Estimation of Activity and Toxicity of Silver Nanoparticles loaded metronidazole against *Giardia Lamblia*.

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**ABSTRACT**

*Giardia lamblia* is one of the most common protozoan cause diarrhoea, and the most health problem in developing countries worldwide. Our work aimed to assess activity and toxicity of metronidazole loaded silver nanoparticles in treatment of acute giardiasis in mice. After inoculated mice with *Giardia* cysts in a dose of 10⁶ cyst for acute infection, treatments were given for eight days. Number *Giardia* cysts in stool were discovered. Toxicity nanoparticles was estimated by measurement oxidative stress markers (GSH and MDA) in liver, kidney tissue homogenate. The results showed single therapy was better effect by silver nanoparticles, highest percentages of reduction in number of cysts *Giardia lamblia* of infected mice treated with silver nanoparticles combined Metronidazole. Also, combination of Ag NPs with metronidazole was the best effect to reduce the toxic effects of this chemotherapy on liver and kidney in tissue homogenate. It seemed significant elevate levels of GSH and decrease level of MDA.

**INTRODUCTION**

*Giardia lamblia* is a flagellated microscopic parasite, *Giardia lamblia* is a flagellated microscopic parasite, that parasitize upper part small intestine of mammals, cattle and pet. It causes the diarrheal disease known as giardiasis, affecting humans worldwide (1). Though giardiasis can affect all age groups, children, infants and immune compromised patients. Giardiasis acquire by ingestion highly infectious cysts from contaminated food, or by fecal-oral transmission (2). Disease causes frequent abdominal pain, vomiting, acute or chronic diarrhea, and malabsorption (3). The prevalence rate is 40% at developing countries which have poor hygiene and sanitation (4).

In United States, approximately 20,000 cases of giardiasis are reported annually (5). Most commonly used drugs to treat giardiasis are Metronidazole, 5-nitroimidazole and tinidazole. Metronidazole resistance has been described, treatment failure occurs in 20% of cases, so trials were designed to test the capacity of different agents for treating giardiasis (6). Currently, the interesting in nanoparticles applied for disease diagnosis, and treatment as drug delivery systems to the specific desired site with reduction in induced tissue damage and reverse effects (7). The main motive is that nanoparticles will be able to be used in treating various diseases in future (8). Ag NPs passess many characteristics that make them attractive as medical device, especially as a therapeutic agent and drug delivery system. Through the years, Ag NPs have undergone numerous tests in vivo and in vitro to provide information about their toxic behavior towards living organisms and their tissues (9). Recently, Ag NPs whose structures are showing remarkably new and special physical, chemical and biological properties such as anti-inflammatory, antifungal, virus, protozoa effects and even have protective effects against colds and influenz (10). Also, antibacterial activity (11). Trials were conducted to verify the effectiveness of AgNPs against leishmaniasis (7) and Cryptosporidium (12). An in vivo study was performed by (13) who compared the activity of AgNPs, Chitosan NPs, and curcumin NPs against *Giardia lamblia* in an experiment in mice.

Another study showed that silver nanoparticles used singly or combined with chitosan nanoparticles have promising anti-Toxoplasma potential (14). A recent study experimentally demonstrated the effect of spiramycin-loaded silver and chitosan nanoparticles on toxoplasmosis (15). Silver nanoparticles was tried for the first time as an anti-*Trypanosoma cruzi* agent (16). The present work is designed to determine the efficacy and toxicity of metronidazole loaded silver nanoparticles as an anti-*Giardia* agent.

**MATERIALS AND METHODS**

**Drug**

- Metronidazole was manufactured and purchased by (Pharonia, Alexandria, Egypt) administered to mice orally 100 mg / kg.
- The preparation of the Nanosilver: Installation was performed according to the method performed by Solomon, 2007 (17). The size of these particles was about (40-60) nm.

