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In vivo genotoxic assessment of silver nanoparticles in liver cells of Swiss albino mice using comet assay

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ABSTRACT

Silver nanoparticles (Ag NPs) has resulted their incorporation into consumer products due to their extensive application in health, electronic, and household products. In particular, the oral toxicity of Ag NPs is of particular concern to ensure public health. For the present study, a genotoxic and cytotoxic approach was employed to elucidate the activity of 5 nm size and spherical shaped Ag NPs in liver cells of Swiss albino mice by using alkaline comet assay. Statistically significant DNA damage raise the concern about the safety associated with the applications of the Ag NPs. The result showed that Ag NPs induced a significant concentration dependent increase in the frequency of tailed nuclei (DNA damage), tail moment, %DNA in the tail, and tail length in the liver cells. Additionally significant histopathological alterations were also observed. The results of present study suggest that exposure to silver nanoparticles has the potential to cause genetic damage. Copyright © 2015 VBRI Press.

Keywords: Silver nanoparticles; DNA damage; liver function; comet assay; histopathology.

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Introduction

Nanosized particles can have remarkable and useful characteristics, but the same properties may be challenging for human health. So it becomes critical to assess the ability of nanoparticles to cause DNA damage. In the earth's crust, occurrence of silver element is rare (0.05–0.1 ppm) but it is deposited at considerable higher concentrations in ores in association with other elements **[1]**. Silver originates from leaching, mining, or anthropogenic sources in the aquatic environment. It is traditionally incorporated in, for example, coins, jewelry, electronics, and photographic manufacturing. In addition, the antibacterial capacity of both nanosilver and silver nanocomposites has expanded its use significantly **[2]**. Silver nanoparticles are being incorporated in a variety of products, including water treatment, textiles, dyes, paints, varnishes, polymers, plastics, food containers packaging, medical applications, wound dressings, bandages and household appliances such as refrigerators and washing machines. They are also used in various consumer applications such as disinfectants, room sprays, cosmetics, cleaning agents **[3,4]**. The antibacterial property of Ag NPs has frequently been used for numerous medical applications and textiles or plastics as they fight both Gram positive and Gram negative bacteria, as well as fungi and viruses **[5,6]**. The actual

mechanism of their bactericide property has not been fully clarified yet. This uncertainty and the high number of applications yield studies on the possible cytotoxic and genotoxic effects of Ag NPs.

Many parameters are important to predict silver nanoparticle genotoxicity. To better understand the genotoxicity mechanisms, as well as the correlations between nanoparticles and their impact on the human health and the environment, this topic needs more studies to be performed. In addition, it must be noted that nanoparticle size is an important parameter to determine particle toxicity. In this context, there is the need to elaborate standard protocols to carefully analyze nanoparticles toxicity in order to decrease the possible discrepancies related to final conclusions. Genotoxicity is a key area governing the risk assessment of chemical substances for human health and two principle modes of genotoxic actions are considered for particles, referred to as primary and secondary genotoxicity. The secondary genotoxicity is a pathway of genetic damage resulting from oxidative DNA attack by reactive oxygen or nitrogen species, generated during particle-elicited inflammation. In case of metal oxide nanoparticles, genotoxicity seems to occur mainly via oxidative stress rather than direct DNA binding with subsequent replication stress **[7]**.

Single cell gel electrophoresis technique, more commonly known as the Comet Assay is a simple, rapid, visual and sensitive technique for detecting and analyzing DNA damage and repair at the single cell level in a variety of organs and cells of mammals **[8]**. It is a simple and effective method for identifying DNA damage in the cells with or without the competency of cell division. Cellular DNA is recognized by the migration of DNA fragments from the cell nucleus through an agarose gel using fluorescent markers/dyes, under the influence of an electric field, resulting in a characteristic comet-like shape. Thus, the assay is also commonly known as the 'Comet Assay'. The comet assay allows any viable eukaryote cells to be analyzed for DNA damage, thus, widely used in biomonitoring and assessments of genotoxicity **[9]**.

The previous in vitro studies demonstrated that Ag NPs caused toxicity in various cell-lines. However, toxicity of Ag NPs in vivo is largely lacking. In addition, the biological effect of nanoparticles depends on the target tissue and the way of their entry in the body. Their further complications in the body are brought about by possible interactions with other biologically active agents. Hence, a detailed study is necessary for each nanoparticle type and cell or tissue type, as well as interacting chemical or physical agents.

