Original Article

Encapsulation of Low Metronidazole Dose in Poly (D,L-lactide-co-glycolide) (PLGA) Nanoparticles Improves *Giardia intestinalis* Treatment

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Conflict of Interest

No conflict of interest.

ABSTRACT

Background: The present study was designed to investigate the anti-giardial efficacy of low metronidazole dose loaded-D,L-lactide-co-glycolide (LMD-PLGA) nanoparticles (NPs) and to compare it with the standard high dose of metronidazole either free (HMD) or loaded on PLGA (HMD-PLGA).

Materials and Methods: PLGA NPs were prepared by single emulsification method, metronidazole (MTZ) was loaded in low and high doses. The nanoparticles were evaluated *in vivo* for mice model. The *Giardia intestinalis* infected mice were treated by LMD and HMD either free or PLGA NPs loaded, the parasitic load and polyclonal anti-giardial serum antibodies (IgG and IgA) were recorded. Histopathological studies on intestinal and liver sections were applied.

Results: MTZ-PLGA NPs was successfully prepared with 81.68% encapsulation efficiency and with an average particle size of approximately 228.00 ± 43.19 nm and -32.28 ± 0.07 mV Zeta potential. Experimentally, it was observed that *Giardia intestinalis* infected animals administered with LMD-PLGA had completely eliminated cyst shedding and trophozoite count compared with *Giardia*-infected mice. Further, it was found that animals belonging to LMD-PLGA group had significantly reduced levels of anti-giardial IgA (0.99 ± 0.05) antibodies in serum compared with *Giardia*-infected. Histopathologically, also animals belonging to LMD-PLGA treated group had intact mucosal epithelium lining, and normal villi with no detection of *G. intestinalis* trophozoites. In addition to the less toxic effect on the liver tissue compared to free HMD, HMD-PLGA and infected-untreated groups using Ishak grading system.

Conclusion: Our study showed that PLGA nanoparticles could be a delivery system for anti-giardial drugs to improve their therapeutic efficacy and minimize their side effects that results from frequent dosing.

Keywords: *Giardia intestinalis*; Metronidazole; PLGA nanoparticles

Author Contributions

Conceptualization: IR, AEA. Data curation:

NAM. Investigation: HN. Methodology: AE.

Supervision: AEA. Writing - original draft:

NAM. Writing - review & editing: NAM.

INTRODUCTION

Giardia intestinalis is the causal agent of giardiasis which is an intestinal infection correlated with poverty and poor drinking water quality. *Giardia* infections affect nearly 33% of people in developing countries, 2% of the adult population in the developed world, and treatment options are limited according to the Center for Disease Control and Prevention [1]. *Giardia* infection causes enterocytes damage and loss of brush border of the epithelial cells of the intestine that leads to shortening of microvilli and altered epithelial barrier function. Drug resistance to common anti-giardial agents and incidence of treatment failures have increased in recent years. [1]. Standard treatment for giardiasis is commonly with 5-nitroimidazole (5-NI) compounds, or metronidazole (MDZ), but a growing number of refractory cases were being reported [2]. Clinicians are increasingly falling back on second-line and less well-known drugs to treat giardiasis [3]. However, these compounds present side effects associated with residual toxicity in the host. Dose-dependent side effects include leukopenia, headache, vertigo, nausea, insomnia, irritability, metallic taste, and central nervous system (CNS) toxicity [4]. Drug resistance to common anti-giardial agents and the incidence of treatment failures have increased in recent years. Therefore, the search for new molecular targets for drugs against *Giardia* infection is essential [1] and a combination of two or more drugs may be a viable approach [3].

Nanoparticles have become highly attractive for their applications in the fields of medicine and biology in the last few years [5].

The polymer-based nanoparticle is prepared from natural or synthetic polymers and can deliver wide kinds of drugs. Nanoparticles permit a targeted direction to a particular cell or organs or controlled drug delivery [6]. By encapsulating these molecules inside a nano-carrier, the stability and solubility of drugs can be enhanced, providing a chance to re-evaluate the therapeutic potential of drugs because of poor pharmacokinetics [7].

