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RESEARCH ARTICLE

Effects of silver nanoparticles on the functional tests of liver and its histological changes in adult male rats

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ABSTRACT

Objective(s): Silver nanoparticles show anti-fungal properties, and is widely used in medicine. In this research, the impacts of silver nanoparticles on the hepatic functional tests and changes in liver tissues in adult male rats were investigated.

Methods: In this experimental study, 28 adult male Wistar rats, each weighing approximately 180-220 g were divided into 4 groups of 7: the control group, and the experimental groups 1 and 2 received silver nanoparticles that were synthesized at 75 seconds interval with doses of 25 and 100 mg/kg intraperitoneally for 14 days, respectively. Experimental group 3 received silver nanoparticles that were synthesized at 300 seconds interval with a dose of 25 mg/kg intraperitoneally for 14 days. At the end of experiment period, blood samples were obtained from their hearts, and serum levels of hepatic enzymes (AST, ALT, ALP), albumen and total protein were measured. In addition, possible histological changes in liver was studied after hematoxylin-eosin staining. The results were statistically analyzed using ANOVA and Duncan test.

Results: The findings reported that the mean serum levels of aspartate aminotransferase (AST), total Protein and albumin in the experimental groups 1 and 3 increased significantly relative to the control group. Similarly, the mean serum levels of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in the experimental group 3 increased significantly relative to the control group ($P < 0.05$). Also, necrosis of the liver tissue was observed in the recipients of silver nanoparticles.

Conclusions: The use of silver nanoparticles can boost the serum levels of hepatic enzymes and Increase liver tissue necrosis as well.

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INTRODUCTION

Nanotechnology is the science of producing Nano particles [1]. The application of silver nanoparticles are generally in the manufacture of medical, household and industrial products [2]. These nanoparticles have antibacterial properties and are widely used in medicine [3]. Studies suggest that silver nanoparticles can induce strong cellular toxicity, pro inflammatory effects and oxidative stresses in human pulmonary epithelial cells [4]. According to some studies, silver nanoparticles can cause *In vitro* hepatocellular toxicity in rat. However, there is little information about their toxic side effects [5].

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Nano-materials induce cellular intoxication by producing reactive oxygen species (ROS) and releasing cytokine [3]. In biological systems, ROSs are removed and produced by endogenous and exogenous antioxidants [6]. Oxidative injury to DNA as well as apoptotic cell death can be induced by increased production of reactive oxygen species(ROS) [3]. Apoptosis is induced by a cascade or successive activities of caspases, which are a group of proteins from the protease family [7]. caspase-3 is an effector caspase involves in internal cell death cascade, and provides different substrates to the cell leading to apoptosis [8].

In a study by Ansar *et al.* (2017), it was shown that silver nanoparticles can lead to testicular inflammation and toxicity in male rats [9]. Also, these particles induce pathological changes in testicular tissues as well [9]. Similarly, Saleiman and colleagues in 2013 demonstrated that silver nanoparticles in Wistar rats before maturation changes the development of reproductive system leading to reduced spermatogenesis and damage to the sperm content in adulthood [10].

Furthermore, Zhang and colleagues (2017) reported that silver nanoparticles can improve biliary atresia syndrome in mice orally inseminated with live attenuated rhesus monkey's Rotavirus vaccine [11]. *In vitro* studies confirm cellular toxicity of silver nanoparticles [12], and show that they can stimulate oxidative stress in human hepatic cells [3]. In addition, the effects of silver nanoparticles on lining of various cancer cells have been reported [13].

Barros *et al.* (2018) determined that silver nanoparticles may be effective against bacterial infections resistant to several drugs [14]. Also, Wang *et al.* (2017) showed that silver nanoparticles induce sperm toxicity and increase damage to DNA through reactive oxygen species production [15].

Furthermore, in a study by Zhang *et al.* (2015), it was shown that silver nanoparticles can cause a number of problems in pregnant mice [16]. The initial exposure to silver nanoparticles has the potential of negatively affecting fetal health during embryonic and postembryonic period through epigenetic changes in placental development [16]. Han *et al.* (2016) also observed that the administration of silver nanoparticles significantly reduces germ cells in male and female mice. These findings reveal that silver nanoparticles have a negative effect on reproduction [17]. Fehaid *et al.* (2018) reported that Silver nanoparticles can reduce apoptotic cell death induced by necrotic alpha factor in the tumoral NCI- H292 cells of pulmonary epithelial cell lines [18].

