Mefloquine loaded niosomes as a promising approach for the treatment of toxoplasmosis

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Abstract

Toxoplasmosis is a disease with a worldwide distribution and significant morbidity and mortality. In search of effective treatment, mefloquine (MQ) was repurposed and loaded with niosomes to treat acute and chronic phases of toxoplasmosis in experimental mice. Mice were orally inoculated with 20 cysts of *Toxoplasma gondii* (ME 49 strain) for the acute phase of infection and 10 cysts for the chronic phase of infection. Infected mice were dosed with MQ solution or MQ-niosomes at 50 mg/kg/day, starting from the second day post-infection (PI) (acute model) or the fifth week PI (chronic model), and this was continued for six consecutive days. The effects of MQ solution and MQ-niosomes were compared with a pyrimethamine/sulfadiazine (PYR/SDZ) dosing combination as mortality rates, brain cyst number, inflammatory score, and immunohistochemical studies that included an estimation of apoptotic cells (TUNEL assays). In the acute infection model, MQ solution and MQ-niosomes significantly reduced the mortality rate from 45% to 25 and 10%, respectively, compared with infected untreated controls, and decreased the number of brain cysts by 51.5% and 66.9%, respectively. In the chronic infection model, cyst reduction reached 80.9% and 12.3% for MQ solution and MQ-niosomes treatments, respectively. MQ-niosomes significantly decreased inflammation induced by acute or chronic *T. gondii* infection. Additionally, immunohistochemical analysis revealed that MQ solution and MQ-niosomes significantly increased the number of TUNEL-positive cells in brain tissue, indicative of induction of apoptosis. Collectively, these results indicate that MQ-niosomes may provide a useful delivery strategy to treat both acute and chronic toxoplasmosis.

Keywords: *Toxoplasma gondii*; Mefloquine; Niosomes; TUNEL; Apoptosis inducer

1. Introduction

Toxoplasma gondii (T. gondii) is a highly infectious parasite. The organism is related to animal origin with cats the definitive host. Ingestion of food contaminated with oocysts is the main route of infection in humans (Lorenzi et al., 2016). Other modes of transmission include the consumption of undercooked meat that harbors tissue cysts or via congenital transmission (Sroka et al., 2010). The infection is widely spread with about 30 to 50% of people worldwide infected with *T. gondii*. This makes it one of the most prevalent infections among humans (Flegr et al., 2014).

T. gondii infection can progress to encephalitis, brain abscesses, and death, especially in immuno-compromised patients (Rostami et al., 2014). Recent investigations have linked male and female infertility with latent toxoplasmosis (Shiadeh et al., 2016) and latent infection may also contribute to neuropsychiatric disorders including schizophrenia, obsessive-compulsive disorder, bipolar disorder, anxiety, and parkinsonism (Sutterland et al., 2015). Additionally, congenital infection of the fetus by *T. gondii* may lead to spontaneous abortion, prematurity, or stillbirth or it can lead to the development of ocular and neurological sequelae like seizures, epilepsy, microcephaly, hydrocephalus, mental retardation, or blindness (Robert-Gangneux and Dardé, 2012).

Currently, the recommended chemotherapy for toxoplasmosis is the anti-folate combination therapy of pyrimethamine and sulfadiazine plus folinic acid. However, the parasite may develop resistance, or these drugs may cause hematologic toxicity, bone marrow suppression, or life-threatening allergic reactions (Antczak et al., 2016). It is noteworthy that chemotherapy for toxoplasmosis is not specific and not effective against both stages of the parasite. It is only able to inhibit replication of the tachyzoites but cannot readily eradicate the bradyzoites which are surrounded by tissue cysts (Kaplan et al., 2009). Therefore, the search for new therapeutic strategies for toxoplasmosis is required, for instance, repurposing medicines used in the therapy of different diseases may be useful in the therapy of toxoplasmosis, such as anti-malarial medicines (Andrews et al., 2014).

Mefloquine (MQ) is a 4-methanolquinoline that is an analogue of quinine. MQ is frequently used in malaria prophylaxis and treatment of chloroquine-resistant *Plasmodium falciparum* (Schlagenhauf, 2010). MQ has proved to possess excellent anti-parasitic activities, especially against apicomplexan parasites such as *Babesia* (Munkhjargal et al., 2012) and *Cryptosporidium parvum* (Aly et al., 2017). Moreover, MQ has shown activity against *Schistosoma* (Keiser and Utzinger, 2012), *Fasciola gigantica* (Shalaby et al., 2016), and *Echinococcus multilocularis* (Rufener et al., 2018), besides its action against some Gram-positive bacteria and multidrug-resistant Mycobacterium tuberculosis (Krieger et al., 2015).

