ASSESSMENT THE IMPACT OF THE USE OF POLYMER NPs IN TREATMENT OF EXPERIMENTAL MURINE TOXOPLASMOSIS

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Abstract

Toxoplasma gondii (T. gondii) is an obligate intracellular protozoan parasite of adverse effect on fetus and immunocompromised host. The potential therapeutic effect of spiramycin-loaded *poly lactic-co-glycolic acid* (PLGA) nanoparticles (NPs) was evaluated for treatment of acute murine Toxoplasmosis. Fifty Swiss albino mice were grouped into five (ten each). Groups 1 and 2 were kept as untreated controls, negative and positive, respectively. Tested mice were subcutaneously injected by the virulent RH strain of *T. gondii* (10^3 tachyzoite/ mouse) and simultaneously treated by spirmycin alone (group 3), PLGA alone (group 4) and spiramycin loaded PLGA (group 5). The treatment lasted for 7 consecutive days. Spirmycin was administered in doses of 200 mg/kg/ body weight once daily while 200μg/kg/day was the dose for PLGA (groups 4 and 5). On the seventh day post infection the animals were sacrificed for evaluation. The toxicity induced by the treatment was estimated by measuring reduced glutathione (GSH) and malondialdehyde (MDA) in different tissues. The immune response associated with the treatment was estimated by measuring interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) and IgM in serum samples. In comparison to the infected groups (non-treated, spiramycin treated and PLGANPs treated) spiramycin loaded PLGANPs showed a significant reduction in the number of tachyzoites (P < 0.05) in peritoneal fluid, liver and spleen, achieved the significant least toxicity of the tissues (liver, kidney and

intestine) as reflected by mean levels of GSH and MDA levels. In spite of significant raising of the mean level of IFN-γ (P < 0.05) in group 5, however the mean levels of IgM, TNF-α were significantly reduced (P < 0.05). This study supports the role of PLGANPs in the optimal performance of spiramycin and confirms its efficiency in achieving treatment of murine toxoplasmosis.

Keywords

Toxoplasmosis, Spiramycin, PLGANPS, GSH, MDA.

1. INTRODUCTION

The apicomplexan parasite *T. gondii* is an obligate intracellular coccidian parasite of humans and other mammals. In spite of almost asymptomatic infection the parasite has an adverse sequel on fetus and immunocompromised patients [1]. The active, rapidly replicating and disseminating tachyzoite form that causes pathological effect is the main target in treatment [1]. However, an ideal drug for treatment would show parasiticidal properties against the different parasitic stages with high efficacy and low toxicity. The proper treatment regimen is subjected to many disputes with no approved options for treatment [2].

Spiramycin is a potent bacteriostatic, established macrolide that has long ago been shown to be effective in murine *Toxoplasma* infection [3]. It has minimal fetal toxicity and it prevents the spreading of the parasite to the fetus through the placenta [4]. The pharmacokinetic data for spiramycin include evidence of its high intracellular concentration, tissue penetration and post-antibiotic effect. These features allow its use in the treatment of a range of infections [5]. The bioavailability of spiramycin as any drug, is affected by many factors such as membrane permeability and solubility [6]. The absorption of spiramycin is incomplete with an oral bioavailability of 33% to 39% (range, 10% to 69%). The rate of absorption is slower compared to erythromycin and is thought to be due to the high negative log of the acid dissociation constant (pKa 7.9) of spiramycin, suggesting a high degree of ionization in the acidic stomach [7]. The greatest disadvantage of the mechanism of action is the developing resistance which occurs quite easily because the bacteria only have to modify their ribosomal enzyme system. Moreover, macrolides carry the problem of cross-resistance: If one bacterium becomes resistant to one macrolide, it is resistant to every other macrolide, too. Hence, spiramycin like mentioned before an outdated medication [8]. Although spiramycin mechanism of action is not fully defined, it is believed to act as an inhibitor of protein synthesis by binding to the 50S subunit of bacterial ribosomes. Like other macrolides, it is bacteriostatic [9].

Nanoparticles have been to date extensively used for various applications including better delivery of molecules or drugs [10,11,13,14,15]. At least in theory, they are a tool to optimize pharmacological data such as drug release, tissue specificity and even cell specificity [12]. Many studies used NPs as vehicles to deliver drugs for improvement of their therapeutic efficacy [13-15]. NPs can act as drug carriers that can modulate pharmacokinetics, increase bioavailability and target release with minimal toxic effects [16].

The use of anti-*Toxoplasma* drugs loaded with NPs has proved its effectiveness in previous research. By reviewing the researchers who used the various NPs to treat the experimental toxoplasmosis by loading them with anti-toxoplasmic drugs or even using them alone, all were positive in favor of treatment of toxoplasmosis. They all supported the efficiency of these molecules in several effects that include higher survival rates of infected animals, reduction of the

inflammatory infiltrate in the liver, reduced the number of brain cysts, ultra-structure deformity of tachyzoites, parasite killing in vitro, inhibition the growth of free tachyzoites and decrease in infectivity, protection from reactivated acute infection beside increased drug bioavailability, controlled drug release, as well as preventing degradation, decreasing toxicity and activating the immune system [17-24]. Biodegradable micro/NPs generated from PLGA have attracted attention due to their clinically proven biocompatibility, especially for immunization purposes [27]. These polymeric particulate delivery systems are able to present antigens and activate both humoral and cellular responses. Particles made from PLGA can be used as a delivery system and also provide adjuvant activity during immunization protocols [25,26].

In an effort to spur the development of treatment regimens with reduced toxicity, the present work was carried out to evaluate the efficacy of spiramycin- loaded PLGANPs versus spiramycin alone in the treatment of toxoplasmosis in the experimentally infected mice.

