Study the synergistic Effect between Nanoparticles and Spiramycin on Immunological Response Against Toxoplasmosis

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Abstract. This investigation was carried out to estimate the antiparasitic potential of silver and Chitosan nanoparticles loaded with spiramycin against toxoplasmosis infected. After mice injected intraperitoneal in a dose 10³ viable tachyzoites for acute infection; then treated with spiramycin, chitosan nanoparticles and silver nanoparticles as a single or combined therapy given for seven days. Peritoneal fluid examination revealed a significant decrease in the number of T. gondii tachyzoites in all treated infected mice compared with infected non-treated. The combined therapy presented better results than the single one. The best effect was observed in a group of mice treated with the combined chitosan nanoparticles and silver nanoparticles loaded with spiramycin. Also, immunological parameters IgM antibody, INF- γ and TNF-α cytokines responses against T. gondii antigens were assessed in serum samples by enzyme-linked immunosorbent assays (ELISA) kits after treatment. The current work is the first time of using Ag NPs and CS NPs loaded with spiramycin as therapeutic agents against experimental toxoplasmosis. It was shown that the highest degree of effectiveness attained by the synergistic action of chitosan and Ag NPs as was indicated by lower parasite count and IFN- γ, TNF-α concentration.

1. Introduction

The obligate intracellular parasite Toxoplasma gondii infects humans and other warm-blooded animals and cause Toxoplasmosis[1]. Three morphologically distinctive infectious forms are exhibited by T.gondii. Acute infection contain tachyzoites, tissue cysts contain the bradyzoites and oocysts contain the sporozoites. Infection is most severe in women if the first infection occurs during pregnancy, in which case it can lead to abortion because of intrauterine dispersal to the embryo. The proliferation of parasite and severity of disease vary depending on the time of infection in mothers. Even though toxoplasmosis is asymptomatic in immunocompetent individuals, it can lead to severe pathological effects in immunocompromised patients (HIV/AIDS, cancer, and transplant patients) [2]. Some antibiotics, including the inhibitory spiramycin, have been found to be effective against T. gondii .Maternal and fetal infections are managed in various ways, depending on the treatment center, and spiramycin has been used to inhibit mother-to-fetus transmission of T. gondii. Even though spiramycin is effective, more than 50% of patients treated with it retained T. gondii DNA in their blood and remained infected [3]. A combination of pyrimethamine and sulfadiazine have a synergic action and is effective against toxoplasmosis (REF). However, this therapy has significant side effects, which include hypersensitivity, bone marrow suppression, and teratogenic effects [4]. Nanoparticles (NPs) have recently gained some attention as antiparasitic drugs given their fewer side effects . In recent decades some methods in delivering antiparasitic drugs have been investigated .
method was the use of Chitosan to prepare nanoparticles in various medical and health applications because of its biodegradable and nontoxic properties. Chitosan effectively improved the reduction of the malaria parasite, Plasmodium berghei, infection in Swiss mice [5]. Another method is silver nanoparticles (Ag NPs), whose structures manifested remarkable chemical, physical, and biological properties. AgNPs manifested antibacterial, anti-inflammatory, anti-viral, anti-fungi and anti/protozoa activities. In a recent study, silver nanoparticles were used singly or combined with chitosan nanoparticles to cure toxoplasmosis [6].

To satisfy the need for new anti-toxoplasmosis agents, this research will focus on the efficacy of nanoparticles in the treatment of toxoplasmosis. This study will investigate both silver nanoparticles and Chitosan nanoparticles singly and combined. This is the first time an investigation is carried out on using Ag NPs and CS NPs loaded with spiramycin as therapeutic agents against experimental toxoplasmosis. The trials were carefully structured to test the efficacy of both agents against toxoplasmosis in mice.