**Samples stool collection**

Stool samples of diarrhea were collected from infected patients referred to Theodore Bilhars Research Institute Hospital Laboratory in Giza. Samples were microscopically screened by direct wet saline smear. Fresh samples were kept in a 2.5% potassium dichromate solution at 4°C until used (18). During the time of inoculation, the feces were suspended by centrifugation, the sediments were washed three times and suspended in a phosphate saline (pH 7.4) in the presence of the antimicrobial penicillin and streptomycin (19).

**Experiment animals**

This study was conducted on 60 male albino mice (ten mice in each group), each weighing between 20-25 g. The animals were maintained under specific pathogen free conditions. The experiment was conducted according to a general rule and the institute responsible for the bio-supply program for animal ethics at Theodore Bilhars Research Institute. Albino mice was infected in a dose 10⁶ viable cyst orally and divided into five groups as follows: Group 1: (non-treated non-infected normal control), group 2 (Non-treated infected control), group 3 treated by AgNPs a dose of 50 μg /mL), group 4 (infected
and treated by metronidazole 100 mg/Kg, and group 5 (infected and treated by metronidazole loaded with silver nanoparticles).

The dose started from the first day until eight days after infection. From the third to the eighth day after infection, the secretion of giardia cysts in the stool was collected daily from each group of infected mice and homogenization in PBS to estimated *Giardia lambia* cysts shedding. Each sample was examined with an iodine-stained smear and the number of cysts was counted and then calculated /gm stool (20). Scarification of all mice was performed by rapid decapitation. The kidney and liver from each mouse were removed and subjected to tissue homogenate and detection of toxicity.

**Measurement of oxidative stress markers**

Determination of toxicity in tissues liver and kidney of all studied groups was by measurement of Glutathione and Malondialdehyde, using colorimetric method:

**A- Measurement of GSH**

Determination of Glutathione was done by using Glutathione Reduced Kit according to the method of (20).

**B- Measurement of MDA**

Determination of Malondialdehyde was done by using Lipid Peroxide (Malondialdehyde) kit according to the method of (21).

The data of the present study were expressed as mean values ±SE. Differences between the groups were statistically analyzed by ANOVA table. A P value < 0.05 was regarded as statistically significant.

**RESULTS**

**Parasitological examination**

In studied infected groups, mice began to shed cysts with their stool in the third day post infection. Average number of *G. lambia* cysts from the group of infected mice on the eight days after treatment. Statistical analysis showed significant differences between all treated groups compared to the control group, P < 0.05. Mean number of cysts was (1350.56±2594) in infected untreated control group. Eight days after treatment with single therapy Metronidazole, the mean number of cysts output became (2435±619). The percent reduction in number of *G. lambia* cysts was (82.3%), which is statistically significant (P<0.05), while in group treatment by nanosilver the mean number of cysts was (845±123), the percent reduction in number of *G. lambia* cysts was (93.8%), which give statistically significant (P<0.001). Combined therapy gave best results than single it detected in group treatment by Metronidazoal with nanosilver the mean number of cysts was (158±22).

The percentage decrease was in the number of *G. lambia* cysts (98.8%), which is statistically significant (P <0.001) (Table 1).

<table>
<thead>
<tr>
<th>Animals Group</th>
<th>Mean ± SE.</th>
<th>% reduction in number of <em>G. lambia</em> cysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected non treated</td>
<td>1350.56±2594</td>
<td>0.0</td>
</tr>
<tr>
<td>Infected treated with metronidazole</td>
<td>2435±619</td>
<td>82.3**</td>
</tr>
<tr>
<td>Infected treated with nanosilver</td>
<td>845±123</td>
<td>93.8**</td>
</tr>
<tr>
<td>Infected treated with metronidazole + nanosilver</td>
<td>158±22</td>
<td>98.8**</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE very significant p<0.001**