2. MATERIAL AND METHODES

2.1. Parasite:

Virulent *Toxoplasma gondii* (RH strain) tachyzoites was obtained from Medical Parasitology Department, Faculty of Medicine, Alexandria University. Maintenance of the strain was done by serial intraperitoneal injection of tachyzoites in Swiss albino mice (6–8 weeks old, 20g weight) every 3–4 days interval. Tachyzoites were collected after 4 days post infection (PI) from the peritoneal exudates and washed three times in phosphate buffer solution (PBS) at pH (7.4). Then, they were diluted with PBS with pH 7.4 and used for infection of mice at a dose of 10^3 tachyzoites per mouse [28-30].

2.2. Experimental animals:

Fifty male albino mice, aged 6 – 8 weeks, weighing 20–25g was provided by the Schistosome Biological Supply Centre (SBSC) in Theodor Bilharz Research Institute (TBRI). Throughout the study, the animals were housed in well ventilated cages with perforated covers (cleaned every day), supplied with standard pellet food and water. Mice stools were examined conventionally to exclude the presence of parasites [31]. The mice were allowed to adapt to the laboratory environment for one week before the experiment. The experimental mice were divided into five groups according to the treatment regimen (10 mice for each) as follows:

Group1: Non-infected (negative-control G1).

Group2: Infected non-treated (positive-control G2).

Group3: Spiramycin treated G3.

Group4: PLGANPs treated G4.

Group5: Spiramycin loaded PLGANPs treated G5.

The study was conducted according to the regulations of the ethics committee of Theodor Bilharz Research Institute (TBRI), based on international regulations of animal care.

2.3. Drugs:

2.3.1. Spiramycin preparation:

The spiramycin drug was manufactured and provided by Pharaonia Pharmaceuticals, Egypt in the form of one and a half milligrams per tablet. Spiramycin 1.5 mg tablet was crushed and dissolved in one ml distilled water. It was orally given to mice in a dose of 200 mg/kg/body weight per mouse [33].

2.3.2. PLGA preparation:

PLGA was supplied by Sigma Chemical CO, Cat No: 805726, USA with an average molecular weight of 46, 7 whose copolymer ratio of DL-lactide to glycolide is 50:50 as indicated. PLGA NPs were prepared according to nanoprecipitation technique by **Fessi et al.,** [34]. It was orally given to mice in a dose of 200 μg/kg/ day per mouse.

2.3.3. Loading spiramycin with PLGANPs:

Spiramycin-loaded PLGANPs was done by the addition of PLGANPs solution to spiramycin solution at concentration (200 mg/ml). Spiramycin –loaded PLGANPs was separated from aqueous suspension by centrifugation at 14,000 rpm and 14°C for 30 minutes. The supernatant was collected and protein content (free spiramycin) in supernatant was determined by the Bradford protein assay spectrophotometric method at 595 nm [35].
Spiramycin-loaded PLGANPs were at dose (200μg/kg/day) PLGANPs containing 200 mg/kg/day spiramycin per mouse.

2.4. Animal infection:

The numbers of *Toxoplasma gondii* tachyzoites were detected and counted by taking 50 μl of peritoneal wash (estimated by micro pipette) and put on a glass slide then examined by light microscope at high power (100x objective lens). It was a must to repeat the counting three time to take the average using this equation (count / 50μl x20) = count /ml [6].
Subcutaneous injection of each mouse by *Toxoplasma gondii* tachyzoite in a dose about 10^3 tachyzoite/ mouse was done according to Eissa et al., [32].

2.5. Drugs administration:

Mice were fasted for 1 h before receiving the drugs. Each drug regimen was administrated orally for each specific group once daily by gavage in a volume of 0.2 ml by means of a feeding needle. The drug regimens started from the first day to the seventh day post infection (PI).

2.6. Animal Sacrificing:

At the end of the seventh days after infection and receiving treatment, the surviving mice were sacrificed by rapid decapitation and autopsied. Blood was collected from each mouse into tubes, and centrifuged at 3000 rpm for 5 minutes. The serum was removed in clean tubes and stored at 20 C°. Each mouse was fixed in dissecting tray; a small slit was carefully made in the mid-line of abdominal wall, muscles and peritoneum. Peritoneal cavity was washed by about 1ml of sterile normal saline using 3ml sterile syringe with withdrawal of peritoneal fluid. Internal tissues (liver, spleen, kidney, intestine) from each mouse were removed in clean tubes and preserved in 10% formalin.

2.7. Evaluation of the efficacy of the tested drug regimens:

2.7.1. Estimation of the mortality rate (MR) Eissa et al., [32]

$$MR = \frac{\text{Number of dead mice at the sacrifice time}}{\text{Number of mice at the beginning of the experiment}} \times 100$$

2.7.2. Parasitological examination:

All the infected mice were subjected to the following:

Estimation of the tachyzoites count in:

- **Peritoneal fluid:** Fifty μl of peritoneal wash (estimated by micro pipette) put on a glass slide was examined by light microscope at high power (100x objective lens) to detect and count tachyzoites. It was a must to repeat the counting three times to take the average. The equation (count / 50μl x20 = count /ml) was used to calculate the count [6].

- **Liver and spleen**: Impression smears were made from liver and spleen, stained with Giemsa stain. Counting of *T. gondii* tachyzoites in different tissue smears were carried out using oil immersion objectives (100×) lens. The mean of ten different fields from each organ of each mouse were calculated [36].

2.7.3. Assessment of drugs toxicity in tissues:

Assessment the drugs toxicity in liver, kidney and intestine were done by estimation of GSH [37] and MDA [38] using colorimetric method. Kit of Bio diagnostic (Cat.No.GR. 25 11, Egypt) was used for estimation of GSH level and kit of Bio diagnostic (Cat. No. MD. 25 29, Egypt) was used for estimation of MDA.

2.7.4. Immunological evaluation in serum samples of tested mice:

- **Determination of gamma interferon serum level (IFNγ)** was done by quantitative sandwich enzyme immunoassay technique using Kit of Quantikine® Mouse IFN-γ (Catalog Number SMIF00, USA & Canada R&D Systems, Inc.). The optical density (O.D.) was read at 450 nm using a micro titer plate reader within 30 minutes.