2. Materials and methods

2.1 Parasites

Toxoplasma gondii RH virulent Type 1, strain (HXGPRT) hypoxanthine-guanine phosphoribosyl transferase genes is maintained in the laboratory of the Medical Parasitology Dep., Faculty of Medicine, Alexandria University, Egypt, by continuous intra-peritoneal passages of albino Swiss mice every three days [7]. For animal infection, tachyzoites were harvested from peritoneal exudates of infected mice on the fourth day of infection, and filtration removed debris and host cells through a sheet of glass wool fibers. The filtrate was washed three times and diluted with phosphate buffer saline (PBS) PH 7.4. Around 10^3 viable tachyzoites/mouse were injected intraperitoneal for acute infection model [8].

2.2. Drug

2.2.1. Spiramycin

Spiramycin was manufactured and purchased by Phraonia Pharmaceuticals 1.500000 IU, Egypt. Spiramycin was administered to mice orally in a dose of 200 mg/kg/body weight once a day. Doses of the drug were administered from the first day to the seventh day post-infection [9].

2.2.2. Preparation of chitosan nanoparticles

The ionotropic gelation technique by Ohya, Shiratani [10] was followed for the synthesis procedures. Nanochitosan was administered orally to mice at a dose of 20 mg/kg/body weight. Spiramycin-loaded nanoparticles were formed by the addition of chitosan solution to Tripolyphosphate TPP solution containing 200 mg/ml. Then, the protein content (free) supernatant was collected and determined using the Bradford protein assay spectrophotometric method at 595 NM.

2.2.3. Preparation of silver nanoparticles

The method by Solomon [11] was employed to carry out the synthesis procedure. Nanosilver was administered orally to mice about 5 µg in 100 µl of PBS/mouse/dose and the size of these particles were about (40-60) nanometer.
2.3. Animal groups
The experiment was carried out on 160 male Swiss albino mice, weighing 20–25 g, aged 6–8 weeks, purchased from Theodor Bilharz Research Institute (TBRI). They were housed in well-ventilated cages with perforated covers (cleaned every day), and supplied with standard pellet food and water. Their stools were examined following standard procedures to exclude the presence of parasites. The experiment was carried out according to the internationally valid guideline and an institution responsible for animal ethics [Schistosome biological supply program (SBSP) at Theodor Bilharz Research Institute (TBRI)]. The normal control group contained 10 mice. The rest of the experiment animals were divided to 7 groups each group contained 15 mice as follows:

- Group 1: Uninfected non-treated (the normal-control group).
- Group 2: Infected non-treated group (the infected-control group).
- Group 3: Spiramycin treated group.
- Group 4: Chitosan nanoparticle treated groups.
- Group 5: Silver nanoparticle treated group.
- Group 6: Chitosan nanoparticles loaded with spiramycin treated group.
- Group 7: Silver nanoparticles loaded with spiramycin treated group.
- Group 8: Chitosan nanoparticles and silver nanoparticles loaded with spiramycin treated group.

2.4. Parasitological study
The peritoneal fluid containing tachyzoites was collected from each group separately seven days after infection and given treatment; 50 µml of peritoneal fluid was prepared on a glass slide and examined by Light Microscope at high power (100x objective lens) for detecting and counting tachyzoites using hemocytometer to determine their number per ml.

2.5. Immunological studies
Blood was collected from each mouse into tubes and centrifuged at 3000 RPM for five minutes. The clear, non-hemolysis supernatant serum was transferred in a clean tube and stored at -20°C until immunological parameter analysis.

2.5.1. Determination of IgM antibodies
Qualitative Mouse Toxoplasmosis Antibody, IgM (TP-IgM) kit (Cat. No: MBS9310461) uses a sandwich enzyme-linked immunosorbent assay (ELISA) to analyze the existence or non-existence of Mouse TP-IgM in samples according to the manufacturer’s instructions.

