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Introducing Silver Nanoparticles as Anti-Giardial in Experimentally Infected Mice: Therapy versus Toxicity

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ABSTRACT

The protozoan *Giardia* continues to be one of the most common causes of parasitic diarrhea and a considerable health problem particularly in developmental countries worldwide. The commercially used drug, Metronidazole (MTZ), used for the treatment of giardiasis is often associated with hazardous side effects and occurrence of refractory cases due to development of resistant strains. Therefore the search for alternative therapies is required. Silver nanoparticles (AgNPs) are considered one of the most important modern medical applications for treating various infections, including infections with parasites. The aim of this study was to assess the in vivo anti Giardial effect of AgNPs used singly or in combination with MTZ versus MTZ. Mice were infected by inoculation of the *Giardia* cysts orally then they were divided into four groups according to the drug taken. Two weeks post infection; mice were given the drugs orally for three days. Parasitological and histopathological assessments of the drug effect were done. Parasitological assessment was done via studying the parasite density and histopathological assessment was done by examining sections of intestine, liver using Hematoxylin and Eosin stain. Mice that were treated with AgNPs and MTZ showed statistically significant reduction in the mean number of the parasite in stool, when compared to the control group (not treated) however, AgNPs and combination therapy show less antigiardial activity compared to MTZ. Light microscopic examination showed that the parasites have been eliminated from the intestine in mice treated with MTZ, while mice treated with AgNPs have shown remained trophozoites in intestine, as well as in combined treatment (AgNPs with MTZ). Various histopathological changes were observed in the intestine and liver included; degeneration, PMN infiltration and associated with a death of cells by necrosis and apoptosis.

Keywords: *Giardia*, silver nanoparticles, metronidazole, *in vivo*, parasitological, histopathological.

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INTRODUCTION

Giardiasis is an infection caused by the flagellated protozoa *Giardia lambila*; synonymous *Giardia duodenalis* and *Giardia intestinalis*; which is considered a major intestinal parasitic disease causing diarrhea worldwide. Transmission of parasites usually occurs by ingestion of the infectious cysts in contaminated food or water (Eissa and Amer, 2012).

The trophozoites can colonize and replicate in the host small intestinal tract, particularly duodenum and adhere via ventral disk and specific receptor ligand to the human intestinal epithelium more specifically to the brush border. Encystation process occurs while trophozoites migrating towards the lower intestine where they are shed to the outside environment as infective cysts (Cotton *et al*., 2011 and Finkand Singer, 2017).

Metronidazole (MTZ) is the commercially used drug for treatment of giardiasis but the development of parasitic resistance and the side effects of the drug make it necessary to search for new therapeutic remedies (Eissa and Amer, 2012).

In recent years, silver nanoparticles (AgNPs) have gained considerable attention and have many applications in various fields, including therapeutic properties and treatment of various infections (Khan *et al*., 2018) The AgNPs are used as a new therapeutic agent with a broad range of effects on microorganisms including bacteria, viruses, fungi and parasite as *Toxoplasma*, *Entamoeba histolytica*, *Cryptosporidium Parvum*, *leishmania*, and *plasmodium* (Khan *et al*., 2018; Baiocco *et al*., 2011; Allahverdiyev *et al*., 2013; Elmi *et al*., 2013; Gaafar *et al*., 2014; Saad *et al*., 2015; Ahmed *et al*., 2017; Alajmi *et al*., 2019 and Brito *et al*., 2020).

AgNPs can be delivered by various routes and are easily taken in by the reticuloendothelial system's macrophages (Singh *et al*., 2010).

The toxicity of AgNPs is still completely unknown, but in cell culture studies they demonstrated a significant toxicity level such as DNA damage, denaturation of proteins and enzymes, and also generated free radicals. In spite that, several studies have been conducted to boost the effectiveness of AgNPs as a therapy to various infections including parasitic diseases (Nilforoushzadeh *et al*., 2012). Because of the adverse reactions and varying medication efficiencies, the research continues to test alternative drugs for treatment of parasitic infections. Therefore, in this study, we aimed to determine the anti-*Giardia* sp. activity of AgNPs and to test the toxicity of AgNPs *in vivo*.

