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Histological alterations and apoptosis in rat liver following silver nanoparticle intraorally administration

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ABSTRACT

Silver nanoparticles (AgNPs) are increasingly used in different applications and can potentially cause adverse effects on organs and tissues. This study investigated the effects of AgNPs administration on the liver´s histology in the rats. AgNPs were orally administered to Sprague-Dawley rats at various concentrations (30, 125, 300 and 700 mg/kg body weight/day) for 28consecutive days. At the end of the experiments, liver tissue was taken for histologic evaluation. The obtained results showed a decrease in diameter of hepatocytes in 125 mg/kg ($P<0.05$) and increase in the Kupffer cells that was more evident in rats exposed to 30 and 125 mg/kg of AgNPs. The histological changes in the liver parenchyma of rats affected by AgNPs included, disruption of hepatic cell cords, polyploidization, hydropic degeneration, presence of inflammation cells, focal lymphocytic infiltration, dilation of sinusoidal space, blood cell aggregation in central vein and sinusoids, binucleated cells, eosinophilic cytoplasm, pyknotic nuclei of hepatocytes. These histological alterations were more evident in rats exposed to 30 and 125 mg/kg of AgNPs. The apoptotic changes were more prominent in 300mg/kg treated group. In 700 mg/kg group, no major changes in the structural component of the liver were observed while occasional foci of inflammatory cell infiltrates were present. Our results suggest that injection of AgNPs could have cytotoxic effects on the structure and apoptosis of livers in rat.

Key words: Silver nanoparticles, Liver, Hydropic degeneration, hepatocytes, Kupffer cells

INTRODUCTION

Nanotechnology is a new field which has extensive potential use in the domestic, industrial, and biomedical fields. Due to the growing number of applications, there is an increasing risk from environmental exposure to nanomaterials[1].Silver nanoparticles(AgNPs) is one of the most commonly used Nanoparticles. Silver has been known from its antimicrobial activity from centuries and used for treating burns, wounds and other diseases

[2].AgNPs can attach to cell membranes, disturbing permeability and respiration. Its adsorption to the negatively charged bacterial cell wall, changes of membrane permeability, deactivating cellular enzymes and generate reactive oxygen species (ROS), which damage lipids, proteins, and DNA[3-5].

Silver compounds can be absorbed through the gastrointestinal and respiratory tract, skin and other mucous membranes[6].They can accumulate in the liver and kidneys and can cause organ damage such as liver failure[7]. Orally ingested silver nanoparticles enter the circulation from the intestine, the next cells affected are those of the liver [8].

Liver is an interesting organ with high regenerative capacity and complex functions. Its strategic location in relation to the food supply via the portal vein, and the unique gene-and protein-expression patterns of hepatocytes, the main functional cells of the liver, allow it to function as a biochemical defense against toxic chemicals entering through the digestive tract and as a reprocessor of absorbed food ingredients[9].

The aim of the present study was to investigate the histological parameters and apoptosis in liver of male rats following 28 days repeated oral exposure of either AgNPs.

MATERIALS AND METHODS

Nanosilver

The silver nanoparticles were purchased from US Research nanomaterials, Inc. (cas:7440-22-4.net weight: 5gr). The purity of the nano silver particles was more than 99%. Ag NPs powder was suspended as described previously[10]. From this stock suspension, AgNPs suspensions with a final concentration of 30, 125, 300 and 700mg/kg were prepared in deionized water. The morphology of the AgNPs was visualized using transmission electron microscopy (TEM). The hydrodynamic diameter of the particles was measured using dynamic light scattering (DLS) (Nano ZS (red badge) ZEN 3600, Malvern Instruments, Worcestershire, UK).

Animals, Conditions and Treatment

50 male adults Sprague Dewily rats (weight 180 to 200 g) were used in this research. The animals were kept on controlled conditions of 22 ± 1°C temperature, 60% ± 10 humidity, 12 h light and darkness, and 12hours free access to water and food . After a 7 day acclimation period, the animals were divided into 5 groups (10 rats per): group vehicle control (deionized water), low-dose group (30 mg/kg/day), middle-dose groups (125 and 300 mg/kg/day), and high-dose group (700 mg/ kg/day).