- **Determination of tumor necrosis factor (TNF-α)** was done by quantitative sandwich enzyme immunoassay technique using Kit of Quantikine® Mouse TNF-α (Cat No: MTA00B, USA & Canada R&D Systems, Inc.). The optical density (O.D.) was read at 450 nm using a micro titer plate reader within 30 minutes.

- **Determination of IgM antibodies** was performed by Indirect ELISA with some modifications according to **Engvall and Perlmann** [39]. The absorbance was measured at 492 nm using ELISA reader within 30minute (Bio-Rad microplate reader, Richomond, Co). *Toxoplasma* antigen was kindly supplied by the Department of Immunoparasitology and Immunology (TBRI).

Statistical Analysis

Data were collected, revised, coded and entered to the Statistical Package for Social Science (IBM SPSS) version 23. The quantitative data were presented as mean, standard deviations. Also, qualitative variables were presented as number and percentages. The comparison between groups regarding qualitative data was done by using *Chi-square test*. The comparison between more than two groups with quantitative data and parametric distribution were done by using *One Way ANOVA test* followed by post hoc analysis using

LSD test. The comparison between more than two paired groups with quantitative data and parametric distribution were done by using *Repeated Measures ANOVA.* P. value < 0.05 was considered statistically significant.

3. RESULTS:

3.1. Mortality rate:

The least mortality rate was in spiramycin treated group3 (10%) in comparison to any of the other infected groups (2,4,5) with 20% for each and 0% for negative group1as shown in **Fig.1.**
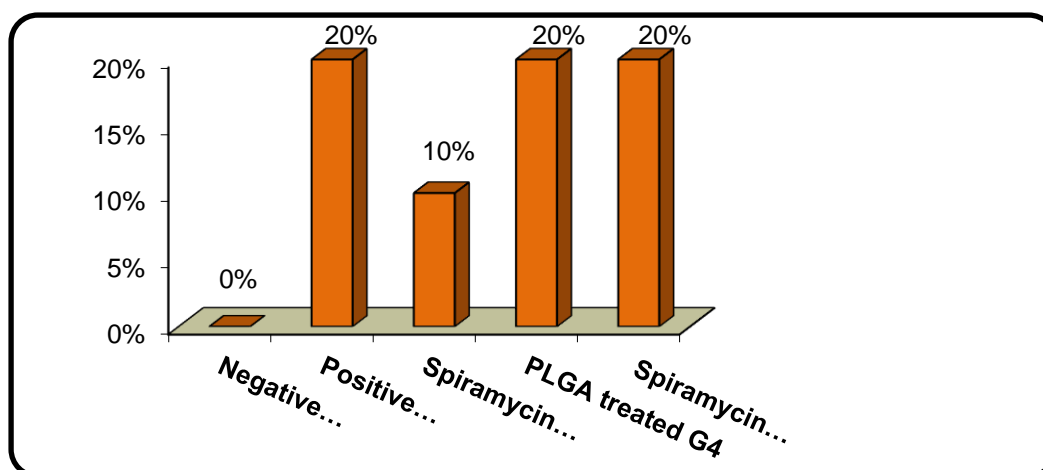

Figure 1: The mortality rate of mice after *T. gondii* tachyzoite infection in all studied groups.

3.2. Estimation of the tachyzoites count in peritoneal fluid, liver and spleen:

The spiramycin loaded PLGANPs treated group5 achieved the lowest mean number (2428.00 ± 345.21, 341.00 ± 42.02, 470.00 ± 66.00 respectively), followed by spiramycin treated group3 (4036.00 ± 624.02, 606.00 ± 70.43, 824.00 ± 101.35 respectively) then PLGANPs treated group4 (7903.00 ± 2240.64, 795.00 ± 160.92, 1287.00 ± 108.22 respectively) as shown in **Fig.2**.
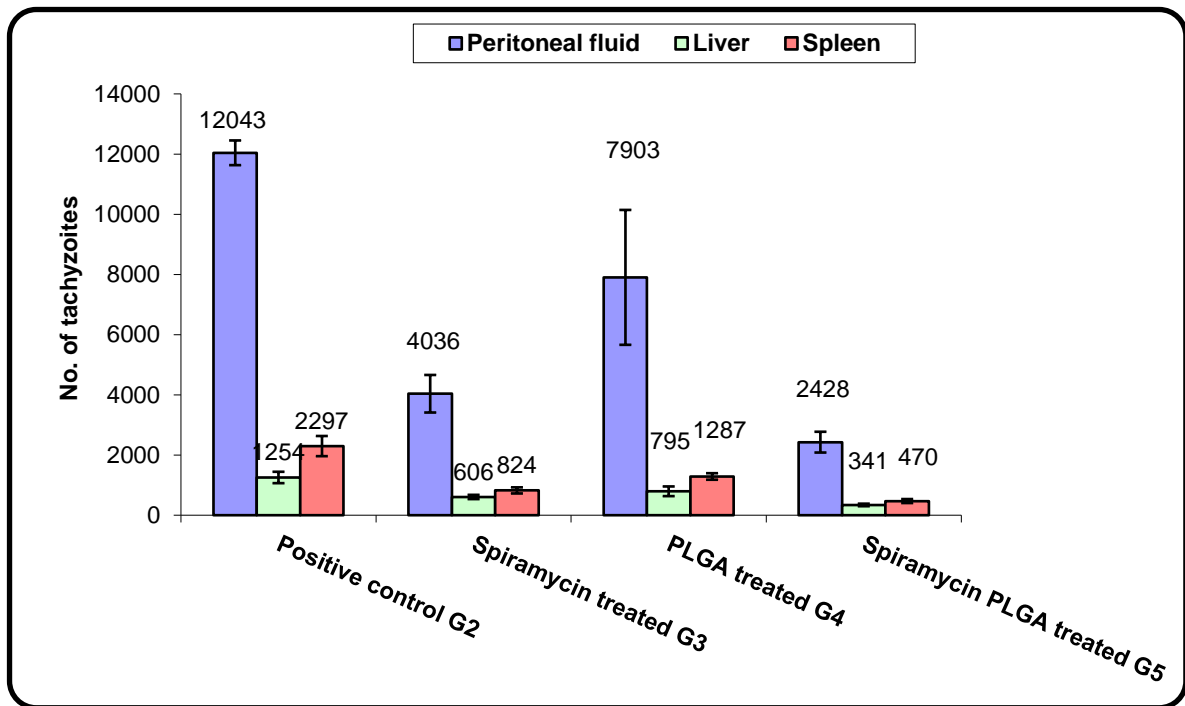
Figure 2: Mean numbers of *T. gondii* tachyzoites in different tissues among infected groups.

