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Research article

Effect of nitazoxanide and spiramycin metronidazole combination in acute experimental toxoplasmosis

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ABSTRACT

Successful treatment of *Toxoplasma gondii* infection is difficult to attain. This study was designed to evaluate the efficacy of sulfamethoxazole-trimethoprim (SMZ-TMP), as the reference drug, nitazoxanide (NTZ), spiramycin (SP) and SP-metronidazole against the virulent RH *T. gondii* strain in acute experimental toxoplasmosis. One hundred Swiss albino mice were divided into control and experimental groups. Each mouse was infected with 2500 tachyzoites. Twenty infected untreated mice were used as control. The experimental group was subdivided into four subgroups (20 mice each); IIa SMZ-TMP, IIb NTZ, IIc SP and IId SP-metronidazole. All drugs were in tablet form, and were administered orally in suspension, for a period of seven days. Assessment of each drug efficacy was achieved through the study of mice survival time, mortality rate, parasite load, viability and morphological studies of tachyzoites by scanning electron microscope (SEM). The obtained results showed that SMZ-TMP, SP and SP-metronidazole were effective against acute murine toxoplasmosis and caused deformities in the tachyzoites ultrastructure. SP-metronidazole gave the best results on both mice survival rate and parasite load in the brain and liver. SMZ-TMP induced formation of prominent filaments extending from the deformed tachyzoites. NTZ showed little effect. In conclusion, all used drugs succeeded to prolong the survival time of the mice. SP-metronidazole gave the foremost effect on both mice survival rate and parasite load in the liver, spleen and brain. As this combination is nontoxic to human, it is promising for the treatment of human toxoplasmosis.

1. Introduction

Toxoplasma gondii (T.gondii) is an obligate intracellular coccidian parasite with a wide range of intermediate hosts [1]. It is one of the most successful parasites on earth, nearly one-third of the human population are exposed to this parasite. Although toxoplasmosis has a worldwide distribution from Alaska to Australia, there is only one species, *gondii*, in the genus *Toxoplasma* [2].Yet, based on the observation of differences in virulence, there are more than one strain of *T.gondii*. A virulent toxoplasmosis strain was discovered in 1941 infecting an asymptomatic six years old boy, who developed neurological signs and died on the 30th day of illness. It was given the initials of the child name and became the famous RH strain [3, 4].

Transmission of *T. gondii* occurs via the fecal–oral route as well as through consumption of infected meat, and by transplacental transfer from mother to fetus [5].It has three biologic infectious stages: sporozoites (in oocysts), tachyzoites (rapidly multiplying form), and bradyzoites (tissue cyst form). Tachyzoites as rapidly multiplying forms are the main cause of the acute phase of toxoplasmosis and are responsible for a wide spectrum of clinical signs [6].Once a host becomes infected, the parasite can survive with complex mechanisms for the whole lifespan within tissue cysts located usually in the skeletal muscles, brain, eyes, and myocardium. In some circumstances, particularly upon suppression of the immune system, latent encysted parasites can reactivate and the symptoms of infection become evident [7].

Till now, it was reported that there was no available gold standard treatment for *T. gondii* infection. The most commonly used drugs for the treatment of asymptomatic *T. gondii* infection among general population or pregnant women are spiramycin (SP), azithromycin and traditional Chinese medicine (Chinese herbs). However, the full therapeutic

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Table 1. Effect of used drugs on the mice survival time of the different studied groups.