Poly (D,L-lactide-co-glycolide) (PLGA) is considered as one of the most successfully used biodegradable polymers due to its hydrolysis leads to metabolite monomers, lactic acid and glycolic acid. As these two monomers are endogenous and simply metabolized by the body through the Krebs cycle, negligible systemic toxicity is associated with the use of PLGA for drug delivery or biomaterial applications [8, 9]. PLGA has a long-standing track record in biomedical functions and well-documented utility for continued drug release in comparison with the conventional devices up to days, weeks or months, and ease of parenteral administration by injection [8].

PLGA-NPs speedily escape the endo-lysosomes and enter the cytoplasm in 10 min of incubation. This helps interactions of nanoparticles with the vesicular membranes leading to a transient and localized deterioration of the membrane resulting in the escape of nanoparticles into the cytosol [9]. It is approved by the United States Food and Drug Administration and European Medicine Agency (EMA) in different drug delivery systems in humans. The polymers are commercially attainable with diverse molecular weights and copolymer compositions. Relying on the molecular weight and copolymer ratio the degradation time can differ from several months to several years [9]. PLGA had proved improvement of the therapeutic efficacy of other antiparasitic drugs [10]. In this study, we aim to assess the efficacy of MDZ-PLGA NPs as anti-giardial treatment to reduce the MDZ dose from (120 mg/kg) to (60 mg/kg) to reduce the possible side effects.

MATERIAL AND METHODS

1. Drugs

1) Metronidazole (MDZ)

MDZ (FLAGYL, Cairo, Egypt) was supplied Sanofi-Aventis Egypt, as suspension. It was administrated in high dose (120 mg/kg) [11] and low dose (60 mg/kg).

2) Preparation of Nanoparticle (NPs)

PLGA with a copolymer ratio of D,L-lactide to glycolide of 50:50 (Mw 40,000 - 100,000 g/mol as indicated by the supplier, Sigma Chemical CO, St. Louis, MO, USA). The NPs prepared from single emulsification process [12] with poly vinyl alcohol (PVA) (87 - 89% hydrolysis degree and molecular mass 12,000 - 13,000 g/mol, Sigma Chemical CO, USA). The organic solvent was methylene chloride (Labsynth Ltd., Sao Paulo, Brazil). As suspending medium, purified water (Milli-Q, Millipore Corporation, Billerica, MA, USA) was used. MDZ was encapsulated either in a high dose (120 mg/kg) or in a low dose (60 mg/kg).

2. Characterization of NPs

1) Particle size and zeta potential

The mean diameter of PLGA NPs in aqueous dispersion was measured utilizing a laser particle analyzer (LAP 3100, Otsuka Electronics, Osaka, Japan) with a photon correlator (LPA 300, Otsuka Electronics). When the diameter was expected to exceed 1 mm, a laser-based time-of-transition system (Cis-1, Galai Production Ltd, Israel) was used. Zeta potential was measured using a clear zeta cell.

2) Entrapment efficiency

Entrapment efficiency of MDZ-PLGA NPs was evaluated according to Stolnik et al. [12]. The supernatant and washing solutions from purification were collected. The drug content was then determined in supernatant and washing solutions spectrophotometrically. The entrapment efficiencies (%EE) of NPs were determined according to the following equation:

$$\text{Entrapment efficiencies (\%)} = \frac{\text{Amount of drug in nanoparticles}}{\text{Amount of drug used}} \times 100$$

All measurements were taken in triplicate.

3. Assessment of anti giardial efficacy:

1) Experimental animals

Laboratory bred male, Swiss albino mice strain (weighing 18 - 20 g), were purchased from Theodor Bilharz Research Institute (TBRI), Giza, Egypt. Experimental animals were maintained for 8 weeks at (21 ± 2°C) and fed dry food (contain 24% protein). The experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee (IACUC); Cairo University, Faculty of Science, Egypt (CUIS/26/16). All the experimental procedures were performed according to the internationally valid guidelines.

2) Extraction of Giardia from stool

G. intestinalis cysts used for infection of mice were obtained from diarrheic patients referred to the outpatient clinic of TBRI. The stool samples of infected patients were collected in sterile clean stool cups. The samples chosen for cyst purification were not only with the highest cysts rate (more than 8 cysts in each microscopic field with the magnification of ×

40) but they were also without any other gastrointestinal parasite or yeast contaminations. After collection of stool samples, homogeneous suspensions in 0.2 M PBS buffer were made that then purified and centrifuged at 500 g for 5 min and the sediments were the subject to purification by modified two-phase sucrose purification method [13].