3.3. Assessment of drugs toxicity in tissues:

3.3.1. Measurement of GSH level:

The highest mean levels of GSH in liver, intestine and kidney tissues are recorded at non infected group1 (4.85 ± 0.52, 3.35 ± 0.4, 3.04 ± 0.43 respectively). These levels have decreased significantly in infected non treated group2 (2.37 ± 0.38, 1.51 ± 0.31, 1.18 ± 0.33 respectively) reflecting the maximum toxicity. It started to rise in spiramycin treated group3 (3.29 ± 0.26, 2.22 ± 0.33, 2 ± 0.32 respectively) then PLGANPs treated group4 (3.96 ± 0.54, 2.32 ± 0.39, 2.05 ± 0.27 respectively) and reached the maximum rise in spiramycin loaded with PLGA NPs treated group5 (4.58 ± 0.42, 2.96 ± 0.5, 2.29 ± 0.25 respectively) with no significant difference of the later in comparison to non-infected group as shown in **Fig.3.**
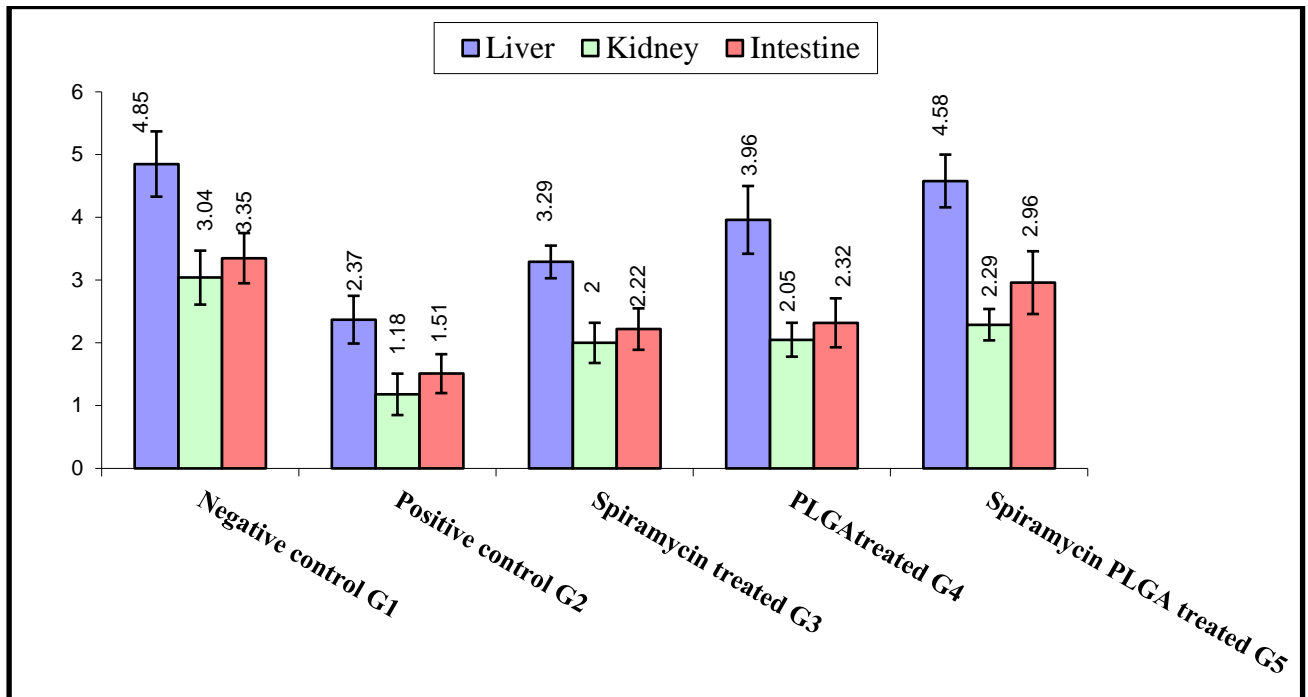
Figure 3: Mean levels of GSH in different tissues in different studied groups.

