Evaluation of Paromomycin Loaded Chitosan Nanoparticles and Oxidative Stress Activities against *Entamoeba Histolytica*

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Abstract: This investigation was carried out to estimate the antiparasitic potential of chitosan nanoparticles loaded with paromomycin against *Entamoeba histolytica* infected. After rats inoculated orally in a dose 10^3 viable cysts for acute infection; then treated with paromomycin, chitosan nanoparticles as a single or combined therapy given for seven days. Stool examination revealed a significant decrease in the number of *Entamoeba histolytica* cysts in all treated infected rats compared with infected non-treated. Combined treatment provided better results than single treatment. The best effect was observed in the group of rats treated with chitosan nanoparticles loaded with paromomycin. Also, the oxidative stress markers Glutathione (GSH) and Lipid Peroxide (Malondialdehyde) (MDA) were assessed in liver tissue homogenate. The current work is the first time of using chitosan nanoparticles loaded with paromomycin as therapeutic agents against experimental amoebiasis. It was shown that the highest degree of effectiveness attained by the synergistic action of paromomycin and chitosan nanoparticles as was indicated by lower parasite count and GSH, MDA concentration.

Keywords: *Entamoeba histolytica,* paromomycin, oxidative stress, chitosan nanoparticles.

1. Introduction

Entamoeba histolytica (*E. histolytica*) is a protozoan that causes amoebic dysentery and is the leading source of health problems in developing countries. *E. histolytica* it can also cause intestinal infections, which can lead to diarrheal illnesses with mucus and blood. Acute amoebic dysentery is the most common symptom of amoebiasis. *Entamoeba histolytica* has two forms in its life cycle: trophozoites and cysts. Trophozoites can coexist in the large intestine without obvious clinical manifestations. However, they can invade the mucous membranes and cause amoebic colitis and spread through the blood. Which may produce extra-intestinal lesions, especially in liver, cause's amoebic liver abscess, also involving pleural lung abscess, acute stomach pain [1]. Treatment of amoebiasis depends on antimicrobial therapy, the most common being the use of 5-nitroheterocyclic drugs, especially metronidazole, and recently nitazoxanide [2]. Paromomycin (also known as aminoglycoside (injection), gabromycin) is an aminoglycoside antibiotic; its range of activities includes many protozoan species. It is generally recommended for the treatment of amoebiasis in asymptomatic and mildly symptomatic patients, although it is included in HIV-infected patients and in the first trimester of pregnancy, because in that case it is considered safe [3]. Chitosan is a polysaccharide (fully or partially deacetylated of chitin), which has been used in the medical field for the past two decades. It is an important material for the preparation of nanoparticles because it is biodegradable and non-toxic.

Many studies have been completed on chitosan nanoparticles (CS Nps) in the treatment of *Giardia lamblia* infection [4], CS Nps has increased the effect of ivermectin as an antifilariasis drug [5], In another study, spiramycin-loaded chitosan nanoparticles were also shown to increase their antiparasitic effect in acute Toxoplasma gondii infection [6]. Oxidative stress is a phenomenon caused by an imbalance between the production and accumulation of reactive oxygen species (ROS) in cells and tissues and the ability of biological systems to detoxify these active products. As a result of the degradation of the antioxidant system, malondialdehyde (MDA) is formed during the peroxidation of polyunsaturated fatty acids by reactive oxygen species. There are two forms of MDA: endogenous (from lipid peroxidation) and exogenous from food [7]. Glutathione (GSH), one of the most important antioxidant molecules in the liver and kidneys. Glutathione has antioxidant properties because the thiol group of its cysteine residue is a reducing agent, which can be reversibly oxidized and reduced [8].

2. Experimental Part

2.1. Drug

2.1.1. Paromomycin

Paromomycin was manufactured and purchased by [PFIZER PHARMA PFE GmbH 250mg,Germany]. Paromomycin was orally given to rat in a dose of (22 to 44 mg/kg/ per day for 7 days by gavage [9].

2.1.2. Synthesis of Chitosan Nanoparticles.

Method of preparing nanoparticles by the chemical method and using the Sol-Gel method with some modifications [10]. Chitosan was dissolved into deionized distilled water and placed in the Ultrasonic path for 30 minutes. The pH was adjusted to 12 by NaoH (1N), and placed on the rotor with a magnetic stirrer for 60 minutes at room temperature. Then the pH was adjusted to 4 by HCL (1N), and also placed on the rotor with a magnetic stirrer for 60 minutes at room temperature. Then the pH was adjusted to 7 with HCL (1N), and placed on the rotor with a magnetic stirrer for 60 minutes at room temperature. Capsules of Paromomycin (Humatin) were diluted in saline then put in ultrasonic bath for 30 minutes. Add solution of chitosan nanoparticles to Paromomycin solution molar ratio (1:1) and was stirrer an hour at room temperature.