The human body is constantly under attack by chemical agents that can cause DNA damage by non-oxidative and oxidative mechanisms, which can cause initiation and begin the process of carcinogenesis. The comet assay is advantageous from the perspective of its sensitivity to DNA damage beyond the body's natural defense and repair processes. It can be applied to a variety of studies including genotoxicity, DNA repair, environmental and human biomonitoring as well as clinical studies. Since most of the toxicity studies of silver nanoparticles are based on their route of exposure, interaction with cellular and nuclear components leading to cell and DNA damage. In this context, genotoxicity of Ag NPs is still limited. The aim of this investigation was to determine the genotoxic potential of silver nanoparticles in liver cells of Swiss albino mice using comet assay.

Experimental

Chemicals

Silver nanoparticles were synthesized freshly for this study. Normal regular melting agarose and Triton-X were obtained from Sigma-Aldrich Corporation (India). Low-melting agarose, ethylene diamine tetra acetic acid disodium salt (EDTA-2Na), NaCl, Hanks' balanced salt solutions, Ethidium bromide and Tris buffer of Himedia Ltd. India were used. Dimethyl sulfoxide (DMSO) was purchased from Merck Chemicals, India.

Animals

Adult, healthy, male Swiss albino mice of proven fertility were used for this study. The mice were initially procured from the animal facilities, IVRI Izzatnagar, Bareilly (U.P.), India and a colony was established in the animal house facility of the department. The subsequent progeny of these mice were used for experimental purpose. The mice housed in groups were kept in polypropylene cages measuring 12" X 10" X 8" under standard laboratory conditions of light-dark cycle (14 -10 h) and temperature (22 ±3^{o}C) and were given water and standard laboratory diet *ad-libitum*.

Silver nanoparticles: Synthesis and characterization

Silver nanoparticles was prepared and characterized as described earlier **[2,4]**.

Experimental design

The Ag NPs were orally instilled using a device for oral intubation at a dose 50mg/kg and 100mg/kg (single instillation groups), 10mg/kg and 20mg/kg once a week for 5 weeks (repeated instillation groups). In the single instillation groups, mice were sacrificed 3 or 24 h after the instillation. In the repeated instillation groups, they were sacrificed 3 h after the last instillation. Six mice per group for each time point were instilled. As a negative control, six mice were given an aqueous solution of Triton X-100 at 0.5mg/kg by a single or repeated oral instillation similar to the Ag NPs. As a positive control, six mice were orally given a single dose of cyclophosphamide at 25 mg/kg at 3 h before sacrifice. In six mice of each group, liver was used for histopathological examination and comet assay.

Preparation of single cell from tissue

Single cell suspensions were obtained from solid tissue by incubation with trypsin, mincing with a pair of fine scissors for a few minutes and by homogenization to release nuclei. During mincing or homogenization, EDTA was added to the processing solution to chelate calcium/magnesium and prevent endonuclease activation, and DMSO as a radical scavenger was also added to prevent oxidant-induced DNA damage.