3.3.2. Measurement of MDA level:

The lowest mean levels in liver, kidney and intestine tissues in non-infected group1 (42.98 ± 1.79, 36.08 ± 3.09, 29.11 ± 1.31 respectively). These levels have elevated significantly in infected non treated group2 (68.9 ± 1.94, 60.02 ± 2.42, 51.03 ± 0.69 respectively) reflecting the maximum toxicity. It started to decrease in PLGA treated group4 (51.53 ± 1.03, 46.15 ± 0.59, 55.01 ± 1.75 respectively) then spiramycin treated group3 (55.78 ± 1.27, 43.14 ± 1.15, 50.98 ± 1.81 respectively) and reached the maximum reduction in spiramycin loaded PLGA NPs treated group5 (46.8 ± 1, 35.88 ± 1.24, 41.02 ± 1.25 respectively) as shown in **Fig.4.**
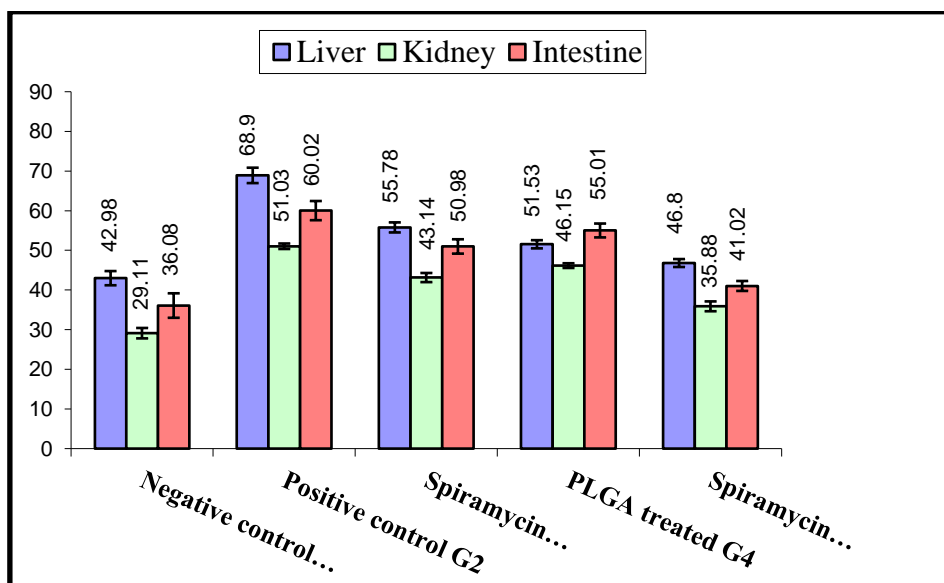

Figure 4: Mean levels of MDA in different tissues in different studied groups.

3.4. Immunological evaluation in serum samples of tested mice:

3.4.1. Determination of IFN-γ:

The mean level of optical density (OD) reading of IFN- γ in the serum samples was at its highest level in the spiramycin loaded PLGA group5(780.30 ± 7.26) in comparison to spiramycin treated group3(691.71 ± 4.62) then PLGA treated group4 (470.04 ± 3.43) and lastly infected non treated group2 (409.84 ± 3.42) while the lowest level was for non-infected group1 (133.65 ± 4.49) as shown in Fig.**5.**
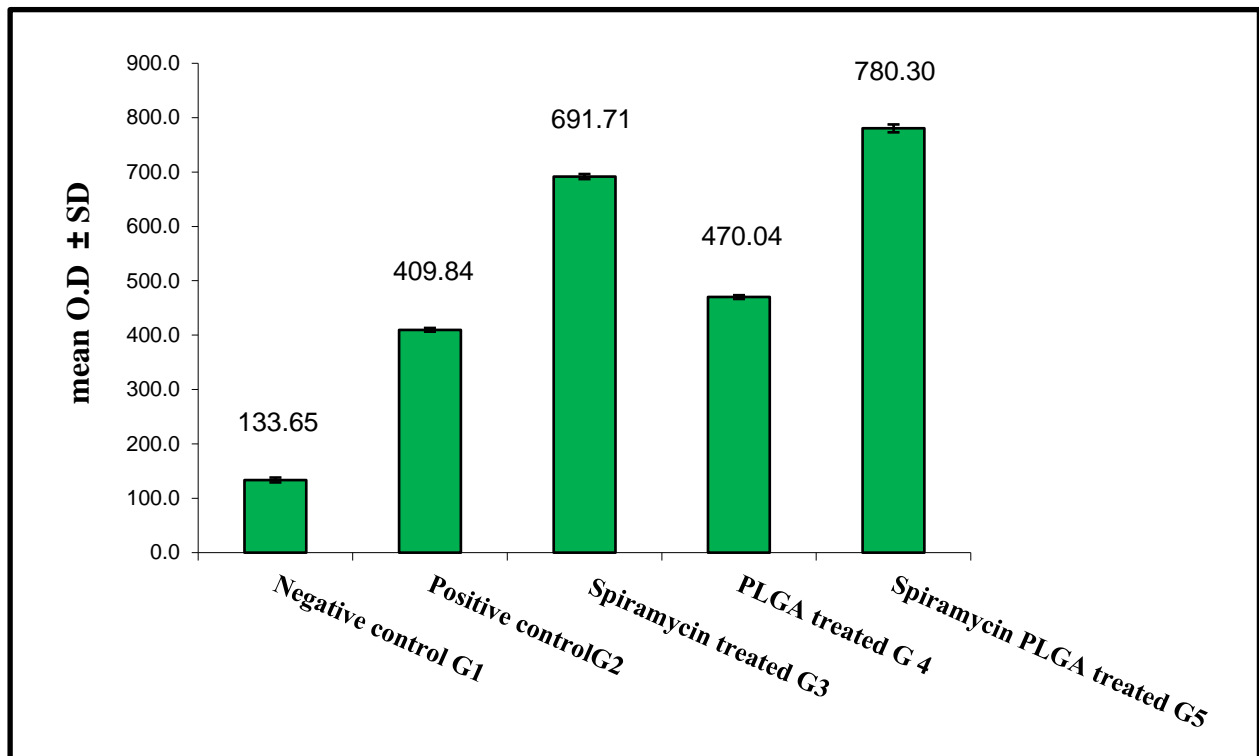

Figure 5: Mean levels of optical density readings of IFN-γ at 450 nm in all studied groups.