METHODS

- Animals

BALB/c mice 8-10 weeks old were proliferated and maintained in the animal house of Department of Biology, College of Science for Women.

- Silver nanoparticles (Ag NPs)

Silver Nano-powder was purchased from market, sky spring nanomaterial's Inc. Houston, TX, the purity of 99.95% and size distribution was 20-30nm. AgNP's solution was prepared in deionized water. The solution was mixed by vortexing for two minutes then it was prepared as100 µg/g(Ahmed *et al*., 2017).

- Parasite collection

Diarrheic stool samples were collected from patients attending the Central Pediatric Hospital, Baghdad, Iraq. The specimens were microscopically screened by direct wet

saline smear. The Positive samples were emulsified in saline solution and filtered through two layers of gauze to remove coarse particles. The suspension was then centrifuged at 500gx for 10 min. After the supernatant was discarded the sediment was containing *Giardia* cysts washed three times and diluted with phosphate buffer saline (pH 7.4) in the presence of antimicrobial (penicillin, streptomycin, and amphotericin (Mahmood *et al*., 2016).

- In vivo assay

Experiments were performed on 20 albino male mice (five mice in each group), 8-10 weeks old and weighing 20–25 g at the onset. Mice were infected in a dose of 1×10^5 viable cysts orally and divided into four groups as follows: Group I (Non-treated infected control), Group II (infected and treated by AgNPs 100µg/g), Group III (infected and treated by metronidazole 100 mg/Kg (Shin *et al*., 2010), and Group IV (infected and treated by metronidazole combination with AgNPs half dose of drugs in combination).

Two weeks post-infection, mice were given the treatment for three days and then the fecal pellets of mice were examined conventionally to detect the parasites at different time periods after 24, 48 and 72 hours.

Mice were sacrificed and the small intestine and liver were removed and fixed in 10% formalin and then embedded in paraffin wax for histological examination according to Bancroft's theory and practice of histological techniques, 2013.

- Index of parasite reduction

Estimated parasite count before and after therapy in stool were calculated using the following formula (Serna *et al*., 2015)

$$\left(\frac{\text{Mean number of cyst in treated mice}}{\text{Mean number of cyst in control mice}} \times 100\right) - 100$$

RESULTS

The present study tested the impact of AgNPs in reducing the number of *Giardia* in infected mice. The direct microscopic examination of the fecal pellets of treated mice that were infected orally by *G. lambila* cyst showed that there was a variation in the parasite percentage of reduction according to the type of treatment.

It was observed that the percentage of reduction in the treated mice with metronidazole after 24 hours was 33.30% while the highest percentage of reduction was 66, 60% when the mice were treated with AgNPs and the second-highest percentage of reduction was 50% when the mice were treated with AgNPs combined with the metronidazole (mixed).

While after 48 hours it was observed that the highest percentage of reduction was with metronidazole (100%) followed by mice that treated with AgNPs(83.30%) and finally the group that was treated with nanoparticles paired with metronidazole (50%), but after 72 hours the highest percentage of reduction was reported in the group that was treated with metronidazole drug (100%), followed by the group that was treated with AgNPs and AgNPs with metronidazole on rate of 83.3% for both, as it is observed in figure (1), which describes the proportions of percentage of reduction after 24, 48 and 72 hours.