Mice group	Mean ± SD	Median	Range	95% CI of mean		Log Rank		p_1
				L L	U L	X^2	P*	
Group I (infected untreated control)	6.9 ± 0.54	7.0	6–8	6.55	7.25	56.722*	<0.001*	0.001*
Subgroup IIa (SMZ-TMP treated)	11 ± 2.37	11.0	8–17	9.45	12.55			
Subgroup IIb (NTZ treated)	8.6 ± 0.80	9.0	7–9	8.08	9.12			<0.001*
Subgroup IIc (SP treated)	8.4 ± 1.02	8.0	6–10	7.73	9.07			0.001*
Subgroup IId (SP/metronidazole treated)	11.2 ± 0.40	11.0	11–12	10.94	11.46			<0.001*

LL: lower limit, UL: upper limit, P_1: P value for Kaplan-Meier test for comparing between group I and all the other. P*: Statistically significant at $p < 0.05$.

potential of these drugs may be hampered by the lack of brain penetration and low bioavailability [8].The current strategy of choice for the treatment of toxoplasmosis is the concept of combination therapy. Combined therapeutics such as pyrimethamine-sulfadiazine (P–S), pyrimethamine-clindamycin (P–C) and atovaquone-clindamycin were commonly used in the treatment of toxoplasmic encephalitis among human. However, the prolonged use of these combinations may cause severe side effects [8].

In this respect, the search for alternative drugs or drug combinations with novel or complementary mechanisms of action should be pursued. The combination of sulfamethoxazole-trimethoprim (SMZ-TMP) has been shown to possess antitoxoplasmic activity in acute cerebral toxoplasmosis and in treating ocular toxoplasmosis [9, 10].SP was coadministered with metronidazole where SP is a macrolid antibiotic, which is effective against acute toxoplasmosis, however, it demonstrates poor penetration across the blood brain barrier (BBB). Metronidazole alone shows no effect on *T. gondii* infection; yet, enhanced SP brain uptake was attained by its co-administration with metronidazole to inactivate the efflux pumps present in the BBB aiming at reaching effective concentrations to eliminate *T. gondii* brain cysts [11, 12].

Nitazoxanide (NTZ) has been used to treat different parasitic infections and was also used to treat the apicomplexan parasite *Cryptosporidium parvum*. Its effect on *T.gondii* was studied in vitro; it was therefore tempting to use NTZ experimentally for the treatment of toxoplasmosis hence increasing the list of diseases that can be targeted by this drug [13, 14]. Accordingly, this work was designed to evaluate the efficacy of SMZ-TMP, NTZ, SP alone and SP combined with metronidazole against the virulent RH *T. gondii* strain in a murine model to promote additional chemotherapeutic options for acute toxoplasmosis.

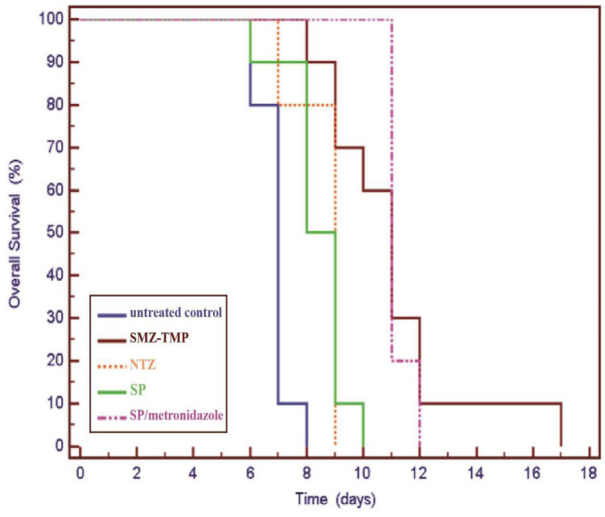

Figure 1. Kaplan-Meier overall survival curve of all groups.

2. Materials and methods

2.1. Experimental design

2.1.1. Ethical considerations

The study protocol was reviewed and approved by the Ethical Committee of the Medical Research Institute, Alexandria University in accordance to the ethical guidelines of animal experiments.

2.1.2. Mice infection and treatment schedule

2.1.2.1. Tachyzoites. Virulent *T. gondii* RH HXGPRT (-) strain was maintained in the laboratory by serial intraperitoneal inoculations of tachyzoites into laboratory bred Swiss albino mice every three days [15, 16].

