The therapeutic efficacy of curcumin nanoemulsion versus Spiramycin in Toxoplasma gondii (ME49 strain) chronically infected mice

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ABSTRACT

Background: Previously, we showed that curcumin (CUR) nanoemulsion exhibited a promising prophylactic effect on acute toxoplasmosis, and decreased parasite burden. Scanning electron microscopy (SEM) of the peritoneal exudates showed deformed tachyzoites in both prophylactic and treated subgroups.

Objective: The present study is designed to evaluate the therapeutic effect of CUR nanoemulsion compared to that of Spiramycin on T. gondii type II, ME49 strain causing chronic toxoplasmosis in experimentally infected mice.

Material and Methods: This case-control experimental study included 30 Swiss albino mice, divided into three equal groups. All mice were infected with avirulent ME49 strain to induce chronic toxoplasmosis. The study included group I (infected non-treated), II (infected treated with CUR nanoemulsion), and III (infected and treated with Spiramycin). The assessment parameters included estimation of the mortality rate, and parasite burden (cyst number and size) in livers and spleens impression smears, and in brains homogenates.

Results: The mortality rate was 40% in the infected non-treated group with no mortality in all treated mice. There was a significant decrease of cyst number and size in livers, spleens, and brains of both treated groups as compared to the infected non-treated mice.

Conclusion: It was concluded that CUR nanoemulsion had a promising therapeutic effect on chronic toxoplasmosis.

Keywords: chronic toxoplasmosis; curcumin; experimental study; nanoemulsion; Spiramycin.

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INTRODUCTION

Toxoplasmosis is a global zoonotic disease, and one-third of the human population is reportedly chronically infected by T. gondii[1]. From the United States, an estimated 11% of the population, 6 years and older, were at risk for acquiring toxoplasmosis[2]. Recently, in a case-control study conducted at Zagazig University Pediatric Hospital, Egypt for estimating Toxoplasma IgG and IgM seroprevalence in 67 children on regular hemodialysis and 50 healthy controls, the total seropositivity was 23%. While anti-Toxoplasma IgM antibodies were not detected, IgG seroprevalence was 16% and 28% in control and hemodialysis groups, respectively with insignificant statistical differences. Significant risk factors included contact with cats or soil, ingestion of semi-cooked meat and blood transfusion[3].

A significant fraction of toxoplasmosis cases is caused by reactivation of existing chronic infections, and T. gondii can persist and encyst chronically in the brain leading to a broad spectrum of neurological complications[4]. In reactivated toxoplasmosis, excysted bradyzoites transform to tachyzoites and proliferate uncontrollably in the brain causing widespread inflammation and critical morbidity. Reactivated toxoplasmosis develops in immunosuppressed patients, such as those afflicted by human immunodeficiency virus (HIV)/AIDS, and solid organ transplant recipients. Reactivation of T. gondii brain cysts often manifests as toxoplasmic encephalitis[5].

Since all currently available therapeutic drugs are only effective against tachyzoites with poor efficacy against tissue cysts, there is much controversy in treatment of chronic toxoplasmosis. As previously reviewed[6], drug resistance developed against several anti-Toxoplasma drugs due to gene mutations. Recent evolutionary technology enabled investigators to understand T. gondii biology system and uncover new
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drug targets. At least 50 promising anti-cyst candidates were identified through screening of compound libraries, target-based drug design, or repurposing of Food and Drug Administration (FDA)-approved drugs[15]. Deficiency of suitable effective drugs that safely eliminate brain cysts encouraged researchers to investigate natural products. Singaporean reviewers tabulated all nutritional products extracted from food plants, phytochemicals, vitamins, and minerals that were evaluated in the last two decades. The reviewers observed that the majority of these nutritional products are promising toxoplasmodial agents without identification of a specific drug target. They also discussed different nanotechnology strategies to increase natural products bioavailability and biosolubility. Due to lack of evidence supporting successful human clinical trials, the reviewers recommended further large-scale clinical trials to confirm the importance of nutritional therapy in prevention and/or treatment of chronic toxoplasmosis by determining the mechanism of action against bradyzoite biology and brain cyst development[19].