The pharmacodynamic effects of MQ are well recognized, including bradyarrhythmia, gastrointestinal disturbances and various neuropsychiatric side effects (Ridtitid et al., 2005). Pharmacokinetics assessment of MQ reported low plasma concentrations due to its poor oral bioavailability, which presumably reflects pre-systemic metabolism in the

liver (Gutman et al., 2009). Hence, there is the potential for variability of the amount of MQ reaching the systemic circulation for subsequent distribution to the target areas including the brain. This may contribute to the high risk of treatment failure. Accordingly, the development of a new drug delivery system for enhanced oral bioavailability of MQ could augment the plasma concentration even in the absence of food. This could reduce variability and increase oral bioavailability and subsequent distribution to the target tissue.

Vesicular carriers have shown promising results for the enhanced oral bioavailability of drugs. Niosomes have gained interest due to their simplicity, stability, and low cost. Niosomes can be formulated using surfactant and cholesterol with the possibility to incorporate membrane fluidizing material (Ag Seleci et al., 2019). Niosomal-encapsulated praziquantel compared to praziquantel displayed superior efficacy against *Schistosoma mansoni* both *in vivo* and *in vitro* (Zoghroban et al., 2019).

Previous studies analyzed the *in vitro* effect of MQ on *T. gondii* tachyzoites (Holfels et al., 1994; Murata et al., 2017). MQ had no inhibitory effect on intracellular *T. gondii* at the tested dose (Holfels et al., 1994) or it had only a 50% inhibitory effect on *T. gondii* growth (Murata et al., 2017). Therefore, this study aimed to examine the potential efficacy of MQ and MQ-loaded niosomes as a potential treatment of mice experimentally infected with *T. gondii* (ME49 strain).

2. Materials and methods

2.1. Parasite

The cystogenic ME49 strain of *Toxoplasma gondii* was used to induce acute and chronic infections in Swiss albino mice. The Medical Parasitology Department, Faculty of Medicine, Alexandria University, Egypt; provided the organism. Cysts were obtained from Swiss albino mice brain homogenates and the strain was preserved in our laboratory, Tanta University by serial passaging in mice according to a well-established protocol (El-Sayed and Aly, 2014). When inoculated with 10 cysts, a consistent infection occurs without mortality, but when inoculated with 20 cysts, mortality occurs (Djurković-Djaković and Milenković, 2001). In order to account for differences in survival, we used inocula of 20 cysts in order to develop acute infection and 10 cysts in order to establish chronic infection in this study.

Animals

One hundred and eighty laboratory-bred male Swiss albino mice (6-8 weeks old and weighing 20–25 g at the time of infection) were selected for the study. Mice were housed according to institutional and national guidelines. The study design and treatment of mice were approved by the Research Ethics Committee, Faculty of Medicine, Tanta University, Egypt (code number: 32598/09/18).

2.2. Drugs

Mefloquine (MQ), pyrimethamine (PYR) and Sulfadiazine (SDZ) were purchased from Sigma-Aldrich (St. Louis, Mo, USA).

2.2.1. Preparation of mefloquine suspension

Mefloquine (350 mg) was dissolved in 1 mL of dimethyl sulfoxide (DMSO) before dispersion in water to a volume of 70 mL to provide a 5 mg/mL suspension of the drug.

2.2.2. Preparation of pyrimethamine and Sulfadiazine mix

This mixture was prepared to contain 1.25 mg of pyrimethamine and 20 mg of sulfadiazine in each mL of the dispersion. Sulfadiazine (1400 mg) and pyrimethamine (87.5mg) were dispersed in 1 mL of DMSO. This was diluted with water to a volume of 70 mL with the aid of bath sonication.