Experiments were carried out on 100 Swiss strain albino mice 6–8 weeks old. All mice were infected intraperitoneally with 2500 tachyzoites of the RH (-HXGPRT) strain. Mice were divided into two main groups:

Group I: infected untreated control group (20 mice).

Group II: infected treated group (80 mice, 20 per subgroup).

Subgroup IIa, SMZ-TMP (Septazole®) (Alexandria Co. for pharmaceuticals and chemicals industries): mice received 100 mg/kg/day [17].

Subgroup IIb, NTZ (Nanazoxid ®) (Pharmed Healthcare for Utopia Pharmaceuticals): mice received 150 mg/kg/day based on previous studies on experimental cryptosporidiosis [13].

Subgroup IIc, SP (Rovac®) (Delta Pharma): mice received 400 mg/kg/day [11].

Subgroup IId, SP co-administered with metronidazole (Rovac®/ Amirazole®) (Amriya for Pharmaceutical Industries): mice of this subgroup first received 500 mg/kg/day of metronidazole suspended in 100 μl of phosphate buffer saline (PBS)/mouse/dose. After 30 min, the mice received 400 mg/kg/day SP suspended in 100 μl of PBS/mouse/dose [11].

Each tablet was crushed into a powder form, weighed and its active ingredient was calculated per mouse per dose, then suspended in 100 μl of PBS and was orally administered by gavage starting on day zero of infection for a total of seven days.

2.1.2.2. Assessment of drug efficacy. Treatment efficacy was determined through the study of:

2.1.2.3. Survival time. Ten mice from each subgroup were not sacrificed but left until they died. Daily observation of mice was done to determine the survival time [18]. Results were compared to the infected untreated control group.

2.1.2.4. Mortality rate (MR %). The mortality rate of the experimental subgroups was estimated at the sacrifice time (7^{th} day post infection) according to the following equation [19].

Table 2. Mean number and percent reduction of tachyzoites/oil immersion field in stained impression smears from the liver, spleen and brain.

Organ/R%	Control group	Experimental group (10 mice each)				F	P
	I Infected untreated	IIa SMZ-TMP	IIb NTZ	IIc SP	IId SP-metronidazole		
Liver	$6.27^{a} \pm 2.67$	$3.47^{b} \pm 2.36$	$4.89^{ab} \pm 1.79$	$3.86^{ab} \pm 1.92$	$2.32^{b} \pm 1.48$	5.136*	0.002*
%R		44.7%	22%	38.4%	64%		
Spleen	$8.09^{a} \pm 3.19$	$5.18^{a} \pm 3.46$	$6.12^{a} \pm 2.55$	$5.44^{a} \pm 3.49$	$5.14^{a} \pm 2.34$	1.653	0.178
%R		36%	24.4%	32.6%	36.5%		
Brain	$0.26^{a} \pm 0.14$	$0.13^{bc} \pm 0.11$	$0.24^{ab} \pm 0.22$	$0.11^{bc} \pm 0.07$	$0.07^{bc} \pm 0.06$	13.721*	0.008*
%R		50%	7.7%	57.7%	73.1%		

F: F test ANOVA test, significance between pairs of groups was done using Post Hoc Test (Tukey's). Mean with different superscripts in the same row are statistically significant at *$p \leq 0.05$. %R: percent reduction.

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

2.2.1. Parasitological studies

2.2.1.1. Parasite load. Giemsa stained impression smears were prepared from the liver, spleen and brain. The smears were examined by oil immersion lens (X1000) and *T. gondii* tachyzoites counted. The mean of ten