Since the therapeutic use of silver nanoparticles has various side effects such as oxidative stresses and the increase in free radicals, and since liver is a vital organ, we tried to examine the possible influence of various doses of silver nanoparticles on hepatic enzyme levels (AST, ALT, ALP), albumin and total protein as well as histological changes in the liver of male rats. So, in case of possible destructive effects of silver nanoparticles on the levels of liver enzymes, biochemical factors associated with liver and liver tissue should be used with caution.

MATERIALS AND METHODS

Synthesis of silver nanoparticle

In this research, silver nanoparticles were produced by electrochemical method in a two-electrode cell using the Sama 500 electro-analyzer system. Nanoparticle synthesis was performed using Controlled Current Coulometry_(CCC) at room temperature [19]. In this method, two plates of platinum are used as cathode and anode. A constant current is applied in a fixed time interval, in which the current range is between 0.001-1 amp and a time interval of 1-65000_sec. One of the electrodes used as cathode and anode is static and the other is rotary (rotational speed of 3000 rpm). The fixed anode electrode is a plate 2 cm long and 1 cm wide. Also, the dimensions of the rotating electrode acting as the cathode are 0.7 cm. In order to achieve a smooth and clean platinum surface, electrodes were electrically polished before each experiment.

The electrolyte solution included 5mM silver nitrate ($AgNO_3$), 0.1 M potassium nitrate ($KNO_{3)}$ and double distilled water, and (20 gr/lit) polyvinyl pyrrolidone (PVP) was added as the stabilizer. To speed up the transfer of synthesized silver nanoparticles from the vicinity of the cathode into the solution and to increase the uniform distribution of nanoparticles in the solution, we used a magnetic stirrer [19].

Animals

The protocol of the study was set up in accordance with the international low on protection of animals in laboratory and approved by the Ethics Committee of Shiraz Islamic Azad University. This experimental study was performed on 28 adult male Wistar rats with an approximate weight of 180-220 g and 2.5-3 months old .Throughout the experiment, all animals had unrestricted water and food , and kept in a special room at 20-22 °c and 12 hours in brightness and 12 hours in darkness.

Animal treatment

Animals were divided in 4 groups of 7 as follows: the control group, which included 7 untreated rats, and the experimental groups 1 and 2 received silver nanoparticles that were synthesized at 75 seconds interval with doses of 25 and 100 mg/kg intraperitoneally for 14 days, respectively. Experimental group 3 received silver nanoparticles that were synthesized at 300 seconds interval with a dose of 25 mg/kg intraperitoneally for 14 days. The used doses, period of time and type of injection

were selected using the previous studies [20-24].

At the end of the treatment period, animals were anesthetized by ether, and blood samples were collected from the left ventricle of the heart. The blood samples are maintained for 20 minutes under laboratory conditions, and centrifuged for 15 minutes at 5000 rpm. Then, serum of each sample was collected and used for the measurement of hepatic enzymes and protein level. AST and ALT levels were Measured by DGKC phosphate buffer, and ALP level was measured by P-Nitrophenyl phosphate AMP method. To measure total protein, the biuret reaction end point method was used. In this experiment, under the alkaline condition, proteins form azure color with tartrate and copper ions, and the color severity is fits with the level of total protein in the sample. To measure albumin, the Bromocresol Green method was used. In this method, the albumin creates a colored complex with Bromcresol, and the severity of the color is fits with the level of albumin in the sample [25 and 26].

Histological experiments

Following blood collection, liver was removed from each animal's abdomen. They were fixed in 10 percent formaldehyde. The dehydration stage was conducted with different concentrations of alcohol (low to high). The clarification step was carried out by placing tissue samples in two containers containing xylan. To substitute sample's water, tissues were placed in three dishes containing molten paraffin (65 °C) for one hour. In the molding stage, the paraffin embedded sample is placed inside with a mold filled of molten paraffin, and in the cross section, the sections of the tissue were cut to 4-5 microns in thickness. In the staining stage, hematoxylin and eosin stains were used. All histological studies were performed under the supervision of a pathologist [27].

Statistical analysis

The results were analyzed by SPSS software (Version 22.0, SPSS Inc., Chicago, IL, USA), and ANOVA and Duncan tests. The statistical inference margin was used to examine significant differences between the experimental groups and the control group. The significance level of the results was considered to be $p < 0.05$., the results obtained in this research are presented in tables along with the corresponding statistical calculations.