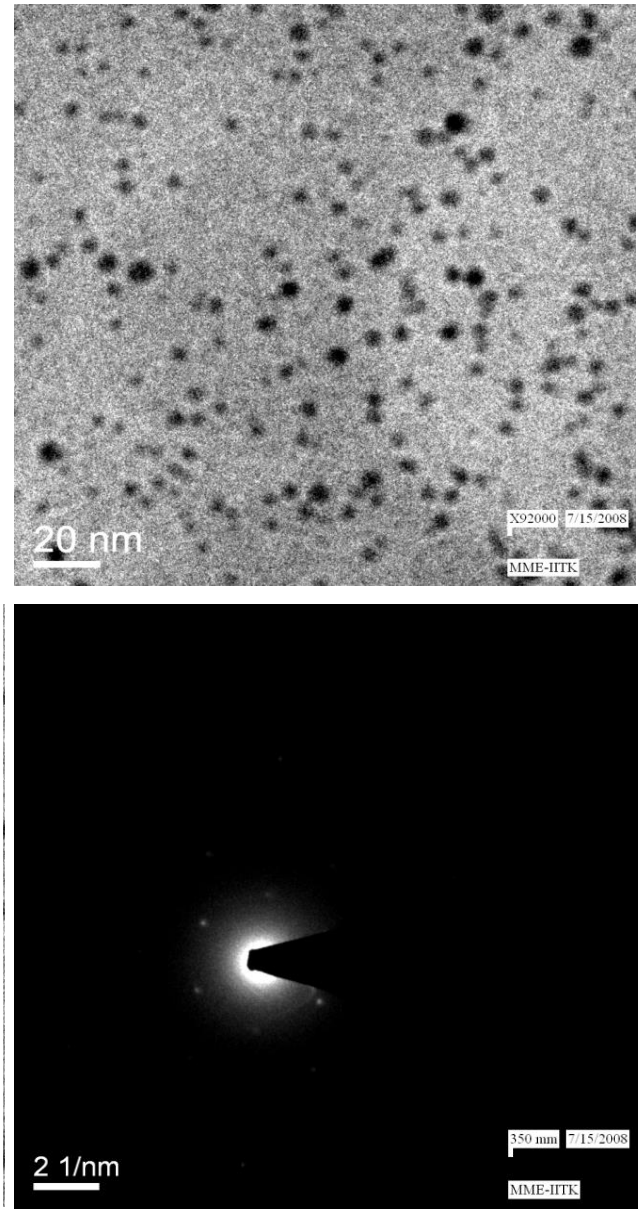

Fig. 1. TEM image of Ag NPs.

Comet assay

Silver nanoparticle induced DNA damage was assessed by single cell gel electrophoresis (SCGE) in accordance with the protocol described earlier **[**9**].** Briefly, a lobe of liver was washed out with homogenizing buffer (Hanks' balanced salt solutions containing 25 mmol/l EDTA-2Na and 10% DMSO) and homogenized in 5ml of the homogenizing buffer. The liver lobes were placed into ice-cold mincing buffer and rinsed sufficiently with the cold mincing buffer to remove residual blood. Cell suspensions were chilled on ice for about 5 min and centrifuged at 1000 rpm for 5 min. After the supernatant was removed, the cells were re-suspended in homogenizing buffer. Ten microliters of the single cell suspension was mixed with 90 ml of 0.5% low-melting agarose gel and 90 ml of the mixture was placed on a slide pre-coated with 1% Normal Melting Agarose (NMA). The slides were placed on ice for 30 min until the agarose layer hardens. Then slides were placed in freshly made cold lysing solution (2.5 M NaCl, 100mM EDTA-2Na and 10mM Tris, pH10.0: DMSO: Triton X-100, 89:10:1) overnight. Next day the slides were placed in the electrophoresis buffer (300 mM NaOH, 1mM EDTA, pH ≥13) for 20 min and electrophoresed for 20 min at the 1 V/cm and ≈300mA in a horizontal electrophoresis unit. Slides were washed 3-4 times with neutralization buffer (0.4M Tris), then dehydrated in chilled absolute alcohol and stored until analysis. To prevent the additional DNA damage all protocol steps were carried out under dimmed light.

Image processing

To analyze the DNA damage, the slides were stained with ethidium bromide (EtBr) (10X stock- 20µg/ml) and examined by randomly selecting 100 cells (50 on each replicate slide) in each experiment group using fluorescent microscope (Axiovert25, Carl Zeiss Micro Imaging Co., Ltd, Berlin, Germany) with an excitation filter of 515-560 nm and a barrier filter of 590 nm. In the present study DNA migration was analyzed by image analysis software (Tritek Cometscore™ version 1.5), calculating the tail length, % DNA in tail i.e., tail intensity (TI) and tail moment (TM). The tail length is the distance from the comet head to the last visible signal in the tail. The percentage of DNA in the tail is calculated from the fraction of DNA in the tail divided by the amount of DNA in the nucleus multiplied by 100. The tail moment, called the Olive tail moment, is the product of the amount of DNA in the tail and the mean distance of migration in the tail **[10]**.

