin-α prevented DNA fragmentation. They also found that apoptosis-related genes (Caspase-3, Bax, and Bcl-2) were regulated by Ag-NPs, which was detected by mRNA and protein level; these results suggest that Ag-NPs induced P₅₃-mediated apoptosis in BEAS-2B cells [46].

CONCLUSION

From these study, it was concluded that the intraperitoneal injection of silver nanoparticles produced changes, gene expressions, of TNF-α, P53 and caspase-3 in the liver tissues and apoptotic effects were indicated. Generally, results of this study confirmed the toxicity of silver nanoparticles.

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Table (B): Primers used in determination of the gene expression of the selected genes:

Gene	Primers	Product length(bp)
p53	F: 5'TCATCTTGGGCCTGTGTTATCT-3'	332
	R: 5'GTGCAGGGTGGCAAGTGG-3'	
Caspase-3	F: GCAGCAGCCTCAAATTGTTGACTA	141
	R: TGCTCCGGCTCAAACCATC	
TNF- α	F 5'-ATGGGGACAATACACAAGGC-3'	421
	R 5'-TCATCTTGTTCCTCGTGGAC-3'	
β -actin	F 5'-TCACTATCGGCAATGTGCGG-3'	260
	R 5'-GCTCAGGAGGAGCAATGATG-3'	

Table (1): Data analysis of mRNA expression of hepatic P⁵³ gene in different groups:

Groups	CT of target gene	CT of housekeeping gene	Δ CT	$\Delta\Delta$ CT	Fold change = $2^{-\Delta\Delta$ CT (Means \pm S.E)
G1 Normal control	23.3	28.4	-5.1	0	1 \pm 0.02 ^c
G2	22.5	28.65	-6.14	-1.044	1.09 \pm 0.08 ^{bc}
G3	22.71	28.91	-6.195	1.095	1.2 \pm 0.03 ^b
G4	22.987	29.2	-6.213	1.113	1.24 \pm 0.06 ^b
G5	21.6	27.9	-6.3	1.2	1.45 \pm 0.07 ^a

Means \pm SE in the fold change column carrying different superscripts are significantly different at (P \leq 0.05)

Table (2): Data analysis results of mRNA expression of hepatic TNF- α gene in different groups

Groups	CT of target gene	CT of housekeeping gene	Δ CT	$\Delta\Delta$ CT	Fold change = $2^{-\Delta\Delta$ CT (Means \pm S.E)
G1 Normal control	24.6	28.4	-3.8	0	1 \pm 0.01 ^d
G2	23.831	28.65	-4.819	-1.019	1.04 \pm 0.06 ^c
G3	24.02	28.91	-4.8864	1.0864	1.18 \pm 0.02 ^b
G4	24.26	29.2	-4.94	1.14	1.31 \pm 0.08 ^a
G5	22.93	27.9	-4.97	1.17	1.37 \pm 0.04 ^a

Means \pm S.E in the fold change column carrying different superscripts are significantly different at ($P\leq 0.05$)

Table (3): Data analysis results of mRNA expression of hepatic Casapase-3 gene in different groups

Groups	CT of target gene	CT of housekeeping gene	Δ CT	$\Delta\Delta$ CT	Fold change = $2^{-\Delta\Delta$ CT (Means \pm S.E)
G1 Normal control	25.6	28.4	-2.8	0	1 \pm 0.08 ^a
G2	24.798	28.65	-3.852	-1.052	1.107 \pm 0.12 ^a
G3	25.015	28.91	-3.895	1.095	1.2 \pm 0.06 ^c
G4	25.243	29.2	-3.957	1.157	1.34 \pm 0.09 ^e
G5	23.876	27.9	-4.024	1.224	1.5 \pm 0.07 ^b

- Means S.E in the fold change column carrying different superscripts are significantly different at ($P\leq 0.05$).