3) Animal infection

The mice were infected by oral inoculation with the isolated *G. intestinalis* cysts in a dose of about $10,000 \pm 1$ cysts/mouse [14].

Animals were divided into the following groups.

Group 1: Negative-control (normal).

Group 2: Positive-control (*G. intestinalis* infected- untreated group).

Group 3 (LMD): *G. intestinalis* infected then treated with low MDZ dose (60 mg/kg) for five consecutive days post infection.

Group 4 (HMD): *G. intestinalis* infected and then treated with high MDZ dose (120 mg/kg) for five consecutive days post infection [11].

Group 5 (PLGA): *G. intestinalis* infected and then treated with PLGA NPs only for five consecutive days post infection.

Group 6 (PLGA-LMD): *G. intestinalis* infected and then treated with low MDZ dose (60 mg/kg) loaded on PLGA NPs for five consecutive days post infection.

Group 7 (PLGA-HMD): *G. intestinalis* infected and then treated with high MDZ dose (120 mg/kg) loaded on PLGA NPs for five consecutive days post infection.

Animals were anesthetized by xylene ketamine 100 mg/kg (4 weeks post infections). Blood was collected individually from Jugular vein. Blood was allowed to stand for 1hr at 37°C, then overnight at 4°C and centrifuged at 2,500 rpm for 15 min (80-1 Electric Centrifuge). The serum was obtained and kept in aliquots at -20°C for immunoglobulin assay.

4) Parasitological examination

Stool samples were examined to all groups to detect *G. intestinalis* cysts to insure the establishment of the infection. Samples were examined using direct smear and Merthiolate-iodine-formaldehyde concentration technique (MIFc) [15].

5) Histopathological studies

Immediately after blood collection, the animals were dissected. Liver and small intestine tissue samples were collected from all mice within the different groups to evaluate the pathological changes according to Drury [16]. Grading of necroinflammation in liver was evaluated according to Ishak scoring system [17]; which assign numbers to the severity of the necroinflammatory features (interface hepatitis, confluent necrosis, parenchymal injury and portal inflammation) and add the numbers to arrive at a grade that can range from 0 to 18.

6) Immunological parameter

Measurement of serum polyclonal anti-giardial IgG antibody and IgA antibody by the Enzyme Linked Immune Sorbent Assay (ELISA). Assays were performed in accordance with the manufacturer's instructions (Sigma Chemical CO, USA).

4. Statistical analysis

The present data were analyzed by IBM SPSS version 22 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to analyse the effect of treatment on the studied parameters. Duncan's test of homogeneity was used to test the similarities between the experimental groups. Correlation coefficient and regression analyses were used to fit the relationships between the studied variables. All the results were expressed as a mean $\pm$ standard error of mean (SE).

RESULTS

1. Characterization of PLGA NPs

PLGA NPs were prepared by single emulsion method. The average particle size of MTZ-PLGA NPs was 228 ± 43.19 nm. The external morphology of the nanoparticles studied by scanning electron microscope (SEM) revealed that nanoparticles were approximately spherical in shape having a smooth surface. Zeta potential was -32.28 ± 0.07 mV and the %EE was 81.68%.

2. Quantification of cysts before (2 weeks post infection) and after treatment (4 weeks post infection)

The infection was confirmed in all groups inoculated with *G. intestinalis* and the cysts count in 1 g of stool before the different treatments was recorded in **Figure 1**. The parasitic load of *G. intestinalis* in mice showed a significant reduction four weeks post infection (p.i.) in all infected treated groups as compared with infected non treated ones. The treatment with LMD induced (74.76%) ($P < 0.0001$) cyst reduction, but a stronger reduction was encountered with the HMD (98.67%) ($P < 0.0001$). The treatment with PLGA was associated with (57.14%) ($P < 0.05$) reduction in cysts number. On the other hand, the administration of HMD-PLGA showed 86.85% cyst count. Interestingly, the LMD-PLGA group recorded a complete eradication of cysts in stool with 100% cyst reduction.

1) Trophozoite count

Trophozoites of *G. intestinalis* were counted along mucosal surface of the small intestine in all the infected-treated groups and compared to the infected untreated group (**Table 1**).