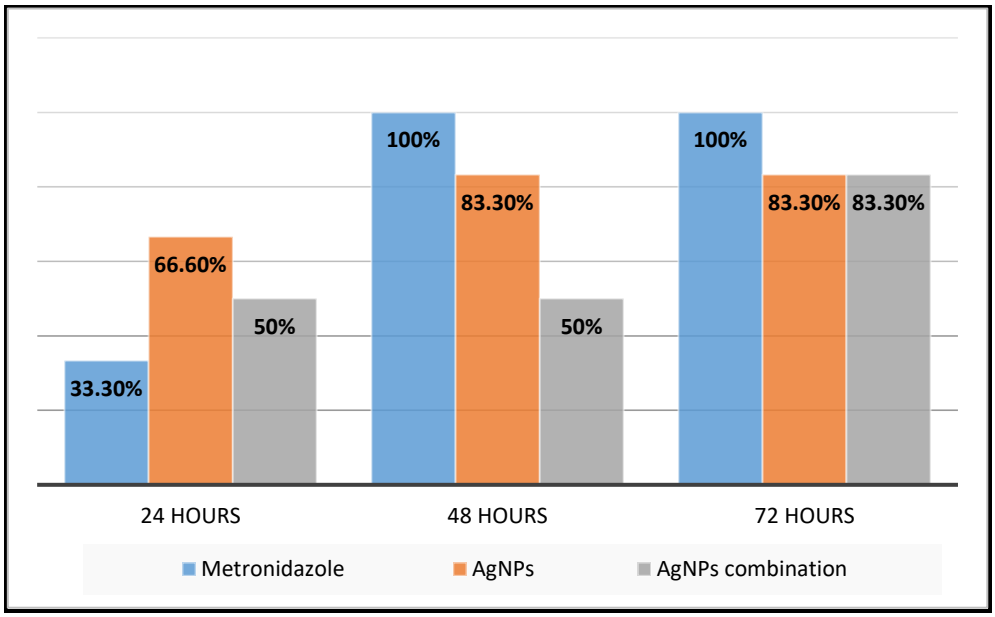

Figure 1: The index of parasite suppression in the stool of infected and treated mice for different time periods.

The figure (2) showed the variation in size, shape and the stage of parasite in mice feces according to treated groups. In infected control group the cyst form showed different development stages (mature and immature) while in metronidazole, AgNPs and combination (metronidazole with AgNPs) treatments groups, reduction in parasite number and cysts are seen. Cysts appear ovoid to ellipsoid

in shape surrounded by cyst wall and have 2 to 4 nuclei, with distinction axostyle running along the midline.

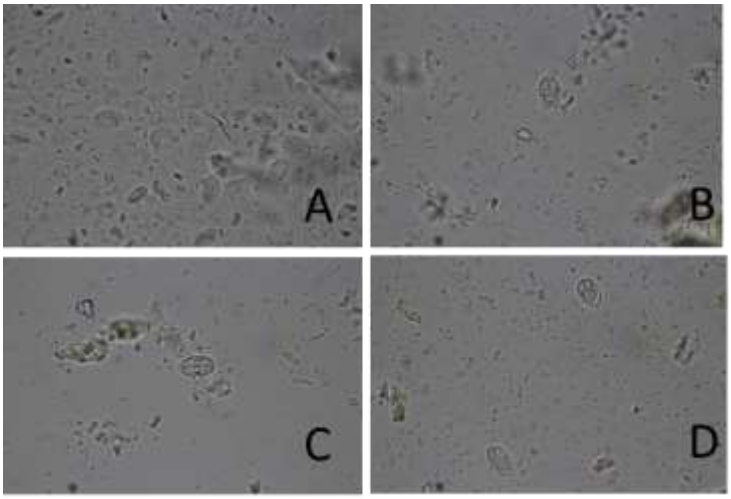

Figure 2: Light microscopy of stool examination after two weeks orally inoculation of *Giardia* cysts, immature and mature cysts are seen, cysts appear ovoid to ellipsoid in shape surrounded by cyst wall and have 2 to 4 nuclei.
A: infected control, B: metronidazole treatment, C: AgNPs treatment and D: combination (metronidazole with AgNPs) treatment.

The results of histopathological study for the small intestine is shown in Figure (3).There were several changes as a result of infection with the parasite. In the intestine of mice infected without treatment many parasites were present with severe degeneration and inflammation, the mucosal architecture tends to be abnormal, with marked shortening of the villi (atrophy in the villi), the ratio of villous elevation to crypt length was decreased and there was necrosis in the epithelial cells, with reductions in goblet cells, and infiltration of lamina propria with inflammatory lymphocytes, and plasma cells. A lot of *Giardia lamblia* trophozoites were founded in the intestine and the parasite was attached at the brush border.

The small intestinal sections of infected mice that were treated with metronidazole showed less histological changes compared with other groups. There was normal villous architecture with columnar epithelial cell, and normal mucosa with villi and unvarying goblet cells and also *Giardia* trophozoites was not found in the lumen.