2.2.3. Preparation of Mefloquine-loaded niosomes (MQ-niosomes)

The niosomal dispersion was prepared according to a published procedure (El Maghraby et al., 2015). Positively charged, gelucire containing niosomes were prepared according to the formulation detailed in Table 1. Briefly, Span 60, cholesterol, gelucire, and stearylamine were heated on a water bath and then ethanol was added. Heating was continued until complete dispersion was accomplished. Mefloquine was added with mixing until solubility was achieved. Water (3 mL) was added with mixing on the water bath until the suspension appeared clear, and it was then mixed away from the water bath to form a proniosomal gel. The proniosomes were diluted with water to form niosomes with shaking. Complete hydration was achieved by storing the niosomes overnight at ambient temperature. The hydrated niosomes were then bath sonicated for 30 minutes.

2.3. Experimental design:

Mice were divided into three experimental groups:

Group I: The control untreated group was comprised of 60 mice equally divided into three subgroups. Subgroup Ia: Non-infected mice. Subgroup Ib: Mice infected by oral administration of 20 cysts/mouse (a control for acute infection). Subgroup Ic: Mice infected with 10 cysts/mouse (a control for chronic infection).

Group II: The acute-treatment group (60 mice), infected with 20 cysts/mouse, was equally divided into three subgroups. Subgroup IIa: Infected, and then treated with mefloquine at a dose of 50 mg/kg/day. Subgroup IIb: Infected, and then treated with mefloquine-loaded niosomes at a dose of 50 mg/kg/day (Müller et al., 2016). Subgroup IIc: Infected, and then treated with pyrimethamine and sulfadiazine at a dose of 12.5 and 200 mg/kg/day, respectively (Romand et al., 1993). Treatment was initiated on the second day post-infection (PI) and continued for six consecutive days. Mice were observed for 30 days after the end of the treatment regimen, and then sacrificed (Djurković-Djaković et al., 2002; Doggett et al., 2012).

Group III: The chronic-treatment group (60 mice), infected with 10 cysts/mouse, was equally divided into three subgroups; Subgroups IIIa- IIIc, which followed the same infection and treatment conditions as Group II. However, treatment was initiated five weeks PI and continued for six consecutive days. Mice were observed for 14 days after the end of treatment, and then sacrificed (Djurković-Djaković et al., 2002; Doggett et al., 2012). The effect of MQ and MQ-niosomes on *T. gondii* ME49 strain was assessed as follows:

2.4. Estimation of mortality rate (MR)

MR was calculated using the following equation

MR = Number of dead mice at the time of sacrifice/number of mice at the beginning of experiment × 100.

The MR was calculated for each of the acute experimental subgroups of mice and compared to their corresponding controls, at the time of sacrifice (El-zawawy et al., 2015).

2.5. Parasite burden

Tissue parasitism was assessed within the brain by histopathological and immunohistochemical studies.

2.5.1. Histopathology

Three histological sagittal brain sections from each mouse, from five randomly selected mice per group, were cut and dehydrated in alcohol with increasing concentrations (from 70% to 100%). The sections were embedded in paraffin before sections of 4 µm thickness were cut, deparaffinized, rehydrated and then stained with hematoxylin-eosin. All of the slides were analyzed, and the total number of cysts was counted using a 40x objective lens according to a published method (Silva et al., 2010).

2.5.2. Immunohistochemistry

Deparaffinized sagittal brain sections were placed in a Coplin jar containing immunoDNA retriever with EDTA and placed on a trivet in a pressure cooker for 15 minutes, before maintaining at an ambient temperature for a further 15 minutes. Next, the sections were bathed in a solution of 5% hydrogen peroxide for 5 minutes at ambient temperature to block non-specific staining (endogenous peroxidase). The samples were then incubated with primary rabbit polyclonal anti-*T. gondii* antibody (BSB 6043, Bio SB, USA) at a 1:2000 dilution with 1% bovine serum albumin (BSA) in tris-buffered saline with tween 20 (TBST) in a moist chamber at 4°C for one hour. The samples were then incubated with a goat anti-rabbit secondary antibody (HRP) diluted in the same buffer in a moist chamber at 4°C for 15 minutes. To visualize the localization of immune complexes, 300 µL of diluted 3,3′ -diaminobenzidine tetrahydrochloride (DAB) solution (chromogen to substrate ratio of 1:40) was added to each sample and incubated for 5-10 minutes at ambient temperature. Finally, the slides were counterstained with Harris hematoxylin for one minute, dehydrated in alcohol solutions with increasing concentrations (70%, 80%, 90%, and absolute), cleared in xylene, and mounted with Canada balsam on

coverslips. The slides were examined with an Olympus microscope (LEICA DFC 290 HD Image Analysis System) and the images were captured using a digital camera. Three slides were prepared for each mouse and the average cyst count was calculated (Bezerra et al., 2011).