This characterization was used to assess the surface topography of chitosan nanoparticles by angstrom advanced Inc. SPM-AA300. U.S., use the AFM communication mode. Five drops of chitosan nanoparticles solution has been added at cover slide. Three-dimensional structure (topography) of the sample surface at a high resolution can be imagined by the reaction between the probe and the forces of the sample. This is accomplished by a raster scan of the location sample with respect to the tip and by recording the height of the probe that corresponds to a constant between the probe – sample interaction. To alter the properties of the sample in a controlled manner, the forces between the tip and the sample can be manipulated. Examples of this include atomic manipulation, local stimulation of cells and scanning probe lithography.

After sample homogenization with KBr , the FT-IR spectra of CS nanoparticles , PM , CS-PM nanoparticles were analyzed, under a vacuum at 100 ° C for 48hours. Potassium bromide (AR-grade) was dried and then mixed 100 mg of KBr with 1 mg of sample separately for KBr pellet preparation. Data was collected between 500-4000 wave number/cm. In Shimadzu-IR affinity-I spectrophotometer, Uv spectra were recorded. The spectra are plotted as intensity versus number of waves.

2.2. Samples Stool Collection

In this study, diarrhea stool samples were collected from infected patients who were referred to some private laboratories. Samples were examined microscopically by a direct wet saline smear. Fresh samples are stored in 2.5% potassium dichromate solution at 4 ° C until use. During inoculation, the feces were suspended and centrifuged. The sediment was washed 3 times and suspended in phosphate saline (pH 7.4) containing antibacterial penicillin and streptomycin. Approximately 10^3 viable cysts / inoculated rats for acute infection [11].

2.3. Experimental Animals

Twenty-five adult male albino rats, (weight. 200-250gm with age 2.5-3 months) were obtained from the biotechnology center/ Al Nahrain University. The animals were maintained under specific pathogen-free conditions. The experiment was conducted according to a general rule and the center responsible for the bio- supply program for animal ethics at the biotechnology center. Animals were divided to five groups each group included 5 rats as follows:

- Group 1: Uninfected non-treated inoculated with (0.1) ml of normal saline (negative control).
- Group 2: Infected non-treated group (a positive control group).
- Group 3: Animals were given Paromomycin (22 to 44 mg/kg/ per day for 7 days) mg/kg [9].
- Group 4: Animals were given Chitosan Nanoparticles (50) mg/kg orally as a single dose per day [12]
- Group 5: Chitosan nanoparticles loaded with Paromomycin treated group.

2.4. Parasitological Study

The secretion of *E. histolytica* cysts in the stool was collected daily from each group of infected rats and homogenization in PBS to estimated *E. histolytica* cysts shedding. Each sample was examined with iodine- stained smear and the number of cysts were counted using hemocytometer and examined by a light microscope to determine their number per ml.

The day after the end of treatment, all animals were sacrificed; the liver was removed and subjected to tissue homogenate and detection of toxicity.

2.5. Measurement of Oxidative Stress Markers

Determination of toxicity in tissue liver of all studied groups was by measurement of Glutathione and Malondialdehyde, using colorimetric method. The level of Glutathione (GSH) was estimated using the Glutathione Reduced Kit according to the method of [13], and determination of Malondialdehyde (MDA) was done by using Lipid Peroxide (Malondialdehyde) kit according to the method of [14].

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

3. Results and Discussion

3.1. Parasites Count

In studied infected groups, rats began to shed cysts with their stool on day four post infection. Start from the four day to the end of the experiment, several symptoms were seen in the present study, a loss of hair coat and slow motion. The highest mortality rate was observed in rats belong to the infected

non treated group, whereas, CS Nps -loaded paromomycin-treated rats had the lowest mortality rate Statistical analysis showed significant differences between all treated groups compared to the affected control group, P <0.05.The mean number of cysts was (2210.61 ± 118.41) in the infected untreated control group. Seven days after treatment with daily single therapy paromomycin, the mean number of cysts output became (820.11± 1.77). The percentage reduction in the number of *E. histolytica* cysts was (80.8%), which is statistically significant (P<0.05), while in group treatment by CS Nps the mean number of cysts was (386.13±71.55), the percentage reduction in the number of *E. histolytica* cysts was (88.6%), which give statistically significant (P<0.001). The combined treatment gave the best results from the single treatment as it was detected in the group treatment by paromomycin with CS Nps and the mean number of cysts was (102±1. 3). The percentage decrease in the number of histological cysts was (98.8%), which is statistically significant (P < 0.001) (Table 1).

Table 1. The mean number and the percent reduction in number of *E. histolytica cysts*.