Figure 1. (A) The oocysts count /1 gram stool. (B) The PR of oocysts count before and after the treatment with free LMD and HMD or loaded on PLGA Nanoparticles in *Giardia intestinalis* infected mice, 2 weeks post-treatment.

^{a,b}Represent significant difference as compared to the corresponding value before treatment, at ($P < 0.05$) and ($P < 0.0001$), respectively. PR is in relation to the corresponding infected untreated group.

PR, percentage reduction; LMD, low metronidazole dose; HMD, high metronidazole dose; PLGA, D,L-lactide-co-glycolide.

Table 1. Effect of free (low and high dose) of metronidazole or loaded on poly (L-lactic acid) copolymer with glycolic acid (PLGA) Nanoparticles on *Giardia intestinalis* trophozoite count in the small intestine, 4 weeks post infection and 2 weeks after treatment

Group	Trophozoite reduction	
	Number of trophozoites/gram tissue (mean $\pm$ SE)	Reduction (%)
Control	0.00 $\pm$ 0.00 ^a	-
Infected-untreated	31.69 $\pm$ 4.07 ^b	-
LMD	4.05 $\pm$ 0.06 ^a	87.20
HMD	2.08 $\pm$ 0.08 ^a	93.44
PLGA	10.76 $\pm$ 1.05 ^c	66.04
LMD-PLGA	0.00 $\pm$ 0.00 ^a	100.00
HMD-PLGA	3.96 $\pm$ 0.36 ^a	87.52

% represents percentage reduction as compared to the infected-untreated group. Means followed by the same letter within the same column are not significantly different ($P > 0.05$) whereas those marked with different ones are significantly differed ($P < 0.05$).

SE, standard error; LMD, low metronidazole dose; HMD, high metronidazole dose; PLGA, D.L-lactide-co-glycolide.

A significant reduction in trophozoite count was recorded in intestinal tissue following the treatment with LMD (87.2%), and a higher reduction (93.44%) ($P < 0.05$) was obtained following HMD treatment. The administration of the high metronidazole dose but loaded on PLGA nanoparticles (HMD-PLGA) lowered the trophozoite reduction (87.52%), this was not statistically significant as compared to the HMD group (93.44%). The maximal anti-giardial effect and the complete cure were recorded in the LMD-PLGA group as the treatment was concurrent with (100.00%) reduction of trophozoite count.

3. Histopathological examination

Histopathological examination of small intestine sections as shown in **Supplementary Fig. 1**. Liver sections from all the studied groups were examined and evaluated according to Ishak score (**Supplementary Table 1**, **Supplementary Fig. 2**). HMD-PLGA was the group that recorded maximal liver injury (grade 6).

4. Measurement of serum IgA and IgG levels by ELISA

Serum levels of IgA and IgG antibody against soluble *G. intestinalis* antigen were measured 4 weeks p.i. by ELISA (**Fig. 2**).

Figure 2. Serum levels of anti-giardial IgA and IgG immunoglobulin antibodies in response to the different treatments.

Data are presented as (mean $\pm$ SE) of OD.

^a($P < 0.05$) significance versus infected untreated group.

^b($P < 0.05$) significance versus LMD group [ANOVA].

IgA, immunoglobulin A; IgG, immunoglobulin G; OD, optical density LMD, low metronidazole dose; HMD, high metronidazole dose; PLGA, D.L-lactide-co-glycolide.

Normal controls showed no detectable levels of IgA and IgG antibodies against *G. intestinalis* (0.25 ± 0.01 and 0.40 ± 0.03 , respectively). The levels of serum IgA and IgG for *G. intestinalis* infected-untreated group were significantly elevated (1.93 ± 0.07 and 2.93 ± 0.07 , $P < 0.05$) as compared to the normal control group. After the treatment with LMD, a significant decrease in the OD values for IgA (1.11 ± 0.02) and IgG (2.33 ± 0.04) antibody levels were recorded ($P < 0.05$). The decrease in IgA and IgG levels was also significant in HMD group (1.09 ± 0.09 and 2.15 ± 0.06 , $P < 0.05$) as compared to the infected-untreated control. In contrast, infected mice receiving only PLGA treatment alone did not elicit significant change in serum anti-giardial IgA and IgG responses ($P > 0.05$) (1.63 ± 0.08 and 2.65 ± 0.24 , respectively) in comparison to infected-untreated mice. The IgA and IgG response of infected mice treated with LMD-PLGA (0.99 ± 0.05 and 2.02 ± 0.05) was comparable to that of the infected mice treated with HMD (1.09 ± 0.05 and 2.15 ± 0.06 , respectively) with no significant difference between them ($P > 0.05$).