2.6. Inflammatory scoring

The total numbers of focal or diffuse inflammatory foci and the cuffing of blood vessels were analyzed in the stained sagittal brain sections of infected mice (hematoxylin and eosin stained). The analysis was performed in a blinded manner by two independent researchers using a 40x objective lens. The inflammatory score was graded in arbitrary units: 0-2 (mild), >2-4 (moderate), >4-6 (severe), and more than 6 (very severe) as described by El-Kowrany et al. (2019).

2.7. Detection of apoptotic cells (TUNEL assay) in brain tissue by immunohistochemistry

The fragmented DNA of apoptotic cells was analyzed by calculating the mean number of TUNEL positive (+) cells in high-power fields (HPFs) of brain tissue sections. Therefore, five randomly chosen HPFs from each section were examined at a ×400 magnification. The counting of TUNEL+ cells was achieved via computer-driven Leica Q 500 image analysis software (LICA Microsystem Corporation, England, UK). This system employs a Java-based image processing system. According to a well-established TUNEL assay protocol for light microscopy examination (Hewitson et al., 2006), deparaffinized rehydrated tissue sections were initially covered with a proteinase K solution and incubated for 15 minutes at ambient temperature. Following proteinase K treatment, endogenous peroxidases were blocked with 2% hydrogen peroxide for 5 minutes at ambient temperature. Next, the sections were covered with Terminal deoxynucleotide Transferase (TdT) reaction buffer and carefully covered with a glass coverslip and incubated in a humidified chamber for 30 minutes at 37°C. Nonspecific binding was then blocked by covering the tissue sections with 2% BSA (bovine serum albumin) solution for 30–60 minutes at ambient temperature. After that, the slides were incubated in Vectastain ABC-peroxidase solution (Vector Laboratories, UK) for one hour at 37°C. Finally, the tissue sections were stained with Diaminobenzidine (DAB) staining solution (Vector Laboratories, UK) at ambient temperature for 20 minutes. Subsequently, they were lightly counter-stained with hematoxylin (Sigma-Aldrich, St. Louis, MO, USA) and coverslips were mounted (Polysciences, Inc., Warrington, PA, USA).

2.8. Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences (IBM SPSS) version 20.0. (Armonk, NY: IBM Corp, USA). Qualitative data were described as numbers and percent. Quantitative data were presented as mean and standard deviation (SD). The Kolmogorov-Smirnov test was used to verify the normality of the distribution of variables. Parametric tests were applied for normally distributed data, such as an ANOVA for comparing more than two means, followed by post hoc Tukey's test for pairwise comparisons. Non-parametric tests were used for non-normally distributed

data, such as the Kruskal Wallis test to compare different groups, followed by Dunn's post hoc test for pairwise comparisons. $P < 0.05$ was adopted as the level of significance.

3. Results

3.1. Efficacy of MQ on acute toxoplasmosis

The effects of a six-day course of treatment with MQ and MQ-niosomes initiated day 2 post-infection were monitored by 37 days survival, brain cyst burdens, inflammatory score and TUNEL assay by the end of the observation period.

3.1.1. Mortality rate (MR)

Figure (1) shows the survival rate expressed as cumulative survival percentage as a function of time. The normal uninfected group showed only 5% death throughout the study period with only one mouse dying 17 days post-infection. In the infected control subgroup (Ib), death of mice started at 9 days post-infection with the death of 10% of the mice. This mortality advanced progressively to reach 45% death at 33 days post-infection. Treatment with MQ solution improved survival with a cumulative mortality of 25% for the study period. Treatment with MQ-niosomes (subgroup IIb) provided the greatest protection with only two mice dying (10% MR). This reduction of MR was statistically significant compared with the infected control group or that treated with the MQ solution ($P < 0.05$). Administration of the standardized therapy (PYR/SDZ) produced mortality of 15% for the study period which was significantly lower than the infected untreated group or that treated with MQ solution but was similar to that for mice treated with MQ-niosomes ($P < 0.05$) (Fig. 1).