Suspension of silver nanoparticles was fed to rat (3ml every day) by gavages for 28 days. After this period the rats were anesthetized and dissected, liver was separated carefully and were put in 10% buffered formalin to be fixed and kept for histopathological experiments. The animal body weights were recorded before and after treatment. The body weight gain was calculated from final body weight minus initial body weight. Hepatosomatic index (HSI) was calculated according to following standard formula:
HSI = liver weight (g)/body weight (g) × 100

Histopathology

The fixed tissues were processed routinely, embedded in paraffin, sectioned, deparaffinized and rehydrated using the standard techniques. The histopatological structure was evaluated by assessing the morphological changes in the liver sections stained with hematoxylin and eosin, using standard techniques. All the images were obtained through a transmitted light microscope with video camera (motic 2000) and motic images 2.0 software for the acquisition of the differences in histomorphology of all studied groups.

Light microscopic analysis of hematoxylin and eosin stained slides was also used to quantify apoptotic cells, which were identified by morphological criteria (increased eosinophilic cytoplasm, darkened nucleus, and pycnotic separation of cytoplasmic membrane from neighboring cells).For counting of the number of apoptotic cells in microscopic sections, apoptotic cells were randomly counted in 10 microscopic fields with magnification of 40.

TUNEL (terminal deoxynucleotidyl-mediated dUTP nick labeling) assay

Cell death in the liver tissues was determined using a TUNEL assay kit (Promega, Co., Madison, WI), according to the manufacturer's instructions. Paraffin-embedded tissue sections were rehydrated by immersing the slide in a

graded series of ethanol solutions, and permeabilized with proteinase K solution. Tissue sections were then incubated with biotinylated nucleotide mix, recombinant terminal deoxynu-cleotidyl transferase, and equilibration buffer for 1 h at 37 ◦C. After incubation, tissue sections were immersed in 0.3% hydrogen peroxide in phosphate buffered saline to block endogenous peroxidase, and then incubated with peroxides solution for 30 min at room temperature. Tissue sections were stained with 3,3-diaminobenzidine substrate and counterstained with hematoxylin. An apoptotic index (AI) was calculated for each sample by counting the number of positively stained hepatocyte nuclei divided by the total number of hepatocytes and expressed as percentage.

Statistical analysis

All data are expressed as means ± SE. Data analysis was performed using one way ANOVA and Tukey test. To compare selected pairs of groups, p-value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Characterization of silver nanoparticles

To identify the diameter of the silver nanoparticles, the AgNPs suspension was subjected to dynamic light scattering (DLS) analysis. It demonstrated a hydrodynamic diameter peak, with an average size of 200-300 nm, which was considerably larger than that indicated in product's information (< 100 nm) as mentioned in our previous publication[10].Transmission electron microscopic(TEM) images of silver nanoparticles confirmed that the nanoparticles types were near spherical (Fig.1.).

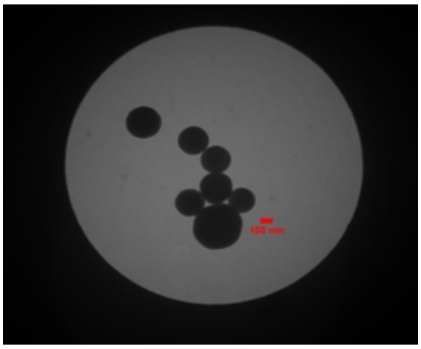

Figure 1 Transmission electron microscopicimages obtained from AgNPs.

Body and organ Weight

Body weight gain significantly decreases in rats fed 300mg/kg AgNPs compared to control group (p=0/002). The rats did not show any significant changes in body weight in other groups during the 28-day experiment (P>0.05).Rats exposed to different concentration of AgNPs showed significant increase (p< 0.05) in the HSI ratio in 125mg/kg group when compared with control (p=0/008) (Table 1). The rats did not show any significant changes in other groups (P>0.05).