Animal Groups	Mean No. of cyst ±SD. (Per ml)	Percent reduction
Group 2	2210.61 ± 118.41	0 – 0
Group 3	820.11± 1.77*	80.8 %
Group 4	386.13± 71.13**	88.6 %
Group 5	102.30±29.30***	98.8 %

In this study, paromomycin was administered orally at a dose of 22- 44 mg / kg / day for 7 days to treat *E. histolytica*. The results showed that the percentage of cyst excretion decreased by 80.8%. Although, [15] studies have shown that the efficacy of paromomycin in a patient provides a treatment efficacy of 80% on the 10th day after treatment. In another study on the results of [16], Paromomycin is used as a usable drug, at a dose of 8-12 mg / kg, orally 3 times a day against *E. histolytica* infection. Several studies on the efficacy of paromomycin against various protozoan parasites such as *Cryptosporidium* calves [17] and *Girdia lamblia* in rats [18]. Paromomycin is an aminoglycoside that is poorly absorbed from the gastrointestinal tract, but is concentrated in the colon cavity. It is only active against intestinal *E. histolytica* cysts. The results support the use of paromomycin as a first-line drug to treat asymptomatic *E. histolytic* infections. The results of this study show that the effective rate of nanochitosan in the encapsulation and excretion of *E. histolytica* is 88.6%. [19], the results of the research carried out showed that iron oxide nanoparticles coated with chitosan oligosaccharides are effective in eliminating the encapsulation of *E. histolytica* in water.

Chitosan nanoparticles have become a new delivery system for many antiparasitic drugs. They are unique pharmacological tools for the treatment of *Cryptosporidium*, *Plasmodium falciparum*, *Leishmania* and *Giardia lamblia*. They also reduced the required dose and reduced the side effects of the drugs used [20].

In the current work, chitosan nanoparticles were tested with paromomycin for the first time in the treatment of acute infection of *E. histolytica* in rats. The highest percentages of decrease in the number of *E. histolytic* cysts were in the group of affected rats treated with chitosan nanoparticles combined

paromomycin while paromomycin was less effective in reducing cyst secretion, and the percentages were respectively 98.8%, 80.8% The combination can improve the bioavailability of paromomycin and prolong its retention time, and maintain its effect on *E. histolytica*. Chitosan nanoparticles are good choices for loading because it has attractive drug delivery properties, and its formulated nanoparticle form has proven to be effective, and its ability to adhere to mucosal surfaces is considered the most the characteristic of attraction, which leads to prolonged existence at the site of drug absorption and increase drug penetration [21]. In a similar study in [22], researchers used paromomycin-loaded mannosylated chitosan nanoparticles to combat leishmaniasis, which affects the two stages of parasites, especially immaculate body. In addition, research [23] proposed a PLGA nanop loaded with mannose thiolated paromomycin for the treatment of visceral leishmaniasis.

3.2. Measurement of Oxidative Stress Markers

Table (2) shows the mean level of hepatic GSH a significant reduction as a result of *Entamoeba histolytica* the infected group showed a decrease to about (5.125±1.137mmol/g) compared to the normal control group was (7.189±1.885mmol/g), which is highly statistically significant ($p<0.001$). The least significant ($p<0.05$) in the mean level of GSH was observed in a group of rats treated with the single therapy paromomycin the level was (5.280± 1.210 mmol/g), while paromomycin plus nano chitosan the level was (6.830±1.390 mmol/g) compared with all treated infected rats groups.

Table 2. Mean concentration GSH in liver (mmol/g) in all studied groups.

Animal Groups	**Mean conc. of GHS ±SD.(mmol/g)**	***p value***	**Sig.**
Group 1	7.189±1.885		
Group 2	5.125±1.137		
Group 3	5.280± 1.210		*
Group 4	6.261± 1.352		**
Group 5	6.830±1.390	p< 0.0001	***

In our study, compared with the normal control group, the significant decrease in GSH activity in the infection group indicates a decrease in GSH concentration. This can be explained as one of the most important cellular antioxidants, which protects cells from oxidative damage reacts with free radicals produced by lipid peroxidation and peroxidase. Table (3) shows that the level of hepatic MDA was highly significant ($p<0.001$) in the infected control group the level was (14.271± 4.966 mmol/g) as compared to this of normal control the level was (9.435±2.225 mmol/g). MDA levels were found to be decreased in all treated groups as compared to infected control group, this was more pronounced in the group treated by paromomycin combined CS NP.

Table 3. Mean concentration MDA in liver (mmol/g) in all studied groups

Animal Groups	Mean conc. of MDA±SD.(mmol/g)	*p value*	Sig.
Group 1	9.435±2.225		
Group 2	14.271± 4.966		
Group 3	13.186.± 3.55		
Group 4	12.923±4.30		
Group 5	10.022 ±4.13	0.0003	**

MDA is a product of lipid peroxidation, an indicator of oxidative damage caused by free radicals, and plays a role in the pathogenesis of many parasitic infections [24]. In this study, compared with the normal control group, the MDA concentration of the infected control group increased significantly. The increase in MDA concentration observed in this study may be due to the excessive production of free radicals and oxidants after infection, or it may indicate a decrease in the enzymatic activity of the antioxidant defense system. This result is consistent with the results reported in [25], it found that albino male rats infected with *E. histolytica* had significantly higher levels of MDA and significantly lower levels of (GSH) when compared to the control group

4. Conclusions

In summary, the effect of the combined therapy is better than the single therapy, which is detected in the group of rats treated with paromomycin combined with chitosan nanoparticles, which has a high degree of statistical significance.

Generally speaking, the combination of CS NP and paromomycin is the best effect to reduce liver toxicity of this chemotherapy in tissue homogenate. It appears to significantly increase GSH levels and decrease MDA levels.

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