Due to its porous structure with high surface area, metal-organic frameworks (MOFs) were utilized as a drug delivery system. An Egyptian study investigated the therapeutic efficacy of CUR@MOFs nanocomposite in treatment of chronic toxoplasmosis in experimentally infected mice with ME49 strain. Parameters evaluated were brain cyst count and histopathological examination of tissues obtained from liver, spleen, and lung. It was concluded that the new nanocomposite significantly decreased the number of brains cysts, and ameliorated the histopathological changes with preserved parenchyma, and stromal tissues in the examined organs[8]. Therefore, the present study aimed to assess the therapeutic effects of CUR nanoemulsion against experimental chronic toxoplasmosis as compared to specific therapeutic medication with Spiramycin.

MATERIAL AND METHODS

This case-control study was conducted at Medical Parasitology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt, during the period from August, 2018 to January, 2019.

Study design: The study included three groups of mice, infected non-treated (GI), infected treated with CUR nanoemulsion (GII), and infected treated with Spiramycin (GIII). All mice were sacrificed at the end of the 9th w post infection (PI) for assessment of mortality rate, parasite burden in livers and spleens impression smears as well as in brains homogenates.

Experimental animals: The study included thirty female Swiss albino pathogen-free mice (Mus musculus domesticus), 6-8 weeks of age and weighing 20-25 g. Mice were purchased from animal house, Faculty of Medicine, Suez Canal University. They were housed under controlled temperature and light conditions, and provided with water and commercial chow ad libitum.

Parasites: T. gondii avirulent strain (ME49) was obtained from the Zoonotic Disease Department, National Research Center, Giza, Egypt. It was maintained by serial oral inoculation of mice every 8 weeks with 0.1 ml of brain suspension containing 20 cysts of previously infected mice[9].

Drugs: Spiramycin and CUR were purchased from Sigma-Aldrich, Germany. Preparation, dose and methods of administration were previously described[10].

Preparation and characterization of CUR nanoemulsion: Previously described[8].

Mice infection: A brain of previously infected mouse was homogenized in one ml saline (0.85% NaCl). Each mouse received orally 0.1 ml that contained 20 cysts[11].

Pilot study: As previously described[10], a pilot study was conducted to determine the least effective dose of CUR nanoemulsion, and a dose of 20 mg/kg/d was established as the lowest effective dose.

Mice grouping: Table (1) describes the study groups.

Mortality rate (MR): This was calculated according to the following equation; MR= (number of dead mice at the sacrifice time)/(number of mice at the beginning of the experiment) X 100[12].

Cyst counts and sizes in livers and spleens: Impression smears from the cut surface of livers and spleens were performed. Liver and spleen of each mouse was cut into two halves exposing a fresh surface that was lightly blotted using a paper towel and then pressed to a clean glass slide once. The impression smears were stained with Giemsa stain and examined under oil immersion lens[13]. Cyst number was counted

| Table 1. Study groups. |
|------------------------|--------------------------|------------------------|
| **Group I** | **Group II** | **Group III** |
| **No.** | **Name** | **Characteristics** |
| 10 | Control | Infected, non-treated. |
| 10 | CUR nanoemulsion | Infected and treated with CUR nanoemulsion 20 mg/kg/d orally once daily for 10 d after 6 w PI[14]. |
| 10 | Spiramycin | Infected and treated with Spiramycin 100 mg/kg once daily for 10 d after 6 w PI[14]. |
in 10 high power fields (HPF) for each mouse, and the mean number of cysts in each group was determined. Cyst size was measured by calibrated ocular micrometer, and the mean cyst size was calculated. Reduction percentage (R%) in cyst count and size was calculated according to the following equation: R% = [(C-E)/C] X 100; where C = mean cyst count or size in the control group (GI), and E = mean cyst count or size in each treated group.

Cyst counts and sizes in brains homogenates: Smears were prepared from a half ml saline homogenate of one half of the brain of each mouse. Air dried smears were stained with Giemsa stain and examined under oil immersion lens. Parasite burden was evaluated microscopically by counting the cysts in 10 HPFs of the brain homogenate smear of each mouse. Similarly, the mean cyst counts and sizes and R% were calculated as previously conducted in livers and spleens impression smears.

Statistical analysis: Results were recorded, tabulated, statistically analyzed by statistical package SPSS. Descriptive statistics including mean±standard deviation (SD) and analytical statistics using ANOVA (F test). Statistical significance was considered at \( P<0.05 \).

Ethical consideration: The study was approved by the ethical committee of Suez Canal University. Animal experiments were carried out according to the National Research Council’s Guide for the Care and Use of Laboratory Animals.