Table 1: Body weight gain measurments (means±SD) in control and treated groups with different concentrations of nanosilver particles.

Feature	Control	AgNp 30mg/kg	AgNp 125mg/kg	AgNp 300mg/kg	AgNp 700mg/kg
Body weight gain	**64.83±12.10**	**54.83±16.31**	**55.33±13.18**	**21.50±19.10***	**53.00±21.15**
Hepatosomatic index	**3.38±0.42**	**4.29±0.74**	**4.84±0.78***	**3.86±0.49**	**4.59±0.83***

**Significant difference vs. control, p <0.05*

The first indication for effect of the administered AgNPs was the decrease in total body weight with significant differences observed in 300 mg/kg group. In the present study, an increase in liver weight, and HSI ratio following the exposure of rats to AgNPs were observed. The HSI reflects the relative liver size and is related to the hepatic activity in the detoxification of compounds and hence can be used as an indicator of exposure to toxicants [11-13]. Increases in liver weight were related to hepatic histopathological alterations, this results partially agreed with Kim [14]and Mahdy[15]who recorded liver-weight changes in rats after 90 and 28 days respectively.

Histopathological examinations

Histopathological evaluations of liver tissues were conducted under light microscopy as shown in Figure 2. Normal morphology was found in the control, that is, compact liver parenchyma, clear and regular hepatic cords, and Polygonal hepatocytes located along sinusoids and contained a large, spherical, central nucleus.

However, adverse histological changes in the liver parenchyma of rats affected by AgNPs included the following: disruption of hepatic cell cords, polyploidization, hydropic degeneration, presence of inflammation cells, focal lymphocytic infiltration, dilation of sinusoidal space, blood cell aggregation in central vein and sinusoids, binucleated cells, eosinophilic cytoplasm, pyknotic nuclei of hepatocytes and apoptosis (Fig 2 and Table2). These histological alterations were more evident in rats exposed to 30 and 125 mg/kg of AgNPs. The apoptotic changes were more prominent in 300mg/kg treated group. In 700 mg/kg group, no major changes in the structural component of the liver were observed while occasional foci of inflammatory cell infiltrates were present. A comparison of histopathologic changes between studied groups are shown in table2,

Table 2. Histopathologic changes induced by different doses of Agnps in liver tissue. Severe (+++), moderate (++), mild (+), none (-)

		Control group	30 mg/kg	125 mg/kg	300 mg/kg	700 mg/kg
disruption of hepatic cell cords		-	+	+	+	-
Hydropic degeneration		-	+ +	+ + +	+ +	+
Mononuclear cell infiltration	portal	-	+ + +	+ +	-	+ +
	periportal	-	+ + +	+ +	-	-
	intrasinosoidal	-	+ + +	+ +	-	-
	intralobular	-	+ + +	+ +	-	-
Pyknosis		-	+ + +	+ +	+	-
Hepatocyte with eosinophilic cytoplasm and pyknotic nucleus		-	+	-	-	-
polyploidization		+	+ +	+ + +	+	+ +
binucleated cells		+	+ +	+	+	+ + +
sinusoidal congestion		-	+ +	+	-	+ + +
Central vein congestion		-	+	-	-	+
Apoptosis		-	+	+	+++	++

Pyknotic nuclei were seen in some hepatocytes of 30, 125 and 300 mg/kg AgNPs treated rats. Karyopyknosis is an irreversible condensation of chromatin in the nucleus of a cell undergoing necrosis or apoptosis[16].

The presence of eosinophilic bodies, shrinkage of hepatocytes, nuclear pyknosis indicate typical non-specific necrotic lesions. These changes were more evident in 125 mg/kg group.