**Measurement of oxidative stress markers**

**A- Measurement of Glutathione (GSH)**

Table (2) shows the mean concentration of hepatic GSH a significant reduction as a result of *Giardia lambia*, the *Giardia* infected group showed decrease to about (5.355±0.016mmol/g) compared to the normal control group was (8.255±0.665 mmol/g), which is highly statistically significant (p<0.001). The concentration was significantly lower (p <0.05) in the mean group of mice treated with metronidazole single treatment (6.110 ± 0.120 mmol / g) compared with all treated infected mice groups. However, groups were receiving nanosilver either treated alone or in combination with metronidazole showed increase in mean concentration of GSH , the concentration was (7.825 ± 0.182 mmol / g) and it was highly significant (p <0.001).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean conc. of GSH ±S.E (mmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.255±0.665</td>
</tr>
<tr>
<td>Infected non treated</td>
<td>5.355± 0.016</td>
</tr>
<tr>
<td>Infected treated with metronidazole</td>
<td>6.110±0.120</td>
</tr>
<tr>
<td>Infected treated with Nanosilver</td>
<td>7.232±0.653**</td>
</tr>
<tr>
<td>Infected treated with metronidazole + nanosilver</td>
<td>7.825±0.182**</td>
</tr>
</tbody>
</table>

The means concentration of GSH in kidney in all groups at the eight days post treatment are shown in Table (3). *Giardia lambia* infection resulted in a significant decrease (P <0.001) mean GSH concentration (4.088 ± 0.004 mmol / g) in the kidneys compared to the normal control (7.954 ± 0.003 mmol / g). However, the groups were treated with nanosilver showed highly increased in concentration of GSH, either treated alone or in combined with other drug when compared to infected non treated control group, this was more evident in the group treated with metronidazole combined nanosilver, the mean concentration of GSH was (7.787± 0.183 mmol/g) a significant reduction (P < 0.001).
The hepatic MDA concentration was significant (P <0.001) in the affected control group, mean concentration was (0.823 ± 0.003 mmol / g) while the normal concentration was (0.557 ± 0.002 mmol / g). It was found that the MDA concentration decreased in all the treated groups compared to the affected control group as shown in table (4). The lowest MDA concentration was in the metronidazole group. While the groups receiving silver NPs showed a profound decrease in MDA concentration, this was more pronounced in the group of mice treated with metronidazole combined nanosilver, and the mean MDA concentration was (0.524 ± 0.003 mmol / g).

Table 3: Means concentration GSH in kidney in all groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean conc. of GSH ±SE (mmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.954 ± 0.003</td>
</tr>
<tr>
<td>Infected non treated</td>
<td>4.088±0.004</td>
</tr>
<tr>
<td>Infected treated with metronidazole</td>
<td>5.830±0.125*</td>
</tr>
<tr>
<td>Infected treated with Nanosilver</td>
<td>7.181±0.632**</td>
</tr>
<tr>
<td>Infected treated with metronidazole + nanosilver</td>
<td>7.787±0.183**</td>
</tr>
</tbody>
</table>

The mean concentration of renal MDA in standard control group was (0.313± 0.002 mmol/g), whereas in infected control, mean concentration was increased to (0.619±0.002 mmol/g). During infection and after the treatment group, the MDA concentration decreased when compared to the standard control group as shown in table (5). The mean concentration (0.474±0.004 mmol/g) was detected in a group of mice treated with nanosilver combined metronidazole.

Table 4: Mean concentration MDA in liver (mmol/g) in all studied groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean conc. of MDA ±SE (mmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.557±0.002</td>
</tr>
<tr>
<td>Infected non treated</td>
<td>0.823±0.003</td>
</tr>
<tr>
<td>Infected treated with metronidazole</td>
<td>0.698±0.001*</td>
</tr>
<tr>
<td>Infected treated with Nanosilver</td>
<td>0.602±0.001**</td>
</tr>
<tr>
<td>Infected treated with metronidazole + nanosilver</td>
<td>0.524±0.003**</td>
</tr>
</tbody>
</table>