2.5.2. Determination of IFN-γ and TNF-α cytokines
According to the manufacturer’s instructions, the Quantizing® Mouse kit (Catalog Number SMIF00, USA & Canada R&D Systems, Inc.) is a 4.5-hour solid phase sandwich ELISA that helps to quantify mouse IFN-γ and TNF-α.
3. Results

3.1. Mortality rate

Start from the fourth day to the end of experiment, the highest mortality rate was observed in mice belong to infected non-treated group, while the lowest rate of mortality rate was observed in mice belong to infected treated with spiramycin+ nanochitosan+ nanosilver (Table 1).

| Animal Groups | Total No. at start exp. | Total No. at the end exp. | Mortality rate |
|---------------|------------------------|---------------------------|----------------|
| Group 2       | 15                     | 7                         | 53.34          |
| Group 3       | 15                     | 8                         | 46.67          |
| Group 4       | 15                     | 9                         | 40.00          |
| Group 5       | 15                     | 11                        | 26.67          |
| Group 6       | 15                     | 10                        | 33.34          |
| Group 7       | 15                     | 12                        | 20.00          |
| Group 8       | 15                     | 13                        | 13.34          |

3.2. Parasites count

The mean number of *Toxoplasma gondii* tachyzoites in peritoneal fluid from infected mice groups at the seven days post-treatment, the mean number of tachyzoites was (3901.33± 173.031) in the infected non-treated control group. Peritoneal fluid examination showed a reduction in the tachyzoites’ number of *T. gondii* in all treated infected mice compared with infected non-treated, which of high statistical significance (p<0.001). The least significant in the mean number of tachyzoites (1500.00 ± 1.606) was observed in a group of mice treated with the single therapy spiramycin; there was a 61.55% reduction in the number of *T. gondii* trophozoites. It was followed by the better effect of the single therapy nanochitosan mean number of tachyzoites (582.22± 8.249). Nanosilver mean number of tachyzoites (280.33± 1.935) percentage of reduction in the number of *T. gondii* trophozoites was 85.08%, 91.79%, respectively. Combined therapy showed better results than a single therapy; spiramycin with nanochitosan was detected as the mean number of tachyzoites (420.00±1.871). In addition, spiramycin with nanosilver mean number of tachyzoites (300.44± 2.095) percentage of reduction in number of *T. gondii* trophozoites was 89.23% and 90.77%, respectively. While the highly statistically significant (p< 0.001) in the mean number of tachyzoites (153.00 ±1.258) was observed in a group of mice treated with spiramycin combined with CS NPs and Ag NPs, the number of *T. gondii* trophozoites was reduced by 96.07% (Table 2).
Table 2. The mean number and the percent reduction in number of *T. gondii* tachyzoite

| Animal Groups | Mean No. of tachyzoites ± SE (Per ml) | Percent reduction |
|---------------|-------------------------------------|-------------------|
| Group 2       | 3901.3 ± 173.031                    | -                 |
| Group 3       | 1500.00 ± 1.606*                    | 61.55 %           |
| Group 4       | 582.22± 8.249**                     | 85.08%            |
| Group 5       | 280.33± 1.935***                    | 91.79%            |
| Group 6       | 420.00±1.871**                      | 89.23%            |
| Group 7       | 300.44± 2.095***                    | 90.77%            |
| Group 8       | 153.00±1.258***                     | 96.07%            |

3.3. Measurement of Immunological parameters in serum levels

3.3.1. Immunoglobulin M (IgM)

Direct ELISA assessed the levels of toxoplasma IgM. The average level of IgM in the infected group was 2.212 pg/ml compared with 0.277 pg/ml in the standard control group, while in groups infected after treatment showed different averages in the concentration level of IgM. Average value of IgM was reduced to 0.481 pg/ml in the group infected and treated by Spiramycin combined CS NPs and Ag NPs approaching the control group as figure 1.

![IgM mean levels in all studied groups](image)

**Figure 1.** IgM mean levels in all studied groups

3.3.2. Interferon-gamma (INF-γ)

The average level of INF-γ in the standard control group was 186.6 pg/ml, while in the infected control group the concentration was increased to 233.7 pg/ml. On the whole, during the infection and after treatment groups, all the levels of INF-γ increased when compared with the standard control group as figure 2. Groups of mice treated with spiramycin and nanochitosan had 234.6 pg/ml and 245.7 pg/ml, respectively, as their levels of INF-γ. No
significance is seen when they are compared with the infected group. However, the groups were treated with spiramycin plus nanochitosan and all groups receiving nanosilver show the most significant level was 401.8 pg/ml, 389.6pg/ml and 464.1pg/ml, respectively. There was pronounced in the group treated by spiramycin combined CS NPs and Ag NPs, and the level of INF-γ was 509.4 pg/ml.