3.1.2. Parasite burden

A cyst count was undertaken after staining the brain sections with H&E, in addition to immunohistochemical staining (Fig. 2). The recorded cyst count data for the sagittal brain sections is shown in Table (2). The infected control subgroup (Ib) had a mean of 2.60 ± 0.70 cysts per sagittal brain section. MQ and MQ-niosomes treated subgroups showed a significant reduction in the number of brain cysts with the magnitude of reduction being 51.5% and 66.9%, respectively, compared to the infected control subgroup (Ib) ($P=0.001$). Interestingly, the efficacy of MQ solution (IIb) was similar to that of the PYR/SDZ treatment (IIc) compared to the infected control (Ib) ($P=0.001$), and there were no significant differences between either the MQ-treated subgroups (IIa and IIb) or when the MQ-treated subgroups were compared with the PYR/SDZ subgroup (IIc) (Table 2, Fig. 2a-c).

3.1.3. Inflammatory score

Figure (3) shows the inflammatory regions within the brain tissue. In general, meningitis and encephalitis were the prominent brain lesions in the infected mice and were characterized by glial nodules, vascular cuffing by lymphocytes and mononuclear cell infiltrates. The inflammatory brain lesions from the infected control subgroup were severe and more prominent than those from the mice of the treated subgroups ($P= 0.001$). Broad areas of parenchymal necrosis and focal

mononucleated cell infiltrations were observed in the control group (Fig. 3a,b), giving a mean inflammatory score of 2.40 ± 0.6. The MQ solution treated subgroup resulted in less pronounced inflammation and fewer infiltrating mononuclear cells, with a mean inflammatory score of 1.26 ± 0.25 (P= 0.001) (Fig. 3c). The MQ-niosomes and PYR/SDZ treatments were even more effective at reducing inflammation, with a mean inflammatory score of 1.13 ± 0.17 for both cases (P = 0.001) (Fig. 3d). Hence, no significant differences were observed between the PYR/SDZ-treated and MQ-treated subgroups or between the two MQ-treated subgroups (Table 3).

3.1.4. Detection of apoptotic cells (TUNEL assay) in brain tissue by immunohistochemistry

Figure (4) shows DNA strand breaks of apoptotic cells in brain sections. Table (4) provides the average number of TUNEL+ cells within viewing fields (under high power × 400) of brain sections of treated and untreated groups. Cells undergoing apoptosis were not observed or were very scarce in brain tissue from the uninfected control mice (Fig. 4a, Table 4). In the infected control subgroups, the fragmented DNA of apoptotic cells was observed with varying intensities throughout the study period. The infected untreated control subgroup (Ib) had an increased mean number of TUNEL+ cells/HPF compared with the uninfected group. Both MQ-treated subgroups (IIa and IIb) showed significantly higher DNA fragmentation ($P < 0.05$) compared with the controls (Table 4, Fig. 4c,d). However, a significantly lower mean number of TUNEL+ cells/HPF was observed in the PYR/SDZ treated subgroup (IIc) compared to both MQ-treated subgroups (IIa and IIb) (P= 0.001). In addition, treatment with MQ-niosomes resulted in a significantly higher number of TUNEL+ cells/HPF compared with the MQ solution-treated group (P= 0.001, Table 4).

3.2. Efficacy of MQ on chronic toxoplasmosis

The effects of MQ and MQ-niosomes, 2 weeks after the end of a six-day course of treatment, were monitored by brain cyst burdens, inflammatory score and TUNEL assay.

3.2.1. Parasite burden

Brain tissue parasitism was assessed after staining the brain sections with H&E in addition to immunohistochemical staining (Fig. 2). Table (2) details the recorded cyst count data for the sagittal brain sections. Treatment with MQ solution reduced the number of brain cysts by 12.3% only compared to the infected control subgroup (Ic). MQ-niosomes and PYR/SDZ significantly reduced the number of brain cysts in subgroups IIIb and IIIc by 80.9% and 76.7%, respectively compared to the infected control subgroup (Ic) ($P = 0.001$). The efficacy of MQ-niosomes and PYR/SDZ was significantly higher than MQ solution (IIIa) ($P = 0.001$) (Table 2, Fig. 2d-f).