RESULTS AND DISCUSSION

In this study, silver nanoparticles were synthesized by electrochemical method. Since one of the parameters affecting the production of nanoparticles in this method is the synthesis time, in accordance with the diagram (Fig. 1), we observed that absorption rose with increasing the synthesis time. Also, the bright yellow color of the solution at 25 seconds changed to light brown in 300 seconds, which reflects the formation of more nanoparticles, or elevation of the synthesized nanoparticle's concentration with time [19].

To study the toxic effects of silver nanoparticles on the liver, the nanoparticles synthesized were used at two different time intervals of 75 seconds and 300 seconds. Since, we required enough silver nanoparticles for 14 days of injection, it was necessary to evaluate shelf-life of these particles with time. Thus, to investigate the sustainability of

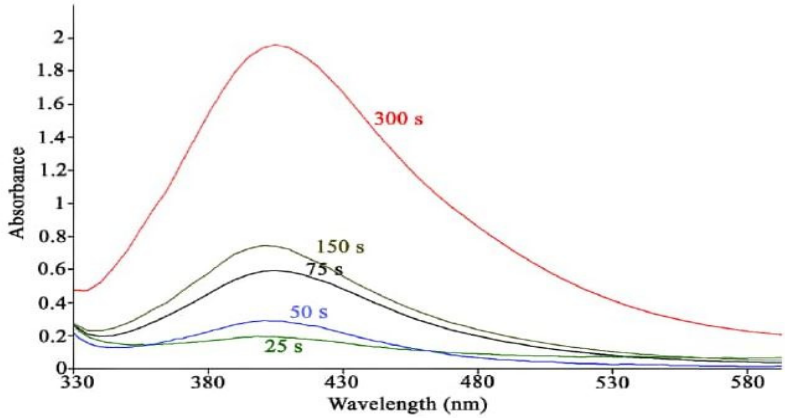

Fig. 1. The ultraviolet–visible spectrum of silver nanoparticles synthesized by electrochemical method in different time intervals of 25, 50, 75, 150 and 300 seconds (1 amp current and rotation speed of 3000 rpm).

synthesized nanoparticles, we examined the color alteration of the solutions and their spectrum with passing time.

The color of silver nanoparticle solution synthesized at 75 seconds became turbid with passing time and formed some sediments. As shown in Fig. 2, after a few days, a little spectrum widening and relatively low absorption intensity is seen in the absorption spectrum of silver nanoparticles synthesized at 75 seconds interval. It can be deduced that this species of nanoparticles sediment with time.

In addition, with time, the color of silver nanoparticles produced at one amp current, rotation speed of 3000 rpm and time interval of 300 second changed from light brown to opaque brown. Also, Fig. 3 shows that the severity of the absorption spectrum of silver nanoparticles synthesized at 300 second interval decreases with passing of time.

The sustainability study of silver nanoparticles indicated that during injection time period (14 days) the absorption intensity of nanoparticle's spectrum declined, and some of them sediment. Hence, silver nanoparticles were synthesized on a daily basis and used for the injections. In this study, the synthesized silver nanoparticles were centrifuged for 15 minutes with 14000 rounds. Then, in order to remove additional chemicals in the final product, the nanotube was washed three times with distilled water [19].

Statistical studies and comparison of mean serum concentrations of AST, ALT, ALP, albumin and total protein were performed in the experimental groups receiving silver nanoparticles compared to the control group. The mean serum

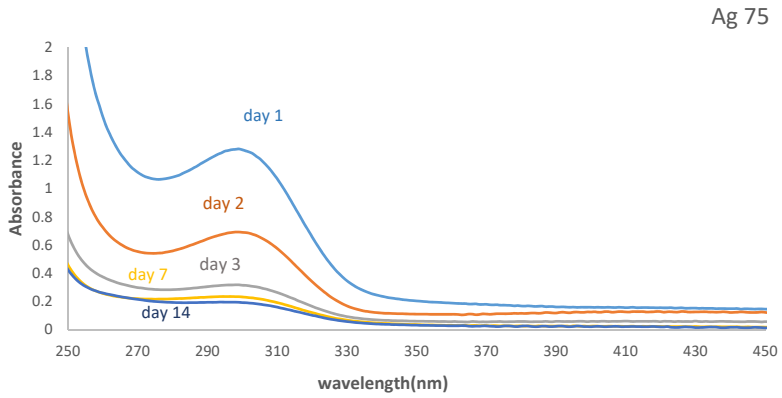

Fig. 2: The absorption spectrum of silver nanoparticles synthesized by electrochemical method at 75 seconds interval in the first, second, third, seventh and 14th days (1 amp current and rotation speed of 3000 RPM).