Increased binucleation and to lesser extent polynucleation were observed in AgNPs treated rats. This change was more prominent in rats exposed to 125 mg/kg AgNPs group. The increased binucleated hepatocytes were related to histopathological alterations. It has been shown in adult rodents that DNA synthesis induced by chemical compounds is associated with changes of polyploid status in the liver[17]. Binucleation seen in the results of the present study might represent a consequence of cell injury and is a sort of chromosomes hyperplasia which is usually seen in regenerating cells[18]. Al Gurabi et al. [19] have reported increased binucleation in in vitro study after AgNPstreatment. Furthermore, it has been shown that the occurrence of binucleated hepatocytes increases with the necro-inflammatory state [20-22]. Similar result obtained in present study.

Inflammatory cells infiltration was seen in the portal, periportal, intrasinosoidal and intralobular zones of 30 and 125 mg/kg AgNPs treated rats. The appearance of inflammatory cells in the hepatic tissue may suggest that AgNPs could interact with proteins and enzymes of the hepatic tissue and leading to reactive oxygen species generation which in turn may imitate an inflammatory response[23].

Sever degree of hydropic degeneration of the hepatocytes was seen and decreased in severity with increasing the dose AgNPs exposure. The cytoplasmic hydropic degeneration as seen in the results of the present investigation might be accompanied by the leakage of lysosomal hydrolytic enzymes that lead to cytoplasmic degeneration[24].

Polyploid nuclei were seen in some hepatocytes of 125 mg/kg AgNPs treated rats than other groups. It has been suggested that the polyploid genome may provide protection against the cellular stress such as toxic exposure that might be helpful for an organ involve drug detoxification[25].