3.2.2. Inflammatory score

Figure (3) shows the inflammatory regions within the brain. The inflammatory lesions in the CNS from the infected control subgroup were severe and more pronounced than those of the mice from the treated subgroups (Fig. 3e,f)

and generated a mean inflammatory score of 4.40 ± 0.25. Treatment with MQ solution reduced the inflammatory score by 22.7% compared to the infected untreated subgroup ($P < 0.005$). MQ-niosomes and PYR/SDZ significantly reduced inflammation (Fig. 3g,h) (subgroups IIIb and IIIc) by 76.1% and 72.7%, respectively, compared to the infected control subgroup (Ic) ($P = 0.001$). Both MQ-niosomes and PYR/SDZ were significantly more effective in the reduction of inflammatory scores compared with the MQ solution ($P = 0.001$) (Table 3).

3.2.3. Detection of apoptotic cells (TUNEL assay) in brain tissue by immunohistochemistry

The fragmented DNA of apoptotic cells in chronic infection showed a similar pattern to that recorded in the acute infection with the treatment protocol showing the same efficacy as in the case of acute infection (Table 4).

4. Discussion

This study employed the *Toxoplasma* ME49/mouse model which is a mouse non-virulent (type-2) parasite strain in the assessment of the efficacy of MQ solution and MQ-loaded niosomes. Using either an acute or chronic model, the infected untreated mice showed signs and symptoms related to the disease confirming successful infection. The recorded changes in these infected mice comply with the published data employing the same strain (Djurković-Djaković et al., 2002; Vidadala et al., 2016).

MQ solution, which was given day 2 post-infection, and coincides with the acute phase of infection, significantly reduced the mortality rates of mice compared to the infected controls (Ib). Regarding the parasite burden, the efficacy of MQ solution resulted in a significant reduction in the number of brain cysts compared with the infected untreated control. MQ solution resulted in less pronounced inflammation and fewer infiltrating mononuclear cells. This reduction was anticipated due to the effective decline in brain tissue parasitism and cyst count (EL-Kowrany et al., 2019). Administration of MQ solution through the chronic phase of toxoplasmosis (five weeks PI) also resulted in a significant reduction in the parasitic load in the brain compared to the infected untreated group but the magnitude of reduction was lower than that shown in the case of the acute therapy group. This discrepancy between the effect of MQ against acute and chronic phases of infection may be explained by being more effective towards tachyzoites that are metabolically active than against the mature dormant bradyzoites which are bounded by a cyst wall, protecting them from the toxic action of the drug (Djurković-Djaković et al., 2002). Moreover, a similar effect has been documented for atovaquone, azithromycin, and spiramycin (Costa et al., 2009; Chew et al., 2012).

The results of previously published *in vitro* studies using MQ were different from the present *in vivo* study since MQ either had no inhibitory effect or only displayed a 50% inhibitory effect on tachyzoites of type I *T. gondii* (RH strain), at the tested doses (Holfels et al., 1994; Murata et al., 2017). The inconsistency between these *in vivo* and *in vitro* studies might be attributed to the use of a different strain (RH, a type I strain) by those authors which is a highly virulent strain in

mice. Furthermore, the *in vitro* investigations employed a different life cycle stage (tachyzoites) which dominates during the acute stage of infection. Other factors also influence the effects of the drug on the host, including drug metabolism and the host's immune response. In keeping with this assumption, Yamashita et al. (1998) demonstrated that the apoptosis in cytotoxic T-lymphocytes decreased *Toxoplasma gondii* parasitic count *in vivo*, but this was not observed *in vitro*, because parasite engulfment by phagocytic cells led to the elimination of parasites containing apoptotic bodies.

Other derivatives of quinolones have shown very promising results against infections caused by *T. gondii*, in both acute and chronic phases. They reduced the overall parasitic burden and could cross the blood-brain barrier, promoting the disintegration and reduction of *T. gondii* cysts containing bradyzoites in mice (Elgawad et al., 2019).

From the present study results, we propose that the mechanism of action of MQ is through induction of apoptosis, in line with previous *in vitro* experiments that have shown that MQ induces apoptotic cell death in *Plasmodium* (Gunjan et al., 2016). Moreover, other recent studies showed that mefloquine induced apoptosis in colorectal cancer (CRC) cells both *in vitro* and *in vivo* (Xu et al., 2018). Therefore, we performed an apoptotic assay; *in situ* DNA fragmentation (TUNEL assay) to evaluate the apoptotic cell death in MQ treated and untreated parasites. This *in situ* DNA fragmentation assay is considered one of the criteria of apoptosis-like cell death in protozoa (Jimenez-Ruiz et al., 2010). Thereby, the TUNEL (Terminal dUTP Nick End-Labeling) method is used to assay the endonuclease cleavage products by enzymatically end-labeling the DNA strand breaks. Terminal deoxynucleotide transferase (TdT) is used to add labeled uridine-5′-triphosphate (UTP) to the 3′-end of the DNA fragments. After that, dUTP can be labeled with a variety of probes to allow its detection by light microscopy as well as fluorescence microscopy or via flow cytometry (Elmore, 2007).