![Figure2. IFN-γ mean levels in all studied groups](image)

### 3.3.3. Tumor Necrosis Factor-alpha (TNF-α)

TNF-α levels in all groups are shown in figure3. The level of TNF-α was highly significant (p<0.001) in infected control group as figure3. The level was 2940.56 pg/ml, whereas that of normal control was 824 pg/ml. TNF-α levels were found to be reducing in all treated groups as compared to the infected control group. The smallest reduction in the level of TNF-α was in the group treated by spiramycin and nanochitosan; the level was 2544.67 pg/ml and 2464.67 pg/ml, respectively. Spiramycin with nanochitosan followed this, and the level was 1717pg/ml. While the groups that received silver NPs showed a profound decrease in their level of TNF-α, the levels of 1207.78 pg/ml and 1501.89 pg/ml were detected in the group of mice treated with nanosilver and spiramycin plus nanosilver, respectively. Moreover, more pronounced in the group treated by spiramycin combined CS NPs and Ag NPs, the level was (1143.67 pg/ml).
Figure 3. TNF-α mean levels in all studied groups

4. Discussion

Toxoplasmosis may have started to become a common risk to well-being as it infects 30-50% of the global population [12]. In this study, spiramycin was shown to be the least effective treatment against *T. gondii* tachyzoites, with a reduction rate of 61.55%. However, Al-Zanbagi [9] found the growth inhibition of *T. gondii* tachyzoites as 91% and 96% on treatment with Spiramycin in 100 or 200 mg/kg/day for seven days, respectively. Although [13] investigated whether the anti-Toxoplasma activity of spiramycin at 100 and 200 mg/kg/day had only an incomplete effect, despite some dependent maintenance of survival, it was effective in protecting mice against death. In our study, better results were seen with spiramycin in a combination therapy with chitosan and silver nanoparticles than a single therapy, while the best efficacy was observed in a group of mice treated with spiramycin combined with CS NPs plus Ag NPs. In Chew, Segarra [14], it was found that spiramycin, when coadministered with metronidazole, was effective in treating chronic toxoplasmosis in a mouse model. Pissinate, dos Santos Martins-Duarte [15] reported that efficiency of this drug against an acute toxoplasmosis in mice was improved by encapsulation of Pyrimethamine (PYR) and that can be considered an alternate for decreasing the dose of PYR, which might, in turn, ameliorate the side effects of the treatment. The present resulting study showed that CSNPs had a better effect of growth inhibition of Toxoplasma tachyzoites, which may be attributable to the adhesive properties of CS nanoparticles. This character prolongs retention time and contact time to the tachyzoites. Chitosan nanoparticles have served as a new delivery system for a good number of antiparasitic drugs. They are a unique pharmacological tool for the treatment of *Cryptosporidia*, *Plasmodium falciparum* and *Leishmania, Giardia lamblia*. They have also reduced the doses required and lowered the toxic side effects of the used drugs[16]. In this investigation, nanochitosan was combined with spiramycin, and they had a better effect of growth inhibition of *T. gondii* tachyzoites. This combination may improve the bioavailability, increase the retention time of spiramycin and sustain its impact on Toxoplasma. The finding is similar to that made in Barrera, Leonardi [17], which suggested that the effect of Albendazole–CS microparticles are useful formulations for the infection by cutting down the number of larvae in liver and treatment of toxocariasis lung. In our study, AgNPs produced the best effect results as demonstrated by parasite count that showed a reduced number of *T. gondii* tachyzoites. Allahverdiyev, Abamor [18] agree with our research. They determined the
antileishmanial effects of Ag NPs on *Leishmania tropica* parasites after an examination of their effects on various cellular parameters of promastigote and amastigote forms. The authors also enhance the effect of Ag NPs by exposure to ultraviolet rays which increased their effects 6.5-fold. The main purpose of using Ag NPs in the previous study was their capacity to produce ROS, to which Leishmanial parasites are known to be susceptible. In addition, the authors hypothesized that their broad surface areas, small sizes, and their ability to bind sulfur- and phosphor-containing groups might increase their antileishmanial effects. Also [19] investigated the effectiveness of silver nanoparticles (Ag NPs) on *Leishmania tropica* parasites in both phases promastigote and amastigote in comparison to pentostam *in vitro* condition. Not departing from these