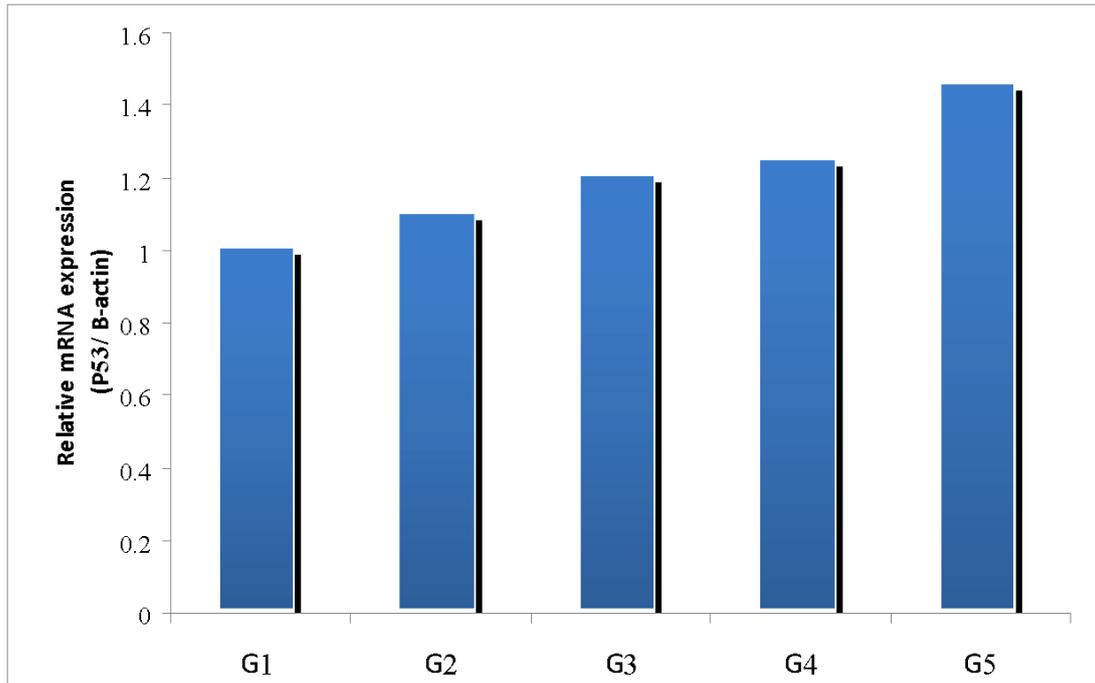


Figure (1): Fold change of P⁵³ gene in relation to β -actin gene

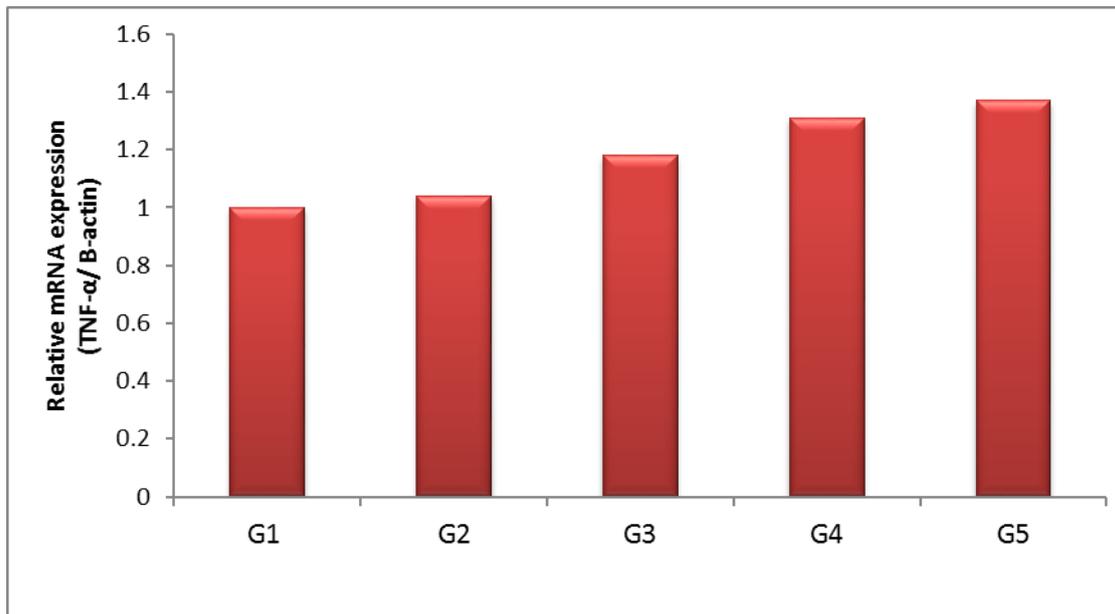


Figure (2): Fold change of TNF- α gene in relation to β -actin gene.

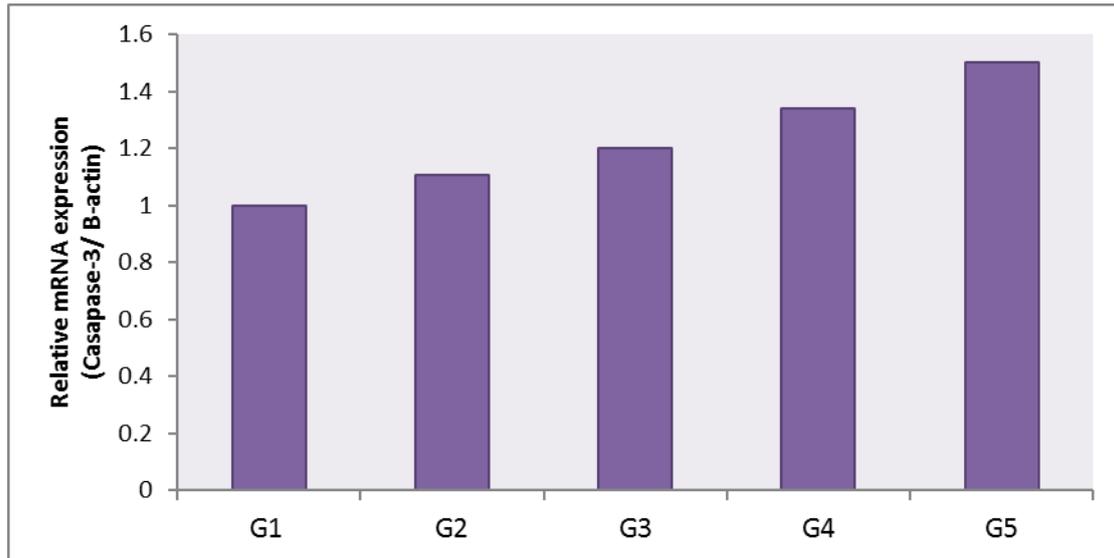


Figure (3): Fold change of Casapase-3gene in relation to β -actin gene