RESULTS

Mortality rate: The highest rate was observed in infected non-treated group (I), that had 40% mortality rate at the end of the 9th week PI whereas zero mortality rates were observed in CUR nanoemulsion and Spiramycin treated groups (II and III, respectively) with statistical significance (\( P<0.05 \)).

Cyst count and size in liver impression smears: The mean cyst count in infected non-treated group (GI) was 20.33±1.53. Comparatively, mean cyst counts of 6.67±1.53 and 6.0±1.0 were reduced due to treatment with CUR nanoemulsion (GII) and Spiramycin (GIII) groups, respectively. Percentage of reduction in both treated groups was consequently calculated as 60.67%, and 60.21%, respectively (Table 2). Mean size of cysts in infected non-treated GI was 26.67±8.51 µ, while mean cyst sizes in GII and GIII were reduced to 8.0±4.58 µ and 8.0±3.61 µ, respectively. Both treated groups had the same percentage of reduction (70%). There were statistically significant (\( P<0.01 \)) decreases in cyst counts and sizes in both treated groups compared to infected non-treated mice (Table 2).

Table 2. The effect of CUR nanoemulsion and Spiramycin treatments on mean cysts count and sizes in livers impression smears (/10HPFs), and R% in \( T. gondii \) ME49 strain infected groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cyst counts</th>
<th>Statistical analysis</th>
<th>Cyst sizes</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Reduction%</td>
<td>F test (P value)</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Infected non-treated (I)</td>
<td>20.33±1.53</td>
<td>--</td>
<td>--</td>
<td>26.67±8.51</td>
</tr>
<tr>
<td>CUR treated (II)</td>
<td>6.67±1.53</td>
<td>60.67</td>
<td>90.17 (&lt;0.01)</td>
<td>8.0±4.58</td>
</tr>
<tr>
<td>Spiramycin treated (III)</td>
<td>8.0±1.0</td>
<td>60.21</td>
<td></td>
<td>8.0±3.61</td>
</tr>
</tbody>
</table>

*: Significant (\( P<0.05 \)).

Cyst counts and sizes in spleens: The mean cyst count in infected non-treated GI was 24.33±2.08. The highest reduction of 75.34% occurred in CUR nanoemulsion treated GI with a mean cyst count of 6.0±1.0. The 73.93% reduction in Spiramycin treated GII was equivalent to a mean cyst count of 6.33±1.53 (Table 3). Mean size of cysts in infected non-treated GI was 21.2±8.1 µ, while in CUR nanoemulsion (GII) and Spiramycin (GIII) treated groups the sizes were reduced to 8.38±1.4 µ and 8.17±2.0 µ, respectively. The percentage reduction of 60.47%, and 61.46% were respectively similar in both treated groups. Significant differences (\( P>0.05 \)) were recorded for both treated groups as compared to the non-treated GI regarding cyst counts and sizes (Table 3).

Table 3. The effect of CUR nanoemulsion and Spiramycin treatments on mean cyst counts and sizes in spleens impression smears (/10HPFs), and R% in \( T. gondii \) ME49 strain infected groups.

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<td>Mean ± SD</td>
<td>Reduction%</td>
<td>F test (P value)</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Infected non-treated (I)</td>
<td>24.33±2.08</td>
<td>--</td>
<td>--</td>
<td>21.2±8.1</td>
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<tr>
<td>CUR treated (II)</td>
<td>6.0±1.0</td>
<td>75.34</td>
<td>129.17 (&lt;0.01)</td>
<td>8.38±1.4</td>
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<tr>
<td>Spiramycin treated (III)</td>
<td>6.33±1.53</td>
<td>73.93</td>
<td></td>
<td>8.17±2.0</td>
</tr>
</tbody>
</table>

*: Significant (\( P<0.05 \)).
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Cyst counts and sizes in brains homogenates:
The mean cyst count in infected non-treated GI was 17.33±2.52. The percentage reduction in CUR nanoemulsion (GII) treated was apparently higher at 73.08% with a mean cyst count of 4.67±0.58, as compared to Spiramycin treated GIII in which percentage reduction was 61.54% and mean parasite count 6.67±1.53 (Table 4). Mean size of cysts in infected non-treated GI was 26.0±8.54 µ while that in CUR nanoemulsion and Spiramycin treated groups were 7.0±2.0 µ and 8.67±2.89 µ, respectively. A higher percentage of reduction (73.08%) was recorded for nanoemulsion treated GII, while that of Spiramycin treated GIII was 66.67%. There was a statistically significant (P<0.05) decrease of cyst counts and sizes in both treated groups as compared to the non-treated group (Table 4).