In all infected subgroups, DNA strand breaks were observed in the brain tissue with differing intensities. Quantitation of the fragmented DNA of apoptotic cells revealed a higher significant number in the MQ-treated subgroup. Further research has supported the role of apoptotic cell death in the treatment of experimental toxoplasmosis by using different drugs such as clindamycin, staurosporine, miltefosine, hydrogen peroxide, camptothecin, and artemether (Nyoman and Luder, 2013; Mikaeiloo et al., 2016). Collectively, the data indicate that *T. gondii* has the capacity to undergo apoptosis and these can be activated by chemotherapeutic agents.

The standard therapeutic protocol (PYR/SDZ) was employed as a positive control in this study. Its administration during the acute phase of infection produced an efficacy similar to that of treatment with the MQ solution with respect to a reduction in the number of brain cysts, but PYR/SDZ was more effective at reducing the inflammation caused by *T. gondii* infection. The standard therapy showed superior efficacy in all measured parameters for the chronic treatment. However, the efficacy of PYR/SDZ was not related to its apoptotic effect as reflected in the low mean number of TUNEL+ cells/HPF. The proposed mechanism of action of the PYR/SDZ combination reflects synergistic blocking of folic acid

biosynthesis through the sequential inhibition of parasite dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) (Maenz et al., 2014).

MQ-niosomes showed a better therapeutic response than the MQ solution. This was evident both in the acute and chronic stages. This superior response could reflect two factors: firstly, the colloidal nature of the niosomes could enhance the MQ bioavailability or its distribution to the mice tissues (Olivera et al., 2013), and secondly, the relatively high ability of the niosomes to provide a transport medium for lipophilic drugs, such as MQ (Mourao et al., 2005). Similarly, El-Mansory et al. (2019) found that niosomal-PYR significantly increased the survival rate of mice when compared with PYR alone. Additionally, these results agree with that documented in previous studies which evidenced that the use of nanosystems enhanced the efficacy of anti-toxoplasmic drugs. For instance, nano-emulsified atovaquone offers useful anti-parasitic activity at reduced doses for *T. gondii*, *in vivo* and *in vitro*, due to improved oral bioavailability and tissue distribution (Azami et al., 2018). Furthermore, the loading of spiramycin on chitosan nanoparticles improves its therapeutic effects in experimental acute and chronic toxoplasmosis (Etewa et al., 2018).

As support to the hypothesis that MQ is an efficient apoptotic inducer, the results of the present work showed that MQ-niosomes were superior to MQ solution, such that there were a relatively higher number of DNA strand breaks in brain tissues of MQ-niosomes treated mice. This was associated with lower parasitism, similar to the effects of resveratrol, which can successfully eliminate intracellular tachyzoites *in vitro*, by facilitating apoptosis (Chen et al., 2019). As the death of the infected cell is usually associated with the death of the infecting agent, self-destruction can stimulate efficient pathogen clearance. Many studies directly addressing the role of apoptosis in murine toxoplasmosis have revealed that inhibition of host cell apoptosis may have evolved to enable the parasite to survive and sustain infection (Besteiro, 2015). Taking into consideration the fact that *T. gondii* can stimulate an anti-apoptotic effect, drugs that induce apoptosis will be beneficial for the eradication of *T. gondii* infection. Hence, the elevation of apoptosis after administration of MQ-niosomes reflect its superiority over the MQ solution (Kim et al., 2006).

In conclusion, this study examined the capacity of niosomes to act as potential carriers for enhanced oral delivery of MQ for the treatment of *T. gondii* infection. The delivery system was efficient both for the treatment of acute and chronic phases of infection. The mechanism of action of MQ is, at least in part, related to an induction of apoptosis, with the niosomal formulation augmenting such a pathway. The efficacy of niosomal MQ was comparable to the standard therapeutic combination of PYR/SDZ. Further research is recommended to assess the potential use of MQ-niosomes for treating congenital *T. gondii* infection and the possibility of the drug becoming a valuable alternative to PYR and SDZ. In addition, further clinical studies are needed to investigate the effect of MQ on infected pregnant women and its role in chemoprophylaxis against recurrent toxoplasmosis. Furthermore, *in vivo* and *in vitro* studies should be conducted to

investigate the efficacy of MQ against *T. gondii* on a wider scale, to precisely characterize its action as anti-*T. gondii* drug and what the requirements will be for a standardized human dose.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest with the production or publication of this manuscript.