The treatment of infected mice with HMD-PLGA also demonstrated a significant reduction of IgA and IgG levels (1.37 ± 0.07 and 1.37 ± 0.07 , $P < 0.05$) as compared to both the infected-untreated and infected-HMD treated groups.

DISCUSSION

Infection with *G. intestinalis* is one of the most common human infections, particularly in developing countries; the infection affects mainly preschool and school children. It contributes to increased mortality in the immunocompromised patients [17]. Several families of drugs with good efficacy are used for *Giardia* treatment, but sometimes dosing regimens are suboptimal and emerging resistance begins to question their clinical value. Moreover, some of these drugs can cause side effects that result in patient discomfort and low adherence to the treatment [18]. MDZ is the central drug of giardiasis therapy; however, it has dose-related CNS toxicity [19]. MDZ has been listed by the United States national toxicology program (NTP) as reasonably anticipated to be a human carcinogen according to WHO International Agency for Research on Cancer (IARC) [20]. The standard metronidazole therapy is (250 - 500 mg/day $\times$ 5 - 10 days) with 60 - 95% efficacy [21]. Bezagio et al. [22] suggested a MDZ therapeutic protocol that was able to maintain the animals free of infection for at least 10 days using (500 mg/kg 3 times a day for 7 days). While a (500 mg/kg once a day for 7 days) therapy failed to completely eliminate the parasite load in infected mice. 120 mg/kg MDZ is the breakpoint below which treatment failure was recorded [21]. So, we applied this dose as the high MDZ dose for the treatment of giardiasis in our model. Furthermore, we tested the validity of the dose reduction to the half (60 mg/kg) in order to minimize MDZ side effects mentioned above.

In the present work MDZ-PLGA NPs were successfully prepared using single emulsification method [12]. MDZ-PLGA NPs sizes observed in our study were smaller than that reported by Singh & Jain [23]. Furthermore, in earlier study Periglaucine A and Betulinic acid were loaded on PLGA NPs with 100 - 500 nm average particle size [10] that was comparable to our result. The ideal range for nanoparticles for drug delivery was found to be from 10 nm to 300 nm otherwise will be eliminated from circulation [24]. The zeta potential and surface charge are also important factors that affect the stability and cell adhesion properties of NPs. The greater the absolute zeta potential is the greater the stability of the NPs. In the present work, MDZ-PLGA NPs recorded a negative zeta potential which can be attributed to the carboxyl on free PLGA-NPs that reported previously [25]. The entrapment efficiency (81.68%) was higher

than that reported by Singh & Jain [23] which was approximately (70.30%) and lower than that observed by Mahboob et al. (98.24%) [10].

At the end of the treatment protocol, both the HMD and the LMD were able to reduce the cyst quantity in stool samples and the trophozoite count. The treatment with MTZ as 120 mg/kg twice daily for 5 successive days was also tested by Aly et al. [11] who recorded (93.9 and 94.2%, respectively) and Fahmy et al. [26] who recorded (93.23 and 92.15%, respectively) reduction rates of cysts and trophozoite count, respectively. As infectivity dose of giardiasis may be as low as ten cysts in contaminated food or drinks [27], the MDZ based treatment applied in this study (HMD or LMD) may be associated with recurrence of infection as it wasn't able to completely eliminate the parasite from the intestine.

NPs have recently gained interest in various biomedical fields. NPs containing therapeutic agents have been used for site-targeted drug delivery and optimization of drug treatment effects [28]. PLGA NPs have several advantages over other natural and synthetic polymers; it is biocompatible, it can be used to synthesize nano/micro-particles with good physico-chemical properties [29]. In this study, we attempted to develop LMD-PLGA and HMD-PLGA nanoparticles and to examine their *in vivo* anti-giardial activity and to compare it with the data obtained after the treatment with free high (HMD) and low (LMD) and metronidazole doses at 120 mg/kg and 60 mg/kg, respectively.