3.4.2. Determination of TNF-α:

The OD mean value of TNF-α in the serum samples of the different studied groups was at its highest level in the infected non treated group2 (2654.90 ± 78.34). Among the treated groups, the lowest significant mean level was recorded for spiramycin loaded PLGANPs group5 (1241.70 ± 38.94) in comparison to in spiramycin treated group3 (1495.20 ± 6.14) then PLGANPs treated group4 (2158.30 ± 44.96) as shown in **Fig.6.**
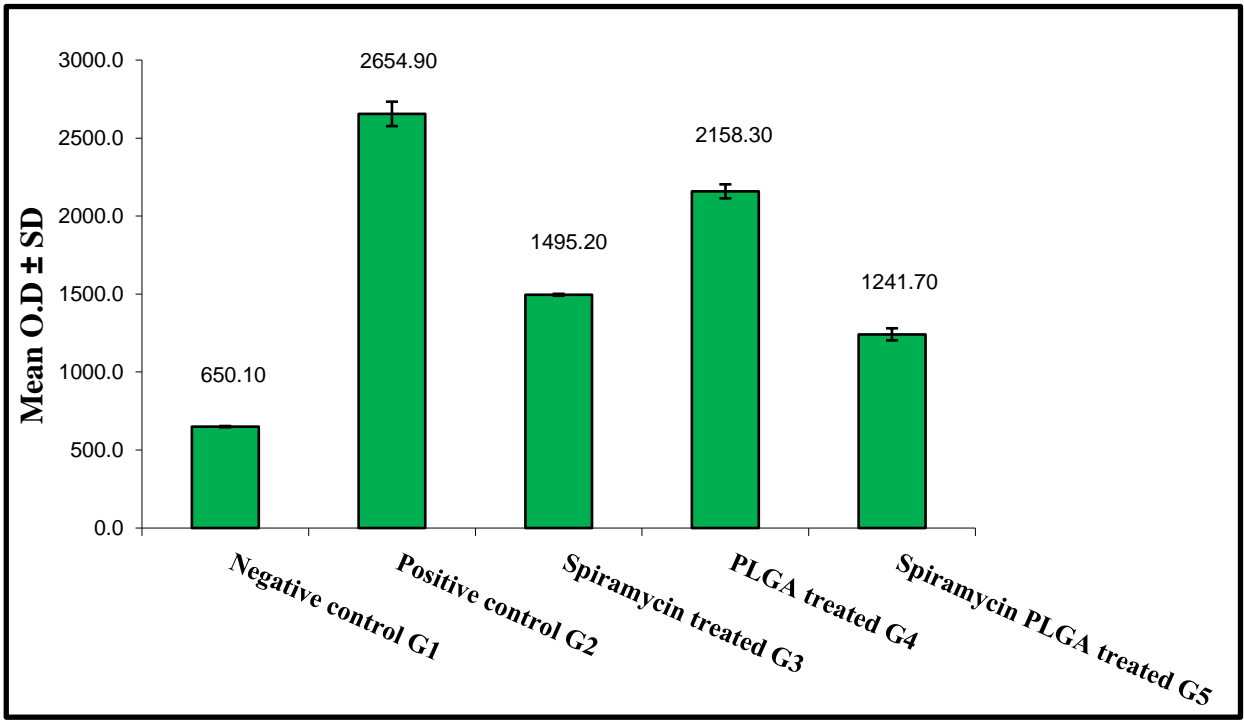
Figure 6: Mean levels of optical density readings of TNF-α at 450 nm in all studied groups.

3.4.3. Determination of IgM:

The OD mean value of IgM in the serum samples of studied mice was at its highest level in the infected non treated group2 (1.40 ± 0.04), while the lowest significant mean level was recorded for spiramycin loaded with PLGA NPs treated group5 (0.60 ± 0.01) in comparison to spiramycin treated group3 (0.74 ± 0.01) then PLGA treated group4 (0.97 ± 0.01) as shown in **Fig.7.**
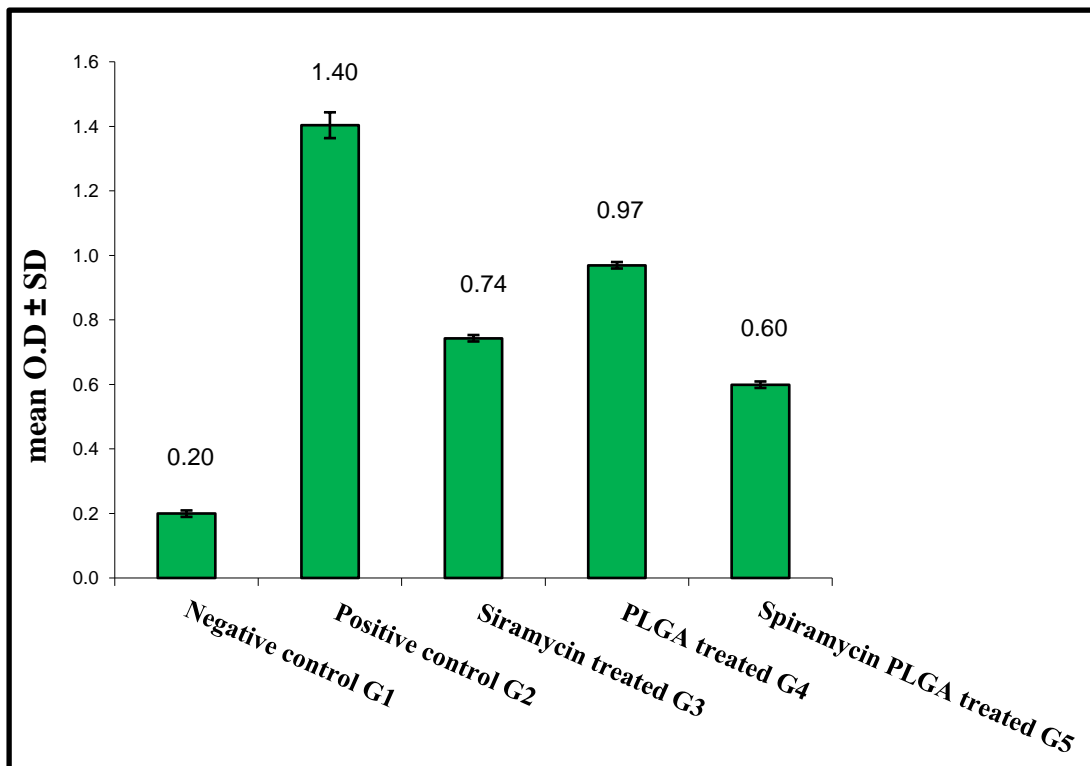

Figure 7: Mean levels of optical density readings of IgM at 492 nm in all studied groups.