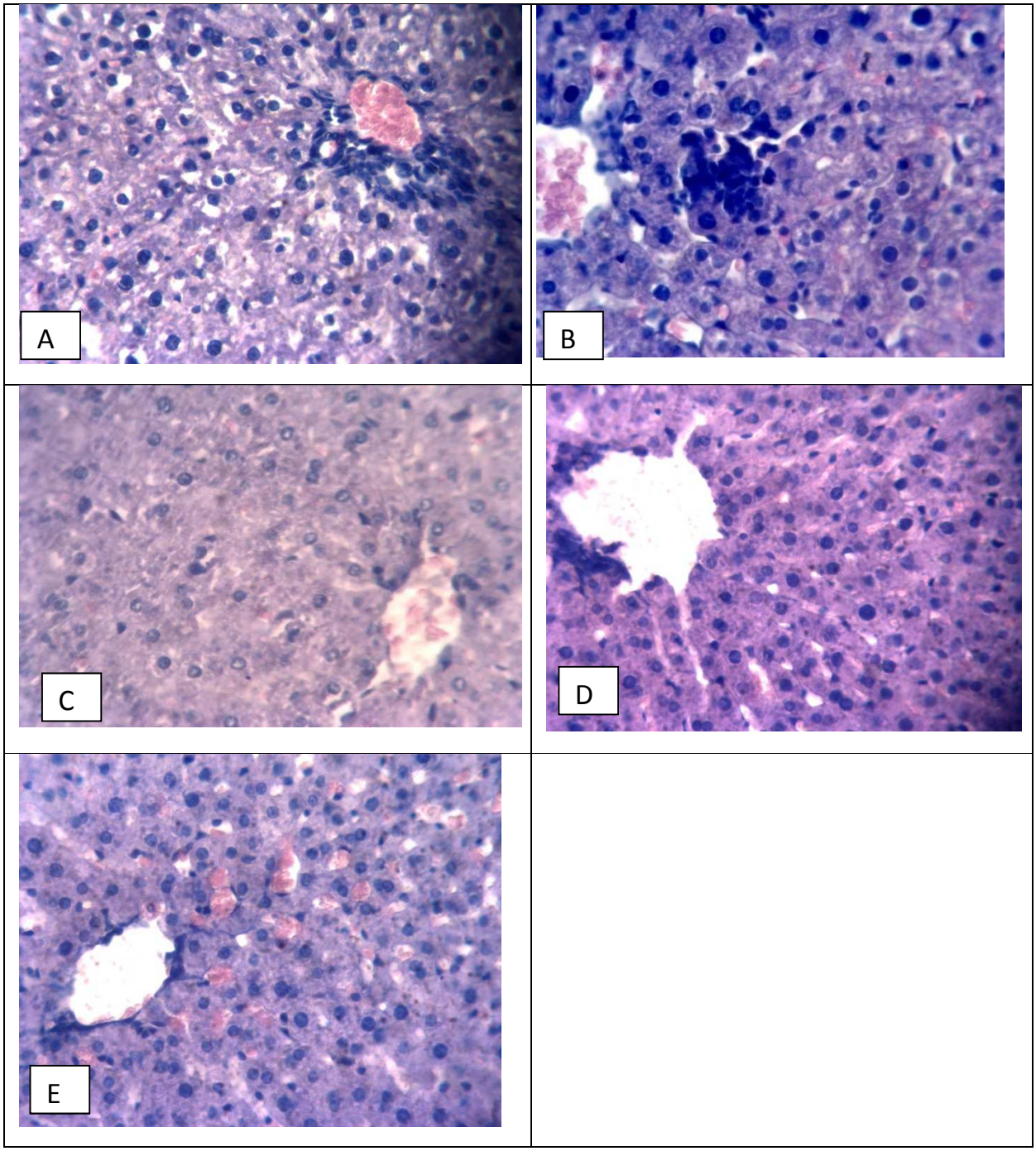

Fig 2. Rat liver (a) 30 mg/kg and (b) 125 mg/kg dose treated groups showing disruption of hepatic cell cords with sinusoidal leucocytosis and Kupffer cell activation and presence of inflammatory cells (H&EX400). (c) 300 mg/kg dose treated group showing mild sinusoidal leukocytosis (H&E ×400). (d) 700 mg/kg dose treated group showing no major changes in the structural component of the liver (H&EX400). (e) Control group

The statistical result from the histomorphometric study of the hepatocytes in the different groups revealed a decrease in the diameter of hepatocytes (Fig. 3). Hepatocytes of rats exposed to the 30 and 125 mg/kg concentrations of AgNPs nanoparticles decreased in size that accompanied to increase in sinusoidal spaces. Such changes indicate progressive hepatocyte degeneration caused by the toxic action of nanoparticles. Agrees with present study Abdelhalim and Jarrar[16]found hepatocytes atrophy and dilation of sinusoidal space in the liver of rats treated with

gold nanoparticles. The decreasing in size and Pyknosis indicate progressive hepatocyte degeneration caused by the toxic action of nanoparticles[26].

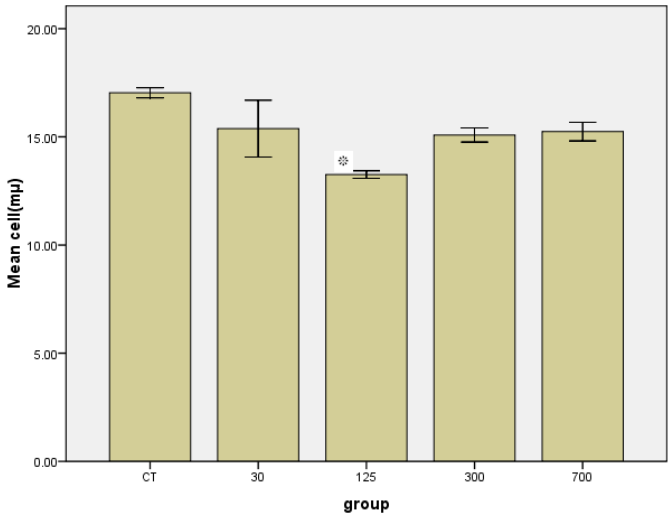

Fig. 3. The diameter of the hepatocytes
**Significant difference vs. control, p <0.05*

The Kupffer cells became prominent and increased in number due to AgNPs exposure. This increase was more evident in rats exposed to 30 and 125 mg/kg of AgNPs (Figure4).