Histopathological examination

For histopathological observations, animals were sacrificed and liver was taken out. Tissue was properly washed, fixed in Bouin's solution and embedded in paraffin. After routine processing 5 µm thick paraffin sections were cut and counter stained in haematoxylin-eosin (H-E) for cytoplasmic contrast. Sections were observed under light microscope (Olympus, CX-21) for histopathological changes.

Liver function tests

Liver functions were examined to assess toxicity induced by Ag NPs. The parameters studied for liver function test were Alkaline Phosphatase (ALP), Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT).

Serum Alkaline Phosphatase (ALP) was estimated by the method given by Henry **[11]** using p-nitrophenyl phosphate (p-NPP). Alkaline Phosphatase cleaves p-nitrophenyl phosphate (p-NPP) into p-nitro phenol and phosphate. p-nitrophenol is a yellow colour compound in alkaline medium and absorbs light at 405 nm.

Activity of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were estimated using the UV kinetic method given by Bergmeyer **[12, 13]** and recommended by the International Federation of Clinic Chemistry (IFCC). The conversion of NADH to NAD+ is proportional to the concentration of AST and ALT in serum and is measured at 340 nm as the rate of decrease in absorbance.

Statistical analysis

The data were expressed as mean ± standard deviation (SD) of three independent experiments. Wherever appropriate, the data were subjected to statistical analysis by one-way analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference (HSD) test for comparison between the treated and control groups and within the treated groups. A value of $p<0.05$ was considered significant and $p<0.01$ level was set as highly significant.

Results and discussion

Silver nanoparticles of size 5 nm and spherical in shape (**Fig. 1**) was prepared by chemical reduction method as described previously **[2, 4]**. Single dose group (Autopsy 3 h after dosing): No clinical signs and changes in body weights were observed in the mice given Ag NPs 50mg/kg and 100mg/kg. The results of comet assays using the liver cells of mice given Ag NPs are summarized in **Table 1**. The value was significantly higher in the 100 mg/kg group than the non-treated control group. No significant difference was observed in % Tail DNA at 50 mg/kg dose. The average % Tail DNA value was 25.06 in the cyclophosphamide treated positive control group, significantly higher than that in the both Ag NPs treated groups.

Table 1. Comet parameters; Tail Length, % DNA in Tail and Tail Moment of different treatment groups.

S. No.	Treatments	Groups	Tail Length (μm)[a]	% DNA in Tail	Tail Moment[a]
1.	Single dose group (Autopsy 3 h after dosing)	Non treated control	8.68±1.33	4.85±1.5	0.31±0.07
		Negative control[b] (Triton X-100)	15.41±2.26	7.04±1.2	0.41±0.38
		Ag NPs (50mg/kg)	15.9±5.4	6.53±1.28	0.34±.24
		Ag NPs (100mg/kg)	21.98±3.32**	14.76±2.41**	2.56±1.00**
		Positive control[c] (Cyclophosphamide)	36.44±3.16**	25.06±2.61**	4.18±1.31**
2.	Single dose group (Autopsy 24 h after dosing)	Non treated control	8.18±1.2	4.23±0.88	0.28±0.13
		Negative control[b] (Triton X-100)	11.63±2.12	7.96±1.20	0.31±0.22
		Ag NPs (50mg/kg)	12.84±3.42	9.22±1.64*	0.42±0.16
		Ag NPs (100mg/kg)	17.56±2.56**	13.23±2.32**	1.88±0.32**
		Positive control[c] (Cyclophosphamide)	26.42±4.76**	23.89±3.81**	3.95±1.18**
3.	Repeated dose group (Autopsy 3 h after dosing)	Non treated control	8.74±1.16	5.12±0.93	0.30±0.19
		Negative control[b] (Triton X-100)	14.52±2.6*	8.56±1.56*	0.44±0.12
		Ag NPs (5mg/kg)	15.36±2.84*	7.32±1.42*	0.53±0.48*
		Ag NPs (10mg/kg)	22.02±3.67**	19.65±2.41**	2.76±0.84**
		Positive control[c] (Cyclophosphamide)	30.18±3.44**	28.45±3.16**	4.05±1.39**

[a]Values are given as the mean±SD.
[b]Triton X-100 was orally administered at 0.5 mg/kg.
[c] Cyclophosphamide was administered intraperitoneally at dose of 25 mg/kg.
*Significantly different from the non-treated control group ($P<0.05$).
**Highly significant difference from non-treated control group ($p< 0.01$).
No. of animals per group: 6.
No. of cells analyzed per animal: 100

Single dose group (Autopsy 24 h after dosing): The result of genotoxicity test revealed that a dose dependent DNA damage at all the concentrations of Ag NPs. These induce significant DNA breakage in mouse liver cells. Higher dose of Ag NPs caused a highly significant increase in tail length, % DNA in tail and tail moment (**Table 1**). The values of TL TI and TM of high dose were comparable to that of group treated with cyclophosphamide.