In infected mice that were treated with AgNPs, there were significant changes such as

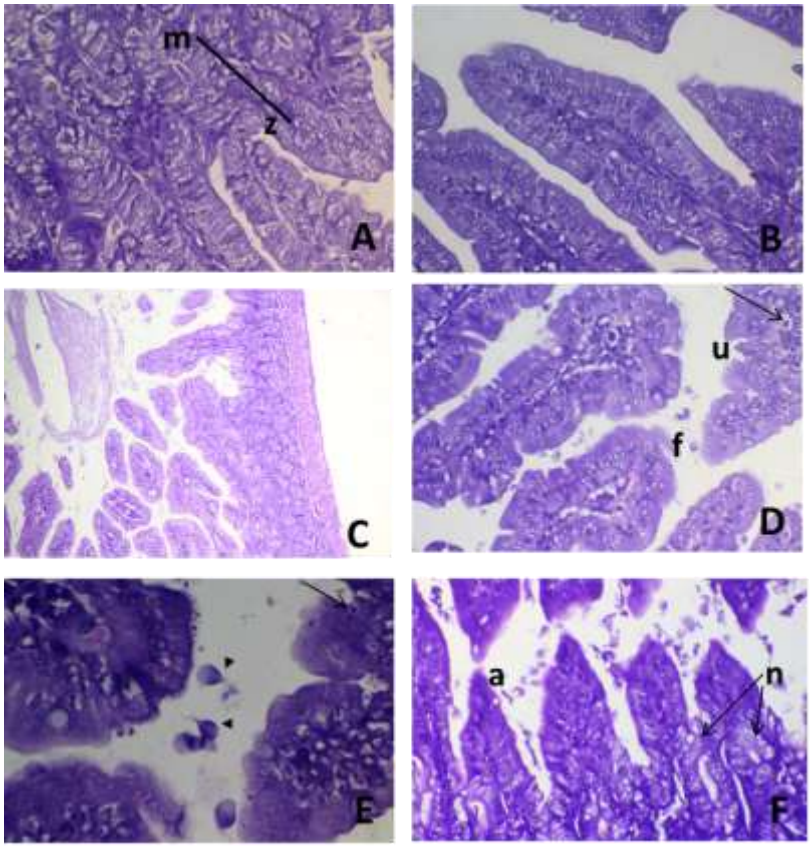

Figure 3: Histopathological findings in small intestines of the infected mice (H & E).

A: untreated group (group I), the ratio of villous elevation to crypt length was decreased, (villous atrophy m-z), the villi are shorter than normal and show loss of cellular differentiation and necrosis was also seen with reductions in goblet cells, infiltration of lamina propria with inflammatory lymphocytes, and plasma cells. In addition, the trophozoites stage of *Giardia* found in the lumen of the small intestine (40X).

B: treated group by metronidazole (group II), normal villous architecture,these include normal mucosa with villi and unvarying goblet cells (40X).

C: (10X), D: (40X) and E: (100X): treated group by AgNPs (group III), various changes are seen due to inflammation disorders, included flattening at the upper surface of villi (f), the columnar epithelial cells have been replaced by polymorphic cells and also ulceration (u) has happened and pyknotic cell (arrow) was also shown, the inflammation process in the lamina propria can also be seen. Binucleate *Giardia* (arrowhead) trophozoites are seen in the lumen and on the brush border of the small intestine associated with the intestinal brush border erosions and sloughing. *Giardia* trophozoites invaded the intestinal mucosa and submucosa, we found trophozoites between epithelial cells, and at the base of empty goblet cells.

F: treated group by a mixed solution of AgNPs and metronidazole (group IV), the initial change is moderate villous atrophy, shortening of the villi, mild chronic inflammation within the lamina propria, and a lot of *Giardia* in the surface of mucosa and between the villi (40X).