Table 5: The mean concentration of MDA in kidney (mmol/g) in all studied groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean conc. of MDA ±SE (mmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.313± 0.002</td>
</tr>
<tr>
<td>Infected non treated</td>
<td>0.619±0.002</td>
</tr>
<tr>
<td>Infected treated with metronidazole</td>
<td>0.588±0.001</td>
</tr>
<tr>
<td>Infected treated with Nanosilver</td>
<td>0.502±0.001</td>
</tr>
<tr>
<td>Infected treated with metronidazole + nanosilver</td>
<td>0.474±0.004</td>
</tr>
</tbody>
</table>

DISCUSSION

*Giardia lamblia* is one of the most common human parasitic diseases worldwide. Infection with *Giardia lamblia* makes finding a safe and effective treatment a huge success. In the current study, metronidazole was shown to be least effective treatment against *Giardia lamblia*, with a reduction rate of 82.3%. This result was in accordance with the study of (24) Those who reported *Giardia lamblia* activity in the experimental infection showed significantly lower ED50 values for metronidazole analogs compared to metronidazole. Also, study (25) who reported Bifidobacterium was more efficient in treating the infection caused by *G. lamblia* than metronidazole.

In our study AgNPs gave the better effect on parasite count that reduction number of *G. lamblia* cysts is higher than that of metronidazole. In agreement with our study of (13) who showed AgNPs exhibited the better reduction rate of *G. lamblia* parasite count in small intestinal sections than single therapy reduction rate. In other hand the drug current study showed best effect of AgNPs combined metronidazole that reduction number of *G. lamblia* cysts.

Silver nanoparticles have various mechanisms for antimicrobial activity. They have the ability to strongly bind to compounds containing sulfur and phosphorous and penetrate into living cells as well as destroy the enzyme containing sulfur and phosphorous containing DNA (26). It is also known to accumulate heavily in mitochondria and is said to impair mitochondrial function through oxidative stress. Another antimicrobial mechanism is the release of silver ions. These ions contribute to cell death by producing amounts of reactive oxygen species (ROS). In other hand, the drug in the current study showed a better effect of AgNPs combined with metronidazole which reduces the number of *G. lamblia* cysts. This synergistic action may in addition to its effectiveness an increase in the bioavailability of metronidazole by increasing the dissolution area, solubility rate, stability, surface, and permeability of the action of metronidazole via absorption into the membrane. Moreover, these results are in line with several other reports that have shown that mitochondria are negatively affected by the overproduction of cellular ROS, as the use of ROS induces cell death by enhancing the processes of apoptosis and activation of cellular autophagy (27, 28). In addition, (29) reported that,
uptake of silver NPs occurred in most cells via endocytosis, which, depended on time, dose and energy. All of these are potential pathways for cell death as a result of AgNPs causing ROS production and / or altered oxidation state. According to the results of the current study it was shown that G. lamblia infection has a significant effect on liver and kidney glutathione (GSH), Malondialdehyde (MDA) concentration.

In our study, the significant decrease in GSH activity in the infected control group compared to the normal control group, and the depletion in GSH concentration, can be explained. GSH which is an one of the most important cellular antioxidants, it defends the cell against oxidative damage by undergoing reaction with free radicals caused by lipid peroxidation and peroxidase (30). However, GSH concentrations in infected groups receiving silver NPs showed significant increase in concentrations of GSH, this was more evident in the group treated with metronidazole combined silver NPs. MDA, a lipid peroxide product and an indicator of oxidative damage that generates types of free radicals, plays a role in causing many parasitic infections (31). In this study, MDA concentrations increased significantly in the affected control group compared to the normal control group. The increase in MDA concentrations observed in this study may be due to increased production of free radicals and oxidizing substances after infection or may be indicative of decreased enzymatic activity of the antioxidant defense system. Combination of metronidazole in with Ag NPs looked to reduce the toxic effect of this chemotherapy on liver and kidney samples of mice.

CONCLUSION
We confirmed the potential therapeutic effect of combination of Ag NPs with metronidazole on Giardia lamblia infection in mice, the percentage decrease in the number of cysts was statistically significant (P <0.001). The best effect of reducing the toxic effects of this chemotherapy on the liver and kidneys is in tissue homogeneity as a promising alternative treatment to common medicines used to combat Giardia.

REFERENCES