The data of the present study showed that AgNPs activates the phagocytic activity of the sinusoidal cells by increasing the number of Kupffer cells. Similar findings were reported by other investigators[27, 28] . This change was more notable at 30 and 125 mg/kg treated groups. Macrophages are responsible for destruction, detoxification, or recycling of endogenous and exogenous materials[29]. Sadauskas et al. [27] reported the presence of nanoparticles in the cytoplasm of Kupffer cells in the in vitro and Sarhan[30]showed nanosilver particle in Kupffer cells in vivo. These findings and our result confirm an important role of Kupffer cells, in scavenging of nanoparticles and explains the increase in their number in the liver of rats exposed AgNPs. When nanoparticles are removed from the liver by Kupffer cells, the generation of free radicals greatly increases [31]. The increase in oxidant production may contribute to damage the cell membrane and ultimately impair liver function [32]. Thus, the number of Kupffer cells can show the amount of damage in liver tissue. The number of kuppfer cells was lower in group 300 and 700 than 30 and 125 mg/kg groups. We have also observed mild hepatocyte degeneration and infiltration of inflammatory cells in portal vein area in 300 and 700 mg/kg groups. These findings indicate that Kupffer cells were involved in the process of inflammation following AgNPs exposure in lower concentrations.

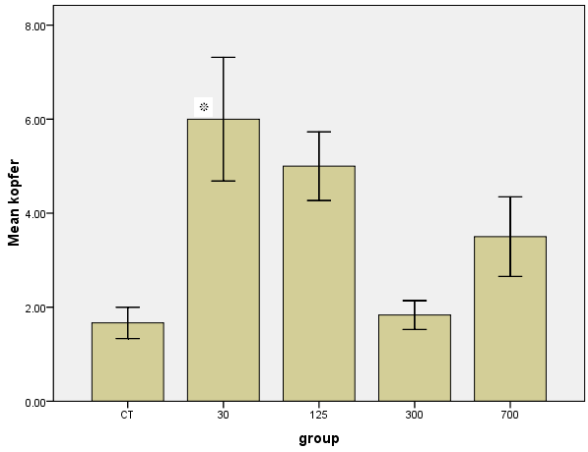

Fig. 4. The mean number of Kupffer cell in 14000 m^2
**Significant difference vs. control, p<0.05*

TUNEL staining

DNA fragmentation analysis in the liver of exposed rats was determined by TUNEL procedure [Figure 3]. It shows that higher TUNEL-positive staining cells in treated 300mg/kg (P=0/001) and 700mg/kg (P=0/001) groups compared with the control, 30 and 125 mg/kg groups (Fig.5).

To assess the extent of cell death by AgNPs, apoptosis was evaluated by H&E staining. Apoptotic changes were observed in the liver tissues of AgNP-treated rats. To confirm that the morphological changes observed were due to apoptosis, the TUNEL assay was performed to detect DNA fragmentation in apoptotic cells *in situ*. Significant increases of TUNEL-positive cells were detectedin the liver tissues of 300 mg/kg AgNP-treated rats. The weak inflammatory response was observed in this group. There is essentially no inflammatory reaction associated with the process of apoptosis[33].

Oxidative stress is an important factor in NP-induced toxicity[34]. A possible role of oxidative stress in the induction of DNA damage and apoptosis has been documented[35]. Our results are consistent with those study demonstrate that AgNPs induce DNA damage, and apoptosis in rats' liver tissues. Many researchers have reported that the AgNPs can dislocate into the rat liver following oral administration of different size and duration[36, 37].