4. DISCUSSION:

Nanoparticles have been used widely in previous studies as vehicles to deliver drugs or vaccines and were tried in helminthic parasites as; *Brugia malayi* and *Echinococcus granulosus* and also in parasitic protozoa as; *T. gondii, Leishmania* major and malaria [13,33,40,41].

Nanosuspensions were prepared from some anti-toxoplasmic drugs in order to overcome their low absorption [42-44]. In this study, spiramycin as a known anti-toxoplasmic drug was loaded with PLGA NPs and its therapeutic potential was estimated in treatment of murine toxoplasmosis.

At the present study, the least mortality rate (Fig. 1) was in spiramycin treated group3 with no significant difference in comparison to any of the other infected groups (2,4,5) and negative group1. Spiramycin (100 and 200 mg/kg/day) was recorded with some dependent prolonged survival rate in mice infected with $2x10^2$ tachyzoites of the RH strain [45]. but not with complete protection against death. In contrast to the present result are Etewa et al; El-Temsahy et al; Sordet et al. [1,15,46] who recorded significant decrease mortality or increase survival rates in murine toxoplasmosis after treatment with drug loaded NPs such as (spiramycin-loaded chitosan NPS (SLCNs), chitosan nanospheres encapsulated with *Toxoplasma* lysate vaccine

and atovaquone-loaded nanocapsules) respectively.

By counting the tachyzoites (Fig. 2) in the peritoneal wash, liver and spleen smears in the examined groups, it was found that spiramycin loaded PLGANPs treated group5 achieved the lowest mean number followed by spiramycin treated group3 then PLGANPs treated group4 with a significant statistical difference using F test and post hoc analysis (P < 0.05) between all studied groups. The highest mean level was recorded for infected non treated group2. This means that spiramycin loaded PLGANPs treated group5 had the highest impact on reducing the number of the parasites in different tissues. Putting in mind that the spiramycin treated group3 achieved a lower significant mean number of parasites in all tissues compared to the PLGANPs treated group4 (P < 0.05). therefore, it could be expected that the reduction of tachyzoites number in different tissues by the combined treatment is mainly due to improved performance of spiramycin after being loaded PLGANPs. This is in agreement with Etewa et al. [1] who noticed that impression smears made from liver and spleen revealed highly significant decrease in the number of *Toxoplasma* tachyzoites in mice groups treated with spiramycin-loaded chitosan NPs as compared to the other subgroups (infected control, infected and received chitosan NPS (CS NPs) and infected

and treated with spiramycin). Also, Gaafar et al. [6] noticed that impression smears made from liver and spleen revealed similar highly significant reduction in the number of *Toxoplasma* tachyzoites in mice treated by silver NPs alone or combined with chitosan NPs.

Nanomaterials associated with molecules or drugs for experimental toxoplasmosis studies has shown improvement in one or more aspects as reducing parasitic load in liver and spleen or even absence of parasites in the blood and brain, deformity in tachyzoites, increased bioavailability of the conjugated drug, amelioration of cellular infiltrate in infected tissues, higher survival rate of the infected animals, in vitro inhibition of the growth of free tachyzoites , decrease the number of infected macrophages and parasites per cell, protection of mice from reactivated acute infection [47]. Among these NPs used alone or loaded with molecule or drugs are silver NPs [6], gold NPs [17,18], polymeric NPs of chitosan [6], polymeric nanocapsules of alginate-chitosan [20], Liposomes [14.22,48], core-shell latex NPs [19], nanosuspensions [4,44, 50,51], core lipid nanocapsules [52], drug-dendrimer complex [53], hydroxypropyl-β-cyclodextrin [23] and cyclodextrin [23,24].

An ideal drug for treatment would show low oxidative stress and accordingly low toxicity. The present study assessed GSH and MDA as keys of oxidative stress related molecules in mice liver, kidney and intestine after administration of different drug regimens.

GSH, an endogen-originated peptide, can be synthesized in the liver without need for genetic data, GSH is an antioxidant, a powerful detoxifier and helps activate the better known antioxidants, vitamin C and vitamin E. It is an important constituent of intracellular protective mechanisms against a number of noxious stimuli including oxidative stress [54]. Oxidative stress is related to systemic glutathione deficiency while protection is indicated by higher levels in the tissues. The normal range for reduced GSH is 3.8 – 5.5 umol/L in plasma, according to the Health Diagnostics and Research Institute [55].

The highest mean levels of GSH in liver, intestine and kidney tissues (Fig. 3) were recorded for non-infected group1. These levels have decreased significantly in infected non treated group2 reflecting the maximum toxicity. It started to rise in spiramycin treated group3 then PLGANPs treated group4 and reached the maximum rise in spiramycin loaded with PLGANPs treated group5 with no significant difference of the later in comparison to non-infected group indicating the minimal toxicity in this group.

MDA is an end-product of lipid peroxidation, a well-established mechanism of cellular injury. So, MDA is used as an

indicator of oxidative stress in cells and tissues [56]. MDA as an oxidative marker - according to the present data – was found with the lowest mean levels in liver, kidney and intestine tissues (Fig. 4) in non-infected group1. These levels have elevated significantly in infected non treated group2 reflecting the maximum toxicity. It started to decrease in PLGA treated group4 then spiramycin treated group3 and reached the maximum reduction in spiramycin loaded PLGANPs treated group5 with significant differences between studied groups upon using F test and post hoc analysis. Similar results are that of Miricescu et al. [57], who assessed oxidative stress by estimation of GSH and MDA levels in healthy rats after acute administration of PLGANPs. The obtained data showed GSH levels were decreased and MDA levels increased in spleen and liver in comparison to their respective controls with no statistical significance between the two analyzed groups as regard GSH but as regard to MDA the absence of significant difference was recorded for spleen only. This current study showed that with the use of PLGA NPs the degree of toxicity decreases and even improves to the extent of approximating that of healthy control. This is in agreement with Semete et al. [58] who observed high doses of PLGA did not lead to any tissue lesions as compared to the same high dose of ZnO (zinc oxide) NPs that led to toxicity in mice.