Shortening in the villi and infiltration of the lamina propria with inflammatory cells. A huge number of *Giardia* trophozoites were seen in the lumen of the small intestine. They were also seen in infected mice that were treated with combination therapy (metronidazole with AgNPs).The trophozoite stage was seen as teardrop-shaped (rounded anteriorly and pointed posteriorly), the internal structures (nuclei, sucking disk, and axostyles) and flagella are also visible. The central portions of trophozoites are showing the big adhesion organ (sucking disk) and attachment to microvilli of the intestinal mucosa. At places where trophozoites have been detached, depression marks become visible.

In the liver, cellular necrosis, congestion, hemorrhage and infiltration of leukocytes were observed in all infected mice and also exposed to treatment dosage. The effect of AgNPs and metronidazole on the liver is showed, there were several changes and also as a result of infection with the parasite these changes include necrosis and apoptosis of hepatocytes and also cell degeneration was found in the liver tissues. Microscopy studies revealed that all groups induced severe morphological alterations to tissue, mainly at the level of the cell, with a huge number of inflammatory cells with granuloma formation. The typical histopathological changes in the liver are shown in figure (4).

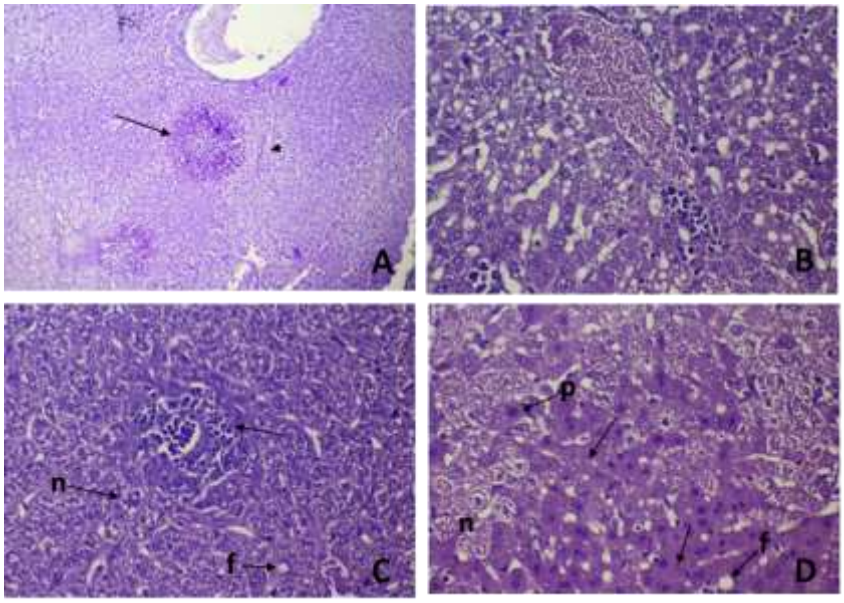

Figure 4: Histopathological findings in the liver of the infected mice (H & E).

A: untreated group (group I), histological alterations within the liver tissue included; acute inflammation (arrow), abundant inflammatory cells infiltrate (PMN), cell degeneration, congestion and hemorrhage (arrowhead) (40X).

B: treated group by metronidazole (group II), various changes are seen like cell necrosis, hemorrhage, and leukocytes infiltration, also show the abnormal hepatic architecture, the parenchyma cell around the portal vein has disappeared and sinusoid disturbed in between(100X).

C: treated group by AgNPs (group III), the micrograph shows the different changes in hepatocytes during their metabolic activities these include spotty necrosis (n), fatty change (f) early granuloma (arrow) (100X).

D: treated group by mixed solution of AgNPs and metronidazole (group IV), shows pyknosis (p), necrosis (n), fatty change (f) and fibrinoid degeneration (arrow) (100X).

DISCUSSION

Protozoan *Giardia* is a flagellated intestinal parasite and a leading cause of human diarrheal disease in the world through ingestion the infectious cysts (Malaya *et al*., 2017). Giardiasis therapy relies on the shown remained trophozoites in intestine, as well as in combined treatment (AgNPs traditionally used drug, metronidazole that has been used as drug of choice. The current treatment has some limitations including unpleasant effects, variable treatment efficacy and parasite drug resistance. Therefore, new anti-Giardial drugs are quested (Eissa and Amer, 2012).