Serum AST and ALT levels in mice were significantly increased; similarly, the treated animals also showed a significant elevation in the ALP activity in both the dose levels in single dose group (**Fig. 2**). Repeated dose group (Autopsy 3 h after dosing): Animals exposed to repeated doses of Ag NPs did not show any changes in general behavior and body weight at both the dose levels. There occurred almost three fold higher values of parameters (TL, TI, TM) at high dose level in comparison to controls. A highly significant increase in TL, TI and TM recorded at high dose level as compared to negative controls. Whereas in comparison to positive control group, both doses of Ag NPs group showed significant decrease in all the values of TL, TI and TM. This showed that Ag NPs induces significant DNA damage in comparison to non-treated and negative controls but this was not comparable to positive controls.

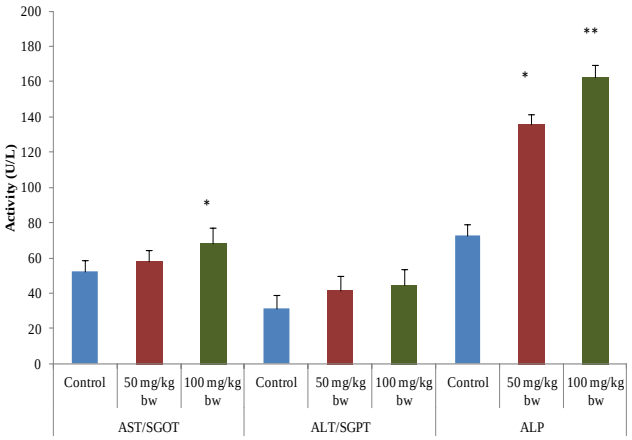

Fig. 2. Activity of AST, ALT and ALP in single dose group.

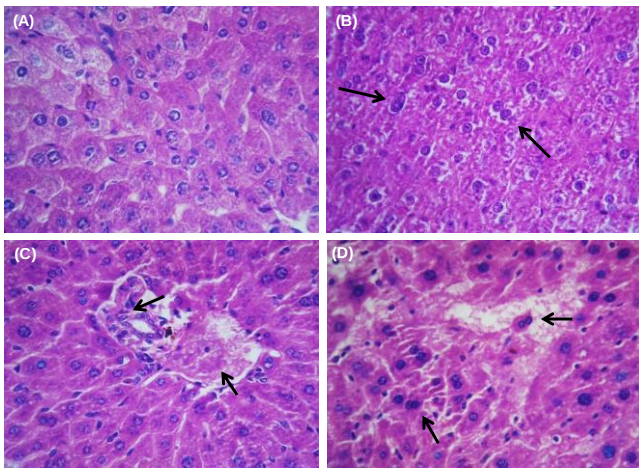

Fig. 3. Liver histopathology images of repeated dose group mice (A) Non treated control, (B) negative control, (C) Ag NPs treated group with 10 mg/kg bw and (D) Ag NPs treated group with 20 mg/kg bw. (Magnification 63X).

Histopathology provides a rapid method to detect effects of irritants in various organs. Treated mice showed hepatic congestion and hemorrhage in liver as compared to non-treated control. Animals administered with the Ag NPs at the repeated dose rate of 10 mg/kg bw cause hepatic and

medullary congestion, leading to mild pathological change in liver tissues (**Fig. 3**).

Serum AST and ALT levels in mice treated with both the doses of Ag NPs in repeated dose group were significantly increased. The treated animals also showed a significant elevation in the ALP activity in this group at both the doses (**Fig. 4**).