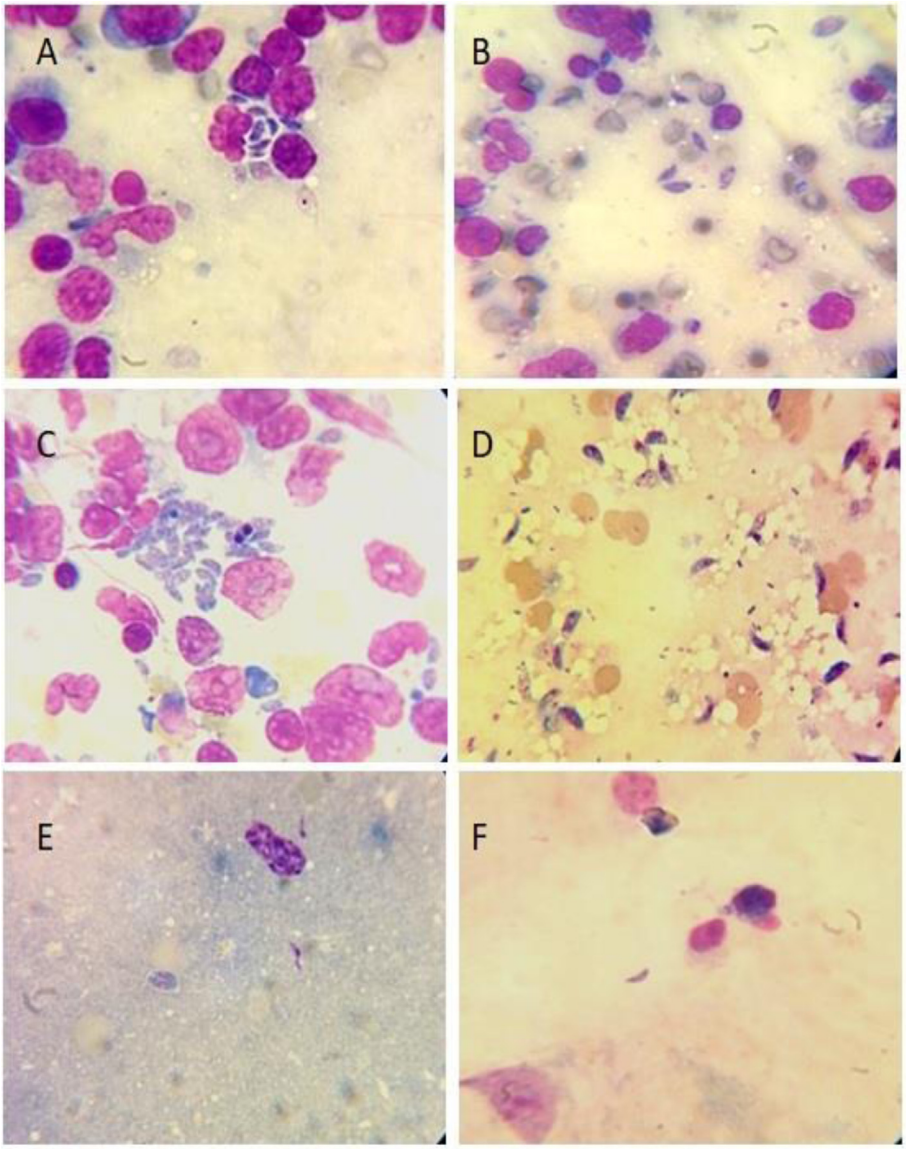

Figure 2. Extracellular *T. gondii* tachyzoites in Giemsa-stained liver impression smear of infected untreated control (A) and infected treated mice (B), X 1000. Extracellular *T.gondii* tachyzoites in Giemsa-stained spleen impression smears of infected untreated control (C) and in infected treated mice (D), X 1000. Extracellular *T. gondii* tachyzoites in brain impression smear of infected untreated control (E) and in infected treated mice brain impression smear (F), X 1000.