Table 4. The effect of CUR nanoemulsion and Spiramycin treatments on mean cysts count and sizes in brains homogenates (7/10HPFs), and R% in T. gondii ME49 strain infected groups.

<table>
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<tbody>
<tr>
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<td>--</td>
<td>26.0±8.54</td>
<td>--</td>
</tr>
<tr>
<td>CUR treated (II)</td>
<td>4.67±0.58</td>
<td>73.08</td>
<td>7.0±2.0</td>
<td>73.08</td>
</tr>
<tr>
<td>Spiramycin treated (III)</td>
<td>6.67±1.53</td>
<td>61.54</td>
<td>8.67±2.89</td>
<td>66.67</td>
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*Significant (P<0.05).

DISCUSSION

Replicating tachyzoites, after invading intestinal epithelial cells, disseminate via the blood stream and lymphatics to all organs including the brain. Inside neurons and astrocytes, the preferred brain cells[9], tachyzoites differentiate into slow-dividing bradyzoites within a cyst containing thousands of bradyzoites. The cyst wall protects bradyzoites against host immune system and therapeutic drugs. Since T. gondii is an opportunistic parasite, brain cysts establish a symptomless chronic infection and become activated by a decrease in host immune response. Excysted bradyzoites transform into tachyzoites that proliferate causing widespread inflammation and critical morbidity. In fact, brain cyst reactivation with toxoplasmic encephalitis (TE) is not commonly reported in immunocompetent individuals with latent toxoplasmosis[9]. In their review[9], the researchers observed that brain cysts were commonly reported in the cerebral cortex, basal ganglia, and cerebellum based on studies conducted on autopsies of AIDS patients with TE[9]. Moreover, the reviewers noticed that latent toxoplasmosis negatively affects neurocognitive functions associated with behavioral changes, e.g. anxiety, depression, epilepsy, and schizophrenia. The strong tropism and persistence of brain cysts was attributed to impairment of a complex of brain functions leading to clinical manifestations that range from long-term behavioral and cognitive changes to lethal morbidity[9]. However, clinical studies attributed the behavioral changes and neurological manifestations to the influence of brain cysts on either neurotransmitter levels[20,21]; or neuroinflammation[22]; or neuroendocrine alteration[23]; or specific brain regions for cognition, mood, and emotion processing[24]. Notably, the long duration of treatment and inability to eliminate brain cysts, combined with frequency of side effects, make current chemotherapeutic options less than ideal and highlight the need for new anti-Toxoplasma chemotherapeutics[25].

In the present study, CUR nanoemulsion exhibited a statistically significant difference in mortality rate in comparison to infected non-treated group, and equivalent to that obtained in Spiramycin-treated group. This lowering of mortality rate was in agreement with two other studies that utilized nanotechnology for treatment[14,26]. In contrast, a study conducted in Alexandria, Egypt investigating the preventive effect of tricosan-liposomal nanoparticles on T. gondii ME49 strain, observed a high mortality rate. It was attributed to the daily stress caused by the twice-oral administration of the drug[27].

In our study, the recorded cyst counts, and sizes showed significant decrease in numbers and size in impression smears of livers and spleens, and emulsion smears of brains in both CUR and Spiramycin treated groups in comparison to the infected non-treated group. Similar results were obtained from two studies that utilized CUR as nanoemulsion[17] and CUR@MOFs nanocomposite[9]. Furthermore, similar results were obtained in several studies that utilized natural products in treatment of chronic toxoplasmosis. Artemisia annua (wormwood) was the first herb investigated in treatment of toxoplasmosis, and artemisinin derivatives exhibited better potency than artemisinin in controlling acute toxoplasmosis and protecting chronically infected mice from reactivated toxoplasmosis[28]. An Egyptian study investigated seed oil of Nigella sativa (black cumin) and showed promising prophylactic and therapeutic effects in chronic murine infection induced by ME49 strain. Results revealed significant reduction in the mortality rate and brain cyst burden that was explained as due to increased level of inducible nitric oxide synthase protecting mice against immunopathology[29]. Another Egyptian study
evaluated the ethanolic extract from Thymus vulgaris leaves (mint) in comparison to combined treatment by sulfadiazine and pyrimethamine. Results revealed significant reduction of the severity of chronic infection as demonstrated by decreased both count and size of brain cysts[30]. Besides, the results obtained from a third Egyptian study revealed significant reduction in the mean number of brain cysts (47.5%) in mice treated with T. vulgaris extract[31].