The study showed an improvement in anti-giardial activity using the LMD-PLGA formulation at lower concentration (60 mg/kg) when compared to unloaded LMD. The LMD-PLGA treatment was 100% efficient with the complete elimination of the parasite cysts and trophozoites. The sustained release and prolonged circulation of MDZ-PLGA NPs in addition to improved bioavailability and absorption influenced by the small particle size could have been responsible for this observation. This is similar to a report on curcumin PLGA-encapsulated nanoparticles and on Periglucine A and Betulinic acid when encapsulated in PLGA NPs [10] which also observed better drug efficacy at lower concentrations when encapsulated with PLGA NPs. The targeted delivery applied using LMD-PLGA may result in a higher concentration of MDZ in intestine, thus simultaneously reducing the total dose administration. So that, cytotoxicity and the probable carcinogenic effects when high concentrations of MDZ were used [30] can be avoided using the LMD-PLGA.

Histopathological examination revealed that preserved villi, reduced chronic inflammatory infiltrate of the lamina propria and the complete elimination of *G. intestinalis* trophozoites were detected with the LMD-PLGA therapy. Ishak scoring system, a very sensitive system to evaluate liver necroinflammation, was also applied as a very good way to indicate the differences in histological response after applying different forms of therapy and to predict any probable disease progression in liver tissue [17]. Using Ishak, the worst liver condition was observed in (HMD-PLGA) group, this confirmed the hepatotoxic effect of prolonged MDZ high dose that was recorded in earlier studies [31]. On the other hand, the LMD-PLGA administration had a less toxic effect on the liver tissue.

The antibody-mediated clearance of *G. intestinalis* infections is suggested to involve prevention of trophozoite attachment to the host intestinal epithelium and also changes in the trophozoite morphology which causes their killing [32]. Secretory antibodies like IgM and IgA isotypes are attractive nominees for immune defense against *Giardia*, because they are secreted in huge quantities into the intestinal lumen and their actions are antigen-specific. Anti-giardial

IgA is important for controlling and eliminating *Giardia* infection. *Giardia* infection leads to a prolonged elevation specific anti-*giardia* IgG response that lasts for many weeks or months [33].

As quantitative and qualitative serum antibody response against *G. intestinalis* infection could be used to assess success of new chemotherapy [34]. Analysis by ELISA, of both IgA and IgG serum levels in the infected groups after the different forms of MDZ treatments recorded a significant reduction in OD values from infected mice. The 100% cure rate in LMD-PLGA treated group was associated with the maximal reduction of IgA specific antibody response. The reduction level of IgA in LMD-PLGA treated group was not significantly different from that of HMD treated group. This finding suggests an enhanced and more sustained effect of the tested drug candidate (LMD-PLGA) over a longer time even with minimizing the MDZ dose to its half (60 mg/kg). The reduction of the IgA level was possibly linked to a reduction in antigen load as a consequence of parasite elimination from intestine [34]. In the present study, IgA antibody response to *Giardia* remained significantly higher than that in the unexposed controls even after the infection has been cleared. This was also reported in previous studies [34-36].

In this preliminary study IgG antibody level was significantly elevated after the infection. Such elevation of *G. intestinalis* specific serum IgG after infection is reported in several previous studies [37, 38]. Also, *G. intestinalis* specific serum IgG was slightly decreased after the different treatments. Jiménez et al. [34] also suggested that the evaluation of specific IgG levels is not a suitable tool for monitoring the humoral response to infection with *G. intestinalis* after chemotherapy. On the other hand, our result was in disagreement with Priest et al. [39] work which reported that serologic IgG level is correlated better than IgA with *Giardia* infection condition.

The present work suggested the efficacy of the PLGA NPs as a delivery system that could help in the reduction of MDZ dose for the treatment of *G. intestinalis*.

In conclusion PLGA NPs were prepared and effectively loaded with LMD (60 mg/kg) and HMD (120 mg/kg). The anti-*giardial* effect of LMD-PLGA and HMD-PLGA NPs were compared to that of free LMD and HMD. The LMD-PLGA nanoparticles could provide a basis for better drug delivery systems rather than free HMD or even HMD-PLGA NPs to make target-specific drugs and may be developed as a promising anti-*giardial* agent that needs further studies.

SUPPLEMENTARY MATERIALS

Supplementary Table 1

The evaluation of the liver necroinflammation level in the different groups according to Ishak grade

[Click here to view](#)

Supplementary Figure 1

[Click here to view](#)

Supplementary Figure 2

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