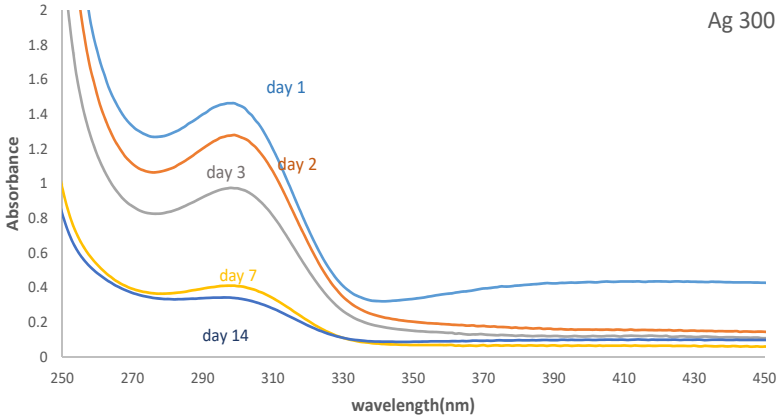

Fig. 3: The absorption spectrum of silver nanoparticles synthesized by electrochemical method at 300 second interval in the first, second, third, seventh and 14th days (1 amp current and rotation speed of 3000 RPM).

concentration of aspartate aminotransferase (AST) enzyme in the experimental groups 1 and 3 receiving silver nanoparticles showed a significant increase compared to the control group (P <0.05; Table 1). Similarly , the mean serum concentrations of alanine aminotransferase (ALT) enzyme and alkaline phosphatase (ALP) in the experimental group 3 relative to the control group increased significantly at the level of P<0.05 (Table1).

Also, the mean serum of albumin and total protein levels in the experimental groups 1 and 3 receiving silver nanoparticles showed a significant increase compared to the control group at the level of P <0.05 (Table 1).

Histological findings

The results of histological studies showed that the liver tissues were completely normal in control group with no cell damage. They had normal cellular order and maintained the radial and natural state observed in normal livers (hepatocytes have one nucleus or two nuclei), and the presence of nucleolus and central venous are two characteristic features (Fig. 4).

Conversely, in the experimental group 1, there was a slight histological variation compared to the control group, and liver tissues changed slightly. In addition, hepatic necrosis and cellular congestion were observed (Fig. 5).

Experimental group 2 showed more damage in comparsion with the experimental group 1. Cellular damaging was seen as swelling, cytoplasmic swelling, nuclear swelling, vacuole formation, hemorrhage and necrosis (Fig. 6).

Moreover, experimental group 3 showed more cell damage than experimental groups 2 and 1 (Fig. 7). As before, cellular damaging was seen as swelling of the cytoplasm, nuclear swelling, vacuole formation, abnormal hemorrhage, cellular congestion and necrosis.

According to the results, serum levels of aspartate aminotransferase enzyme, albumin and total protein in experimental groups 1 and 3 showed significantly increased relative to the control group. Likewise, mean serum levels of alanine aminotransferase and alkaline phosphatase

Table 1: Comparison of mean concentrations of ALP, AST, ALT, albumin and total protein between the experimental groups receiving silver nanoparticles and the control group.

All groups	ALP (U/L) ($\overline{X} \pm SEM$)	ALT (U/L) ($\overline{X} \pm SEM$)	AST(U/L) ($\overline{X} \pm SEM$)	Total Protein (gr/dl) ($\overline{X} \pm SEM$)	Albumin (gr/dl) ($\overline{X} \pm SEM$)
Control group	48.73±760.14	10.61±107.29	9.45±138.29	0.13±6.37	0.12±3.65
Experimental group1	108.63±987.57	23.26±152.57	* 26.11±201.86	*0.27±7.47	*0.14±4.19
Experimental group2	57.56±768.57	8.19±99.14	17.30±193.14	0.34±6.39	0.20±3.50
Experimental group3	* 131.5± 1061.50	* 27.47±166.50	* 29.12±224.67	*0.28±7.35	*0.16±4.10

* There is a significant difference between the experimental groups receiving silver nanoparticles and the control group at the level of p<0.05. Values are based on the mean ± mean error.