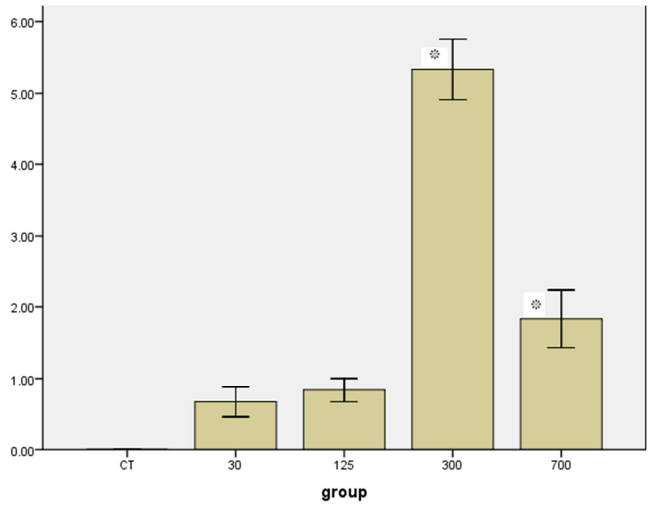

Fig 5:Apoptotic index (Percent of apoptotic cells in control and Agnps treated rats)
**Significant difference vs. control 30 and 125 mg/kg groups, p <0.05*

In the present study, it seems that obtained more sever histological changes of liver in rats administrated 30 and 125 and slight liver damage in 300 mg/kg/day be due to translocation of AgNPs into liver. Our obtained results showed no notable histological changes in group 700 mg/kg/day in comparison with the control group. It may be due to the agglomeration of AgNPs used in this study (~250 nm hydrodynamic diameter agglomerates).

After oral administration, the small intestine is the first site for absorption of nanoparticles. The large agglomeration size of AgNPs in high concentration may also have prevented their intestinal absorption and so resulted in an insufficient amount of AgNPs being available to the liver and interact with hepatocytes. Similar changes were observed by Kim et al. [14]. However, they showed some significant dose-dependent changes in the alkaline phsophatase and cholesterol values rats exposed to AgNPs and indicated that exposure to over more than 300 mg/kg of silver nanoparticles may result in slight liver damage.

CONCLUSION

In conclusion, as shown in the results of the present study, histological changes by AgNPs exposure could be due to AgNPs toxicity. One might conclude that these alterations are dose-dependent with lower ones induced more damage. It seems that AgNPs interact with metabolic processes of the hepatic tissue interfering with the antioxidant

defense mechanism and leading to reactive oxygen species production which in turn may induce stress in the hepatocytes.