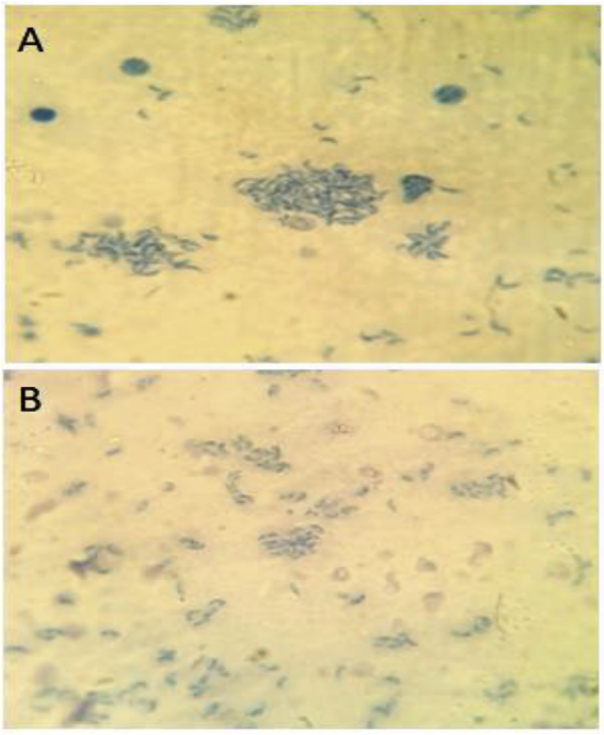

Figure 3. Extracellular *T. gondii* tachyzoites of the virulent RH strain from the intraperitoneal exudates (Trypan blue stain X1000). Dark blue are not viable (A). Light blue color with clear cytoplasm indicating viability (B).

different oil immersion fields from each studied organ of each mouse was calculated then the mean of the subgroup was determined [19, 20].

2.2.1.2. Parasite percent reduction (%R). The reduction in parasite burden in the studied organs of the different subgroups was estimated according to the following equation [21].

$$\%\ R = \frac{\text{Mean number of tachyzoites in the control group} - \text{Mean number of tachyzoites in the infected group} \times 100}{\text{Mean number of tachyzoites in the control group}}$$

2.2.1.3. Viability test. Trypan blue stain was used to detect the viability of tachyzoites. The tachyzoites were collected from the intraperitoneal cavity of all studied subgroups and stained with 0.4% trypan blue. Viable tachyzoites appeared with clear cytoplasm of light blue color while dead tachyzoites appeared as dark blue cytoplasm with unrecognized internal structures [22, 23].

2.2.1.4. Morphological study. Light microscopy (LM) and scanning electron microscopy (SEM) were used in the examination of *T. gondii* tachyzoites collected from the peritoneal cavity on the 7thday post infection from each group. The specimens were processed for SEM according to Klaineret al.(1973) [24] and examined using a Jeol-JSM6360LA, Japan scanning electron microscope.

3. Statistical analysis

Data were analyzed using IBM SPSS software package version 20.0 [25].Quantitative data were described using mean, standard deviation, median, minimum and maximum. ANOVA (F) test followed by Post Hoc Test (Tukey's) were performed to compare variables between different studied groups and significance was set at $p < 0.05$ [26].Kaplan-Meier survival curve, log rank test, Cox regression were done for the significant relation with progression free survival and overall survival [27].Abnormally distributed data and comparison between two independent groups were made using Mann Whitney test [28].

4. Results

4.1. Animal model studies

4.1.1. Survival time

In infected untreated mice the maximum survival time was 8 days and the mean was 7 days. The highest mean survival time of 11 days was observed in mice receiving combined treatment (SMZ-TMP IIa and SP-metronidazole subgroups IId). As to the infected treated mice, the maximal survival times were 17 and 12 days after SMZ-TMP and SP-metronidazole respectively. There was a significant difference in the survival time between all treated subgroups as compared to the untreated mice (Table1 and Figure 1).

4.2. Mortality rate (MR%)

At the sacrifice time on the 7thday post infection, the MR of the infected untreated group was 90%. By that time, none of the mice died after SMZ-TMP or SP-metronidazole. Two mice (20%) had died after NTZ. On the sixth day one mouse from the spiramycin group had died (Figure 1).