Chemical reduction method was used to synthesize silver nanoparticles. The silver particles were spherical with 5 nm average diameter **[2, 4]**. The alkaline version (pH>13) of comet assay is being increasingly used in in vivo genotoxicity testing of substances such as biocides, agrochemicals, food additives, industrial chemicals and pharmaceuticals. Computable study for DNA damage has generated several parameters, including Tailed Nuclei, Tail Length, % DNA in the Tail, and Tail Moment in the comet assay **[8, 9]**. With regard to the interpretation of results, when DNA damage is detected in an organ by treatment with test substance, it is important to confirm whether the damage is attributable to genotoxicity or cytotoxicity.

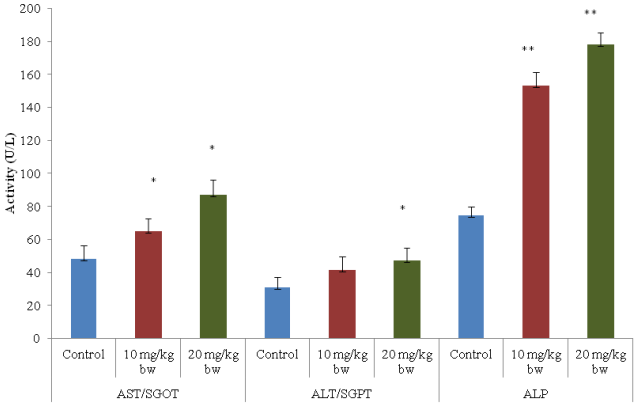

Fig. 4. Activity of AST, ALT and ALP in repeated dose group.

A number of previous studies employing various kinds of cultured cells and animal models suggest that both genotoxicity and apoptosis are important mechanisms for Ag NP induced toxicity. In the present study genotoxicity of Ag NPs was evident in liver cells of mice by comet assay. Statistically a highly significant DNA damage was found at high dose level in cells in all exposure period. These results are in concomitance with other studies shows biochemical and molecular changes related to genotoxicity, initiated by Ag NPs in cultured cells. Ag NPs induced DNA breakage was detected in cell lines using the DNA comet assay **[14-17]**. Furthermore, an enhanced level of histone g-H2AX, which forms at the sites of DNA double-stranded breaks, was observed in Ag NPs treated human hepatoma cells **[18]**, immobilized T cells **[15]** and mouse embryonic fibroblasts and stem cells **[19]**.

The integrity of genomic DNA is constantly under threat, even in healthy cells. Evidence of this study and those of similar previous studies indicate Ag NPs possesses the potential to interact with DNA and cause alterations in mammalian cells *in vivo*. In general, harmful alterations in the genetic material include chromosomal aberrations and point mutations involving a change in a single base. Endogenous ROS or errors in replication or recombination, as well as environmental toxicants can also damage Cellular DNA **[20, 21]**. The main molecular mechanism of nanomaterials induced DNA damage is considered to be the induction of oxidative stress resulting from the generation of ROS. This has been shown that a wide range of nanomaterials including silver nanoparticles are capable to generate ROS both in vitro and in vivo **[4, 6, 22, 23]**.

DNA damage triggers a complex signaling network that functions to activate cell cycle checkpoints and mediate DNA repair. It has been reported that Ag NPs increased the expression level of Rad51, a DNA damage repair protein, in mouse embryonic fibroblasts and stem cells **[19]**. Checkpoint arrested cells resume cell cycle progression once the damage is repaired. Cells with damaged DNA accumulate in the gap 1 (G1) phase, the DNA synthesis (S) phase or the gap 2/mitosis (G2/M) phase. DNA damage give rise to the accumulation of apoptotic cells in the sub-G1 phase **[24]**. A concentration and time dependent increase was also reported in the proportion of A549 cells in the sub-G1 and S phase of cell cycle after treatment of Ag NPs **[22]**. Moreover, DNA lesions such as bulky DNA adducts and DNA double-stranded breaks trigger apoptosis. The formation of bulky DNA adducts has been detected by 32P postlabeling in human lung carcinoma cells exposed to Ag NPs **[25]**. The level of adducts was diminished by pretreatment with N-acetylcysteine, an antioxidant, indicating that ROS initiated DNA adduct formation. Formation of micronuclei, a marker for cellular genotoxicity, was also induced by Ag NPs in human hepatoma cells **[26]**, as well as in lung fibroblasts and glioblastoma cells **[14]**, which send signals downstream to p53, a major effector of the DNA damage checkpoint p53 then induces activation of pro-apoptotic factors, such as B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax) and p21 **[20]**.