In this study, the anti-Giardial effect of AgNPs in experimentally infected mice was demonstrated. In addition, we tried to investigate the toxic effect of AgNPs as well as the adverse effects of AgNPs and metronidazole that were used oralley to treat *Giardia*- infected mice.

In this study, mice that were treated with AgNPs and MTZ showed statistically significant reduction in the mean number of the parasite in stool, when compared to the control group (not treated) however, AgNPs and combination therapy show less antigiardial activity compared to MTZ. Light microscopic examination showed that the parasites have been eliminated from the intestine in mice treated with MTZ, while mice treated with AgNPs have with MTZ).

In agreement with our results, (Said *et al*., 2012) reported that AgNPs were effective against the experimental *Giardia* infection in rats.

The anti-giardial activity of gold nanoparticles was tested in vitro by

(Bavand *et al*., 2013) who concluded that the effect of gold nanoparticles at concentration 0.3 mg/ml is similar to the effect of metronidazole.

Some researchers have studied the in vitro and in vivo activity of AgNPs against some parasite such as *Toxoplasma*, *Trypanosoma*, *Entamoeba histolytica*, *Cryptosporidium Parvum*, *leishmania*, and *plasmodium* (Baiocco *et al*., 2011; Gaafar *et al*., 2014; Saad *et al*., 2015; Ahmed *et al*., 2017; Alajmi *et al*., 2019; Brito *et al*., 2020). Some of these studies agree with us in that AgNPs show less antigiardial activity compared to MTZ and some of these studies recorded parasitic growth inhibition by AgNPs.

The disease-causing trophozoites have flagella and bind to intestinal epithelial cells by sucking disc in the ventral surface, causing cell damage and disrupting cellular junctions, with other histopathological changes, then disturb the absorption and secretion of the intestine (Ma'ayeh *et al*., 2017).

Attachment of *Giardia* trophozoite to epithelial cells (via its sucking disc) is crucial for colonization of the parasite to the intestine resulting in intestinal epithelium damage. Various parasitic factors are known to be involved in this attachment

including surface proteins (ex: lectins, giardin) and protease activities (ex: cysteine proteinases) (Cotton *et al*., 2011).

The (Muller and von Allmen, 2005) mentioned that intestinal colonization by the *Giardia* tends to cause villous shortening, flattening or atrophy. These abnormalities, possibly in combination with other pathological mechanisms may be a direct cause of diarrhea in giardiasis.

In agreement with our results, (Cotton *et al*., 2011) reported that giardiasis pathology occurs without invasion of trophozoites or any overt inflammatory cell infiltration, except for a minor rise in lymphocytes. Moreover, (Cotton *et al*., 2011) mentioned that there are increased apoptosis of intestinal epithelial cells, presence or absence of villous atrophy and disruption of microvilli of brush border that may lead to reduction of the intestinal absorptive surface area.

This also agrees with other studies such as (Buret, 2008) who mentioned that the malabsorption occurs mainly from shortening of epithelial microvilli, epithelial apoptosis which leads to loss of the function of epithelial barrier and increased numbers of lymphocytes infiltration.

In the present study, the histological changes observed during giardiasis in all infected groups is villous atrophy and other signs of mucosal injury with an increase in enterocytes apoptosis and increased immune cell numbers and that agrees with (Fink and Singer, 2017; Ismail *et al*., 2017).

The pathogenesis in giardiasis involves induction of immunological reactions (infiltration of inflammatory cells and CD8/T-cells) that coincide with villus shortening and flattening (Muller and von Allmen,2005).

(Cotton *et al*., 2014) Stated that infection with *Giardia* may decrease Polymorphonuclear leukocytes (PMNs) accumulation and tissue infiltration because PMNs are essential elements in inflammatory responses and crucial to host defense against many pathogens.

Entry of *Giardia* may occur via the base of the goblet cells through which the parasite crosses to the lamina propria and migrates to deeper sites of the villi (Reynoso-Robles *et al*., 2015).