Both Innate and adaptive immunity play critical roles in protecting against *Toxoplasma gondii* infection. A strong cellular immune response was elicited during toxoplasmosis showing significant production of IFN- γ and IL-2 associated with Th1 type response [59]. IFN-γ is the first cytokine implicated in resistance to *T. gondii* and remains the keystone of protective immunity to *Toxoplasma* [60]. The secretion of INF-γ increases the phagocyte activity of macrophages and the cytotoxic activity of CD8 TL. However, IFN-γ triggers the conversion of tachyzoites into bradyzoites at the same time preventing their rupture [61].

This study showed higher significant production of the IFN- γ (Fig. 5) in infected non treated group2 (409.84 ± 3.42) in comparison to the non-infected group1 (133.65 ± 4.49). This is in agreement to Filisetti & Candolfi, [60] who stated that increased production of IFN-γ is strongly correlated with parasite virulence and enhanced apoptosis. Our data showed that the mean level of OD reading of IFN- γ in the serum samples (Fig. 5) was at its highest level in the spiramycin loaded PLGA group5 in comparison to spiramycin treated group3 then PLGA treated group4 and lastly infected non treated group2 while the lowest level was for non-infected group1. This indicates an enhancement of immunity of spiramycin loaded PLGA group5 which showed a significant better level of IFN- γ than other groups. The Increase in IFN- γ could be

correlated with the significant highest tachyzoites reduction in the same group. On support of the present results is Liu et al. [61] who found that PH – responsive PLGA NPs have impact in stimulating cellular and humoral immune response that allowed it to be used in prevention of immune system disorders and inflammatory processes. Mouse immunization with pH- responsive PLGANPs induced greater lymphocyte activation, more antigen-specific CD8 T cells, stronger cytotoxic capacity (IFN-γ and granzyme B), enhanced antigen-specific IgG antibodies, and higher serum IgG2a/IgG1, indicating cellular immunity. Also, Hamad et al. [62] found that the highest level of IFN- γ was in the mice group treated by Chitosan NPs plus spiramycin compared with infected control group. Other NPs administration as Ag NPs alone or combined with CS NPs achieved the highest level of IFN- γ in treated mice [6].

In toxoplasmosis TNF-α would appear to be essential for macrophage activation and inhibition of parasite replication, but this action can only be exerted in synergy with IFN-γ. This protective action is exerted in mice in both the acute and chronic phase of the disease. In addition, TNF-α - like IL-12, another monocyte macrophage product - stimulates the production of IFN-γ by NK cells, which play a crucial role in the early non-specific response during toxoplasmosis [64].

In the present study, the OD mean value of TNF-α in the serum samples of the different studied groups (Fig. 6) was at its highest level in the infected non treated group2 (2654.90 ± 78.34). This result agrees with the findings of Khalil & Rashwan. [65] who reported acute infection was associated with the highest levels of TNF-α indicating that it plays a role in the pathogenesis of acute toxoplasmosis [66]. Among the treated groups, the lowest significant mean level was recorded for spiramycin loaded PLGANPs group5 in comparison to in spiramycin treated group3 then PLGANPs treated group4. On support of our explanation is Hamad et al., [67] who recorded reduction in level of TNF- α cytokine in mice infected by toxoplasma tachyzoites after treatment with spiramycin plus chitosan. In contrary to the present results is Liu et al. [68] who reported that Ag NPs are potent enhancer to the immune system. They recruit leukocytes and increase both TNF- α and IFN- γ in mouse abdominal cavity and also up-regulate the MHC II molecules expression of peritoneal macrophages.

IgM are the first antibodies to be promoted after *T. gondii* infection. These immunoglobulins are the best activators of the complement system. They enable excellent agglutination and have a high level of cytotoxicity. The major target antigens of these IgM are the surface proteins of the parasite [61]. Couper et al. [69] Demonstrated an important inhibitory role for IgM in limiting tachyzoite host cell invasion and

systemic dissemination during virulent *T. gondii* RH infection. With specific relevance to *T. gondii* infection, IgM has been shown to increase tachyzoites killing by neutrophils and is a potent activator of complement [60].

This study recorded the same gradient observed of OD mean value TNF-α for OD mean value of IgM among all studied groups (Fig. 7) where the OD mean value of IgM in the serum samples of studied mice was at its highest level in the infected non treated group2, while the lowest significant mean level was recorded for spiramycin loaded with PLGANPs treated group5 in comparison to spiramycin treated group3 then PLGA treated group4. Consistent with the result of this proposition the previous research of Brinkmann et al. [71] who found that *Toxoplasma*-specific IgM and IgG levels were lower in sulfadiazine treated immunocompetent and B-cell-deficient mice than untreated mice following infection of all with 20 cysts of Me 49 (avirulent strain of *T. gondii*). This study found that the treatment with spiramycin loaded with PLGA did not improve levels of TNF-α and IgM in the infected treated mice. If we link the reduction of the number of tachyzoites achieved by treatment with the levels of TNF-α and IgM we could assume that the immediate treatment after infection had a potent lethal effect on injected tachyzoites thus limited the stimulant antigenicity with weaker immune response. This limiting effect was more pronounced in spiramycin loaded with PLGA group5 in

comparison to other infected groups (2, 3, 4) with significant difference (P < 0.05).

To our knowledge, this is the first research that has been exposed to the effect of spiramycin loaded with PLGA nanoparticles in the treatment of murine toxoplasmosis.

In conclusion, the study supports the effectiveness of spiramycin after being loaded by PLGA NPs in controlling the acute murine toxoplasmosis with the minimal toxicity.

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