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REFERENCES

[1] Colvin VL. *Nature biotechnology*. **2003**;21(10):1166-70.
[2] Rai M, Yadav A, Gade A. *Biotechnology advances*. **2009**;27(1):76-83.
[3] Yin J, Yang Y, Hu Z, Deng B. *Journal of Membrane Science*. **2013**;441:73-82.
[4] Ma S, Lin D. *Environmental Science: Processes & Impacts*. **2013**;15(1):145-60.
[5] Jiang HS, Qiu XN, Li GB, Li W, Yin LY. *Environmental toxicology and chemistry*. **2014**;33(6):1398-405.
[6] Panyala NR, Peña-Méndez EM, Havel J. *J Appl Biomed*. **2008**;6(3):117-29.
[7] Stensberg, Matthew Charles, et al. "Toxicological studies on silver nanoparticles: challenges and opportunities in assessment, monitoring and imaging." *Nanomedicine*, **2011**; 6(5): 879-898.
[8] Liu J, Wang Z, Liu FD, Kane AB, Hurt RH. *ACS nano*. **2012**;6(11):9887-99.
[9] Michalopoulos GK. *Journal of cellular physiology*. **2007**;213(2):286-300.
[10] Pourhamzeh M, Mahmoudian ZG, Saidijam M, Asari MJ, Alizadeh Z. The Effect of Silver Nanoparticles on the Biochemical Parameters of Liver Function in Serum, and the Expression of Caspase-3 in the Liver Tissues of Male Rats. *Avicenna Journal of Medical Biochemistry*. **2016**(In press).
[11] Li Z-H, Zlabek V, Li P, Grabic R, Velisek J, Machova J, Randak T. *Ecotoxicology and environmental safety*. **2010**;73(6):1391-6.
[12] Amacher DE, Adler R, Herath A, Townsend RR. *Clinical chemistry*. **2005**;51(10):1796-803.
[13] Niazi JH, Sang B-I, Kim YS, Gu MB. *Applied biochemistry and biotechnology*. **2011**;164(8):1278-91.
[14] Kim YS, Kim JS, Cho HS, Rha DS, Kim JM, Park JD, Choi BS, Lim R, Chang HK, Chung YH. *Inhalation toxicology*. **2008**;20(6):575-83.
[15] El Mahdy MM, Eldin TAS, Aly HS, Mohammed FF, Shaalan MI. *Experimental and Toxicologic Pathology*. **2015**;67(1):21-9.
[16] Abdelhalim MAK, Jarrar BM. *Lipids in Health and Disease*. **2011**;10(1):1.
[17] Gentric G, Desdouets C, Celton-Morizur S. *International journal of hepatology*. **2012**;2012.
[18] Jarrar BM, Taib NT. *Saudi journal of biological sciences*. **2012**;19(2):203-10.
[19] Al Gurabi MA, Ali D, Alkahtani S, Alarifi S. *OncoTargets and therapy*. **2015**;8:295.
[20] Galon J, Pages F, Marincola FM, Angell HK, Thurin M, Lugli A, Zlobec I, Berger A, Bifulco C, Botti G. *Journal of translational medicine*. **2012**;10(1):205.
[21] Grizzi F, Chiriva-Internati M. *Medical hypotheses*. **2007**;69(2):258-61.
[22] Saller R, Meier R, Brignoli R. *Drugs*. **2001**;61(14):2035-63.
[23] Nagy A. Characterization and Interactions of Nanoparticles in Biological Systems: The Ohio State University; 2010.
[24] Aimaretti G, Ambrosio MR, Di Somma C, Gasperi M, Cannavo S, Scaroni C, Fusco A, Del Monte P, De Menis E, Faustini-Fustini M. *The Journal of Clinical Endocrinology & Metabolism*. **2005**;90(11):6085-92.
[25] Guidotti J-E, Brégerie O, Robert A, Debey P, Brechot C, Desdouets C. *Journal of Biological Chemistry*. **2003**;278(21):19095-101.
[26] Abdelhalim MAK, Jarrar BM. *Lipids in Health and Disease*. **2011**;10(1):1.
[27] Sadauskas E, Wallin H, Stoltenberg M, Vogel U, Doering P, Larsen A, Danscher G. *Particle and Fibre Toxicology*. **2007**;4(1):1.
[28] Priprem A, Mahakunakorn P, Thomas C, Thomas I. *Am J Nanotechnol*. **2010**;1:78-85.
[29] Agius C, Roberts R. *Journal of Fish Diseases*. **2003**;26(9):499-509.
[30] Sarhan OMM, Hussein RM. *International journal of nanomedicine*. **2014**;9:1505.
[31] Jain TK, Reddy MK, Morales MA, Leslie-Pelecky DL, Labhasetwar V. *Molecular pharmaceutics*. **2008**;5(2):316-27.
[32] Aslan M, Ryan TM, Adler B, Townes TM, Parks DA, Thompson JA, Tousson A, Gladwin MT, Patel RP, Tarpey MM. *Proceedings of the National Academy of Sciences*. **2001**;98(26):15215-20.
[33] Elmore S. *Toxicologic pathology*. **2007**;35(4):495-516.
[34] Li N, Xia T, Nel AE. *Free Radical Biology and Medicine*. **2008**;44(9):1689-99.

[35] Chandra J, Samali A, Orrenius S. *Free Radical Biology and Medicine*. **2000**;29(3):323-33.
[36] Loeschner K, Hadrup N, Qvortrup K, Larsen A, Gao X, Vogel U, Mortensen A, Lam HR, Larsen EH. *Particle and fibre toxicology*. **2011**;8(1):1.
[37] Choi JE, Kim S, Ahn JH, Youn P, Kang JS, Park K, Yi J, Ryu D-Y. *Aquatic Toxicology*. **2010**;100(2):151-9.