Apoptosis is a distinctive mode of programmed cell death that involves the genetically determined elimination of cells. In this process, cells destined to die, activate enzymes that degrade the cells' own nuclear DNA, in addition to nuclear and cytoplasmic proteins. It occurs as a defense mechanism when cells are damaged beyond repair, especially when disease or toxicant mediated damage affects cellular DNA, and cellular repair mechanisms are unable to deal with the injury. It has been observed that silver nanoparticles lead to DNA damage, apoptosis, and necrosis **[25]**.

Different other studies, supporting the results of this present study, showed that other nanomaterial induced histopathological alteration in liver of the experimental animals **[6, 27]**. The consistent changes were seen in the liver of animals, of all treatment groups, including varying degrees of degenerative changes and vascular changes.

The major routes of Ag NPs entry into the body are via skin, respiratory system and gastrointestinal tract **[28]**. Absorbed Ag NPs from the gastrointestinal tract enter in the liver through the portal vein and might have an effect on the liver since the liver functions as the first check point for everything absorbed before it becomes systemic. Absorbed Ag NPs bind to plasma proteins and can enter the cells. They are then distributed in organs such as liver, kidneys, heart, lymph nodes, brain, lungs, stomach and testicles **[29]**. Liver is able to actively remove these compounds from the

blood and transform them to chemical forms that can easily be excreted.

Preliminary step in detecting liver damage is to determine the presence of certain liver enzymes. The activity of these liver enzymes is generally used to assess the liver function. Under normal conditions, these enzymes reside within the liver cells. However, when the liver is injured, these enzymes are spilled into the blood stream. The most sensitive and widely used liver enzymes are the aminotransferases, they include AST and ALT. Their level is increased in cases of liver cell death resulting from shock or drug toxicity **[30].** In the present study, the physiological effects of Ag NPs have been evaluated on serum ALT, AST, ALP in mice. A significant increase in the levels of AST, ALT and ALP were noticed in silver nanoparticles exposed groups in comparison to the controls. Hepatic damage induced by gavaging Ag NPs to mice possibly caused severe irritation of the oxidant system in these cells. The smaller the diameter of the NPs, the more is its influence on cells, thereby increasing its molecular effects on the intracellular mechanisms. With changing the diameter of NMs, their distribution and effects on body tissues also change **[29]**. Free radicals induced by nanoparticles can cause destruction of red blood cells **[31]**. In fact, the free radicals generated by Ag NPs attack the hepatocytes and release ALT stored in them into the blood serum, whereas the immune response of mice to an external factor has been reported to be an increase in the number of WBCs for phagocytosis of NPs **[32]**. Similar results were obtained in the present study as ALT in all treatment groups was found to be elevated when compared to control. As a result, the rate of nanomaterial metabolism in the liver is supposed to be dependent upon the dose administered, percent initial dose taken up by the liver and the cellular distribution within the liver. Considering the importance of hepatocytes in detoxification, any changes in their structure and number can cause severe physiological changes in the animal.

In the present study an increase in ALP levels in mice in all the experimental groups in comparison to controls were observed. The probable reason for this increase could be the inflammatory process and the destruction of hepatocytes. Repeated oral doses of Ag NPs for 5 weeks induced liver toxicity as shown by increase in serum activity of ALP.

Conclusion

Results of present study indicate that the silver nanoparticles induced DNA damage in liver cells was attributable to hepato-cytotoxicity in mice. The DNA damage was more severe at 3 h than 24 h after treatment. This might indicate that the decrease in the DNA damage was due to detoxification or repair or turnover of the cell. Further characterization of their systemic toxicity, genotoxicity and carcinogenicity is also essential.

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Author Contributions
The design of the study was proposed by KKA and PJJ. KA was responsible for the synthesis and characterization of Ag NPs. KKA, RV and AA carried out subsequent experimental procedures as well as statistical analyses. The manuscript was drafted by KA, IS and PJJ. All authors read and approved the final manuscript.

Conflict of interest statement
All authors declare there is no conflict of interest.

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