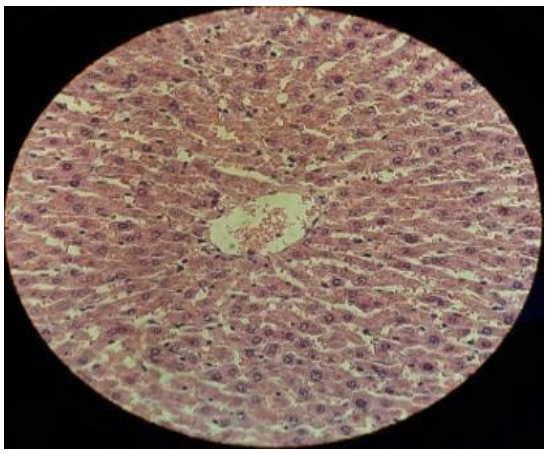

Fig. 4. Photomicrograph of rat liver tissues in the control group. In this group, the hepatic tissues appear completely normal and do not show cellular damaging. All hepatocytes are healthy. (Hematoxylin-eosin staining, magnification 400×).

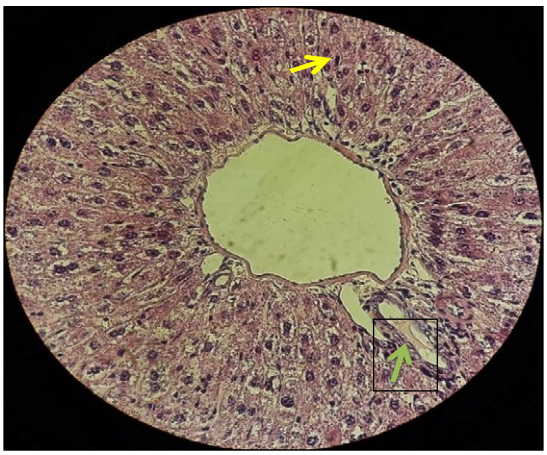

Fig. 5: Photomicrograph of rat liver tissues in the experimental group 1. In this group, there was a slight change relative to the control group. Necrosis is shown with a yellow arrow, and cellular congestion is indicated by a green arrow. (Hematoxylin-Eosin staining, magnification 400 ×).

enzymes in experimental group 3 showed showed significantly increased relative to control group. Also, in the experimental groups receiving silver nanoparticles liver tissue necrosis was observed.

Blanco and colleagues (2018), shown that oral administration of silver nanoparticles would increase oxidative stress symptoms in male' rats liver [28]. These nanoparticles increased the superoxide dismutase and catalase activity in the liver of male rats. In addition, silver nanoparticles at a concentration of 200 mg / kg elevate the production of ROS, followed by cellular damage [26].Also, Kim and colleagues (2010) showed that silver nanoparticles lead to hyperplasia of bile ducts without or with necrosis or fibrosis or pigmentation in treated animals [29].

Moreover, Al Gurabi and colleagues in 2015, the potential effects of silver nanoparticles on apoptotic cell death and DNA damage in albino mice in vivo conditions were investigated [30]. It was shown that silver nanoparticles induce a significant increase in symptoms of hepatic damage such as elevation levels of ALP, ALT and AST enzymes. Additionally, damage to DNA was observed in mice treated with silver nanoparticles, and these particles caused cell death in the liver and DNA fracture in lymphocytes. Silver nanoparticles at a dose of 7.8 mg / kg caused a significant DNA damage and cell death [30].

Guo *et al.* (2016) found that intravenous induction of silver nanoparticles lead to intoxication of the liver and kidney organs through reducing the endothelial interconnections related to intracellular ROS [31]. In addition, abnormalities of endothelial cells induced by silver nanoparticles can mediate general peripheral inflammation in the liver and kidneys through intravenous exposure [31].

Also, in a study by Ramadi *et al.* (2016), systemic exposure to silver nanoparticles was shown to induce liver toxicity and NLRP3-dependent inflammation [32]. These nanoparticles increased levels of AST, ALT and LDH as well. Additionally, about 24 hours after induction of silver nanoparticles, a dose-related increase was observed in the use of peritoneal neutrophils and up-regulation in the expression of several pro-inflammatory gene mediators, including tumor necrosis factor alpha and interleukin B. Moreover, the findings of the liver tissue pathology showed hepatic necrosis and increase in the sinusoidal kupffer cells and granulomatosis 24 hours after the induction of silver nanoparticles [32].

Fatemi and colleagues in 2017 reported that oral administration of silver nanoparticles to mothers may induce oxidative stress and apoptotic cell death in the livers of rat's neonatal [33]. Additionally, these nanoparticles significantly reduced the activity of glutathione peroxidase and levels of glutathione, while the caspase 9 expression and malondialdehyde concentration were significantly increased. However, changes in caspase 8 content in neonatal liver were not significant [33].