Nanotechnology has paved the way for potential clinical trials to be used as drugs for treatment of parasites. The metallic nanoparticles such as AgNPs are rapidly taken up by the macrophages of the reticuloendothelial system (RES). And some reports demonstrated AgNPs as pharmaceutically sound and non-toxic to humans, according to these, it has also been used as an anticancer, antiviral, bactericidal, fungicidal, and antiprotozoal agent (Khan *et al*., 2018; Singh *et al*., 2010).

In our study we evaluate the possible oral toxicity of AgNPs and their effects on the parasite and the animal tissue. AgNPs induced severe histopathological changes and tissue damage in small intestine and liver in all mice exposed to them.

These AgNPs caused hepatic injury with hepatocyte necrosis, serous inflammation, inflammatory cell infiltrates and other pathological observations also confirmed liver damage, these findings are identical to that of (Kim *et al*., 2010) who reported oral toxicity of AgNPs on liver and intestine.

Moreover, we observed degeneration in the small intestinal epithelial cells, necrosis of epithelial cells, which indicate that AgNPs have severe toxicological effects on tissue.

The toxicity of AgNPs on the tissues of rats such as; hemorrhage, cell necrosis, and apoptosis was demonstrated by (Sardari *et al*., 2012) and these findings are identical to our results in mice tissue.

Our results disagree with (Said *et al*., 2012) who mentioned that AgNPs did not exhibit any toxicity and the accumulation of AgNPs in different organs was within the safe limit.

It is known that any foreign material like AgNPs may be released into the blood and can accumulate in the reticuloendothelial system such as the liver and may cause tissue toxicity that leads to death. Therefore the pathological findings in the liver in our results suggest that some AgNPs are absorbed in the intestine and enter the blood circulation to the liver.

The toxic effects of AgNPs have been tested by many studies; however, there is scarcity of the available data that demonstrate these toxic effects and the safety of using AgNPs is still controversial.

It has been reported that a low amount of silver has excessive potential against microorganisms, while the AgNPs at high concentrations (more than 10 μM) are toxic to mammals. AgNPs had a strong affinity towards extracellular membranes and can accumulate in tissues in excess amount for a long time causing several toxic effects to normal cells (Khan *et al*., 2018).

The toxic effects of AgNPs had been demonstrated on liver and macrophages. Upon uptake by the macrophage, silver metal can be further dissolved to Ag+ ions in the lysosomes due to the lower pH. These Ag+ ions are extremely toxic to mitochondria and cause apoptosis. Moreover, the AgNPs diplay toxic effects to all kinds of cells by interfering with their metabolic pathways, induction of apoptosis, and producing superoxides, genotoxicity, or other cytological consequences (Khan *et al*., 2018).

(Sardari *et al*., 2012) suggest the ability of silver ions to attach thiol groups in livers, triggering reduction reactions, transferring of glutathione to bile and reducing the concentration of glutathione available for biochemical reduction reactions. It should be noted that reducing glutathione is important to remove peroxides.

The damage to tissue was caused by the increase of free radicals and the stimulation of oxidative stress. In addition (Jin and Zhao, 2009) mentioned that the reactive oxygen species (ROS) cause damage to DNA and RNA strands in cells via involvement in the process of cell apoptosis. AgNPs increased the concentration of ROS due to the redox reactive property of noble metals and thus induced cell death.

While the (Wuyz and Zhou, 2013). studied the effect of AgNPs on medaka fish and demonstrated the toxicity of AgNPs in aquatic organisms. The highest accumulation of AgNPs was in gill and intestinal tissues, with the highest levels found in the liver tissue. Also (Wuyz and Zhou, 2013) suggested that the histopathological changes may be due to the oxidative stress caused by exposure to AgNPs. Gastrointestinal epithelium can be an important route for

the introduction of AgNPs to fish, contributing to the transportation of AgNPs into the blood from the sites where AgNPs are absorbed and accumulation of AgNPs in liver tissue.

AgNPs can be used as apoptogenic factors which can display reduced mitochondrial function and cell viability and can produce lytic enzymes which can cause apoptosis and necrosis (Panyala *et al*., 2008).

Thus , AgNPs can cause potential toxic effects such as cell activation, the producing of reactive oxygen species, when it bind to various tissues and which are more toxic to tissue, inflammation, and finally, all these processes gradually lead to cell death.

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