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Legends of figures

Fig. 1 Kaplan Meier survival curves of different subgroups of acute infections throughout the study period (37 days). Ia: non-infected mice, Ib: infected with 20 cysts/mouse and non-treated, IIa: infected and treated with MQ at a dose of 50 mg/kg/day, IIb: infected and treated with MQ-niosomes at a dose of 50 mg/kg/day, IIc: infected and treated with PYR/SDZ at a dose of 12.5 and 200 mg/kg/day, respectively.

Fig. 2 a) A section of brain tissue stained with H&E showing *T. gondii* cyst (arrow) in infected control mice (Ib), infected with 20 cysts/mouse, at day 37 PI (× 400), b) A brain tissue section stained with H&E showing a *T. gondii* cyst (arrow) in infected control mice (Ib) at day 37 PI (× 1000), c) A section of brain tissue stained with immunoperoxidase (immunohistochemistry) method showing a *T. gondii* cyst (arrow) in infected control mice (Ib) at day 37 PI (× 400), d) A section of brain tissue stained with H&E showing a *T. gondii* cyst (arrow) in infected control mice (Ic), infected with 10 cysts/mouse, at day 56 PI (× 1000), e) A section of brain tissue stained with immunoperoxidase method showing a *T. gondii* cyst (arrow) in infected control mice (Ic) at day 56 PI (× 1000), f) A section of brain tissue stained with immunoperoxidase method showing *T. gondii* cysts (arrows) in control mice of the infected control subgroup (Ic) at day 56 PI (× 1000).

Fig. 3 Sections of brain tissue stained with H&E showing inflammation score of the three experimental groups a) large area of necrosis surrounded by palisading mononuclear cell infiltrate (arrow) in infected control mice, infected with 20 cysts/mouse (Ib) at day 37 PI (× 100), b) severe inflammation in the meninges and the underlying brain tissue with

mononuclear cell infiltration (arrows) in infected control mice, infected with 20 cysts/mouse (Ib) at day 37 PI (× 400), c) mild to moderate encephalitis with dilated vessels containing inflammatory cells (arrows) in mice of the MQ-treated subgroup (IIa), at a dose of 50 mg/kg/day at day 37 PI (× 400), d) mild meningitis and encephalitis in mice of the MQ-niosomes treated subgroup (IIb) at a dose of 50 mg/kg/day at day 37 PI (× 400), e) severe vascular cuffing by mononuclear cells (arrow) in infected control mice (Ic), infected with 10 cysts/mouse at day 56 PI (× 400), f) severe encephalitis with mononuclear cell infiltration (arrows) in infected control mice (Ic) at day 56 PI (× 400), g) moderate encephalitis with perivascular cuffing (arrows) in mice the PYR/SDZ-treated subgroup (IIIc), at a dose of 12.5 and 200 mg/kg/day, respectively at day 56 PI (× 400), h) mild meningitis and encephalitis with vascular congestion, perivascular edema and scattered areas of gliosis in mice of the MQ-niosomes treated subgroup (IIIb), at a dose of 50 mg/kg/day at day 56 PI (× 400).

Fig. 4 Section of brain tissue of mice stained by immunoperoxidase method and counted by computerized image analysis system of the acute-treatment group, infected with 20 cysts/mouse on day 37 PI (× 400) showing a) one TUNEL + cells /HPF from the uninfected control subgroup (Ia), b) six TUNEL + cells /HPF from the infected control subgroup (Ib), c) 45 TUNEL + cells/ HPF from the MQ-treated subgroup at a dose of 50 mg/kg/day (IIa), d) 73 TUNEL + cells/ HPF from the MQ-niosomes treated subgroup at a dose of 50 mg/kg/day (IIb), e) 17 TUNEL + cells/ HPF from the PYR/ SDZ-treated subgroup at a dose of 12.5 and 200 mg/kg/day, respectively (IIc).