Similarly, Salvia miltiorrhiza (red sage) plant proved to be a promising therapeutic candidate against tachyzoites and bradyzoites in vitro. The investigators recommended further studies to assess its effects against brain cysts in vivo[32]. An Egyptian study was conducted to investigate the therapeutic effects of Araucaria heterophylla (conifer) resin extract and its major component (13-epi-cupressic acid) in chronic toxoplasmosis compared to cotrimoxazole. Results revealed that both agents exhibited higher toxoplasmicidal activity in vitro, and significant reduction in brain cyst burden (96% and 98%, respectively) in vivo[33]. Two studies investigated the ethanolic extract of Myristica fragrans (nutmeg) as a prophyllactic agent for protection against brain cyst development[31], and acute cerebral toxoplasmosis[34] in experimentally infected mice with ME49 and RH strains, respectively. Although the first study showed that M. fragrans extract exhibited mild reduction (0.8%) in brain cyst count, the second study demonstrated that myrislignan, isolated from M. fragrans peels, had potent anti-T. gondii activities both in vitro and in vivo. These activities were attributed to the interruption of mitochondrial function[34].

Lately in 2020, the ethanol extract of Anogeissus leiocarpa bark (African birch) was evaluated against acute and chronic toxoplasmosis. It showed significant inhibitory activity on RH strain tachyzoites, with significant reduction on host cell invasion in vitro. Besides, it was able to increase mice survival rate in chronic infection with ME49 strain[35]. Pomegranate is a rich source of Urolithin-A, a powerful protecting agent against neurodegeneration. Its administration significantly reduced brain cysts number and size in chronically infected mice than those in untreated animals[35]. One year later, three studies were conducted also investigating natural products[36,37,38]. Essential oils of Pelargonium X. asperum showed significant reduction in tachyzoites burden as demonstrated by their inability for growth and invasion[36]. Those of Myrtus communis (flowering myrtle) exhibited significant prophylactic results and improvement of innate immunity[37]. Leaf oil extracts of Rosmarinus officinalis (rosemary) showed a therapeutic rather than prophylactic potential, with reduction rates of cyst burden (81%) and cyst viability (23%). Ultrastructurally, tachyzoites with multiple depressions, protrusions, obtained from brain cysts with irregularities on cyst wall surface were unable to produce successful secondary infection after inoculation into naive mice[38].

Other natural products targeting Toxoplasma were suggested. In one in vitro study, CUR was utilized to promote a detoxification pathway producing inhibition of T. gondii glyoxalase 1 (TgGlo1). The investigators recommended further studies utilizing drug delivery nanotechnology system to overcome CUR bioavailability and biosolubility aiming to explore CUR potential inhibitory effect on TgGlo1 enzymatic activity[39]. Other studies hypothesized that constituents of some natural products inhibited heat shock proteins 70[40] and 90[41]; bradyzoites biosynthesis pathways for unsaturated fatty acid[42]; calcium homeostasis and mitochondrial physiology[43]; and cyst wall integrity[44]. However, inhibition of tachyzoites differentiation into encysted bradyzoites remains the most commonly reported mechanism of action of natural products in chronic toxoplasmosis[40].

In conclusion, CUR nanoemulsion proved to be a safe novel therapeutic agent against chronic toxoplasmosis, and we recommend further studies to evaluate its prophylactic effects for administration to immunocompromised patients either alone or in combination with the currently available drugs targeting tachyzoites growth and viability. On the other hand, due to the variable virulence among T. gondii strains and different incubation periods for chronic infection among host species, no single animal model can reflect the development and progression of chronic toxoplasmosis in humans. Since natural products are safe without side effects even for prolonged periods of administration time, extensive optimization, standardization as well as assessment of the pharmacokinetics are required to transfer experimental studies to clinical human trials.

Author contribution: Rageh EA performed mice infection, all parasitological parameters and analyzed the results. El-Gayar EK and Alabassy MM proposed the study topic, suggested the study design, interpreted the results, and wrote the draft. Barakat AM maintained the strain and shared Rageh EA in mice infection and practical assessment of the parasitological parameters. Abaza SM supervised and finalized the article for publication. All authors agreed on the authorship and final version of publication.

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