Also, Jia *et al.* (2017) demonstrated that pro-inflammatory activity of kupffer cells induced by silver nanoparticles causes liver inflammation, and

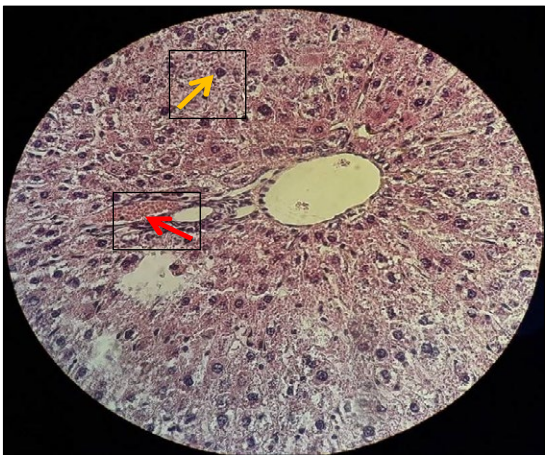

Fig. 6: Photomicrograph of rat liver tissue in experimental group 2. In this group, necrosis was more intense (yellow arrow), and There were also signs of hemorrhage (red arrow). (Hematoxylin-Eosin staining, magnification 400 ×).

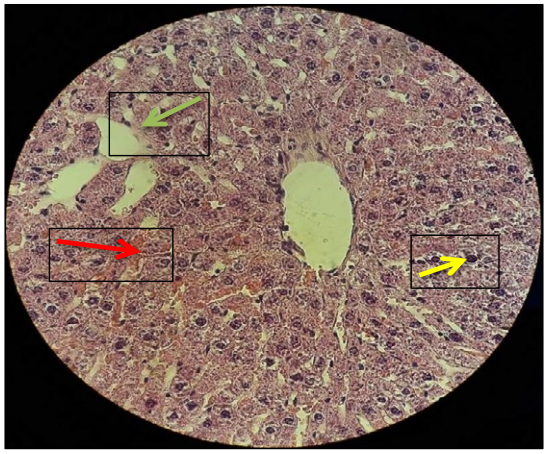

Fig. 7: Photomicrograph of rat liver tissue in experimental group 3. In this group, more severe cell damage was observed compared to other experimental groups. Excessive hemorrhage, necrosis and liver cell congestion were observed (Hematoxylin-Eosin staining, magnification 400×).

reduction in fatty acid oxidation is a key factor in these mechanisms [34]. In this study, it was found that silver nanoparticles did not cause general poisoning in healthy mice, but derived the fatty liver disease from steatosis to steatohepatitis only in mice with high weight [34]. In addition, Teodoro and colleagues (2016) showed that long-term exposure to very low doses of silver nanoparticles can result in changes in liver mitochondrial function in rats [35].

Susan *et al.* (2009) also reported that the different effects of nanoparticles with diameter and their dispresion in body tissues were directly related [36]. In fact, the free radicals of silver nanoparticles attack hepatocytes, and release their stored ATP into the bloodstream, and through immune response to an exogenous factor, the mice increase white blood cell count to engulf silver nanoparticles [37].

In addition, Kim and colleagues in 2009 showed that intoxication caused by silver nanoparticles cannot be achieved solely by the presence of silver in a nanoparticle solution [38]. Silver and silver nanoparticles can induce oxidative stresses associated with genetic and cellular intoxication [39]. Fange *et al.* (2015) also suggested that cell death induced by silver nanoparticles in rats appears to include necrosis and apoptosis [40], and occur in plans related to exposure period and doses. These information provides direct evidence that increased mitochondrial ROS plays a major role in the destructive effects of silver nanoparticles.

It seems that the results of this study appear to be in line with the results of other studies by the researchers; that is, silver nanoparticles stimulate the elevation of serum levels of hepatic enzymes and other biochemical factors associated with liver through the induction of oxidative stress and increased reactive oxygen species, eventually leading to hepatic injuries. According to the results obtained, it can be suggested that factors including the size, dosage and the coating of nanoparticles play a decisive role in their poisoning. Thus, one can reduce the destructive effects of silver nanoparticles in the liver by adjusting their concentration.

CONCLUSION

Overall, the finding of this study showed that silver nanoparticles have adverse effects on liver functional tests and hepatic tissue in adult male rats. Consequently, special tips should be considered while using these particles.

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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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