studies, [20] investigates the effect of Ag NPs on *G. lamblia* parasites. They gave the best results on the parasite either alone or in combination with other native or nanoforms. In addition, [6] showed that silver nanoparticles used singly or in combination with chitosan nanoparticles have promising anti-toxoplasma potentials. This was demonstrated in impression smears made from the liver and the spleen of a growth inhibition of *T. gondii* tachyzoites. On the other hand, this study demonstrated better effects of AgNPs combined with spiramycin which reduced the number of *T. gondii* tachyzoites. While the combined therapy between spiramycin, CS NPs and Ag NPs showed highly significant and higher reduction rate of *T. gondii* tachyzoites. This synergistic action may, besides their effectiveness, bring about an increase of spiramycin bioavailability by increasing the stability, the solubility area, the dissolution rate, and the surface and permeability of spiramycin action through the absorption into membranes. Natural IgM is a crucial relationship between the specific and innate immune responses through its capability of trapping and raising the immunogenicity of pathogens early through infection and by enhancing filtration in the spleen[21]. Also, this result demonstrated that the average level of IgM increased in the infected control group compared to the standard control group. Groups infected after treatment showed various averages in the concentration level of IgM; the average value of IgM was reduced in the group affected and treated by Spiramycin combined CS NPs and Ag NPs approaching to control group. The result was same as those of Couper, Roberts [22], which demonstrated a crucial inhibitory role for IgM in limiting the tachyzoite host cell invasion and systemic dissemination during virulent *T. gondii* RH infection. With particular relevance to *T. gondii* infection, IgM has been shown to increase the killing of tachyzoite by neutrophils; it is also a potent activator of a complement that may be toxoplasmosis. Gazzinelli, Xu [23] reported that IFN-γ was showed to play an important role both in acquired immunity to acute infection toxoplasmosis and in control of parasite growth in chronically infected animals. The average level of production of INF-γ in the infected group had higher production of the cytokine compared with the output in the standard control group. An increased was found in the INF-γ secretion of infected mice when compared with non-infected mice, demonstrating that infected group mice attempted to overcome the infection correlating with the number of tachyzoites. This result agrees with that of [24] who found higher production of IFN-γ to correlate strongly with parasite virulence and improved apoptosis. However, following the administration of Ag NPs, alone or combined, the highest level of IFN-γ was achieved because they improved immunity, and this was more pronounced in the group treated by Ag NPs plus CS NPs plus spiramycin compared with the infected control group. Furthermore, our results were by[6] who found that IFN-γ level went up after the Toxoplasma infection. However, after the administration of Ag NPs, alone or combined with CS NPs, the highest level of IFN-γ was achieved as a result of their enhancement of immunity. In this study, TNF-alpha mean level production in an infected control group of *T. gondii* showed increased production of the cytokine compared with output in the standard control group. The findings of Khalil and Rashwan [25] agree with this result. They found that acute infection was associated with the highest levels of TNF-alpha, indicating that it plays a role in the pathogenesis of acute toxoplasmosis. Also, this result agrees with Kulkarni, Deshpande [26] who reported that what may increase TNF–α production by activated macrophages could bring about an improvement in the
production of lipid peroxidation and oxygen radicals in the pathogenesis of tuberculosis. However, all the groups that got silver NPs showed profound decreases in the level of TNF- α; this was more noticeable in the group treated by spiramycin combined CS NPs and Ag NPs. This result agrees with that of Adeyemi, Murata [27], who reported that treatment toxoplasmosis with AuNPs, AgNPs, and PtNPs depleted the parasite mitochondrial membrane potential, and further strengthens our indication for intracellular ROS production and its liability in the antiparasitic action.

In this study, the synergistic action between NPs and spiramycin demonstrates the highest degree of effectiveness as proved by parasite count and IFN- γ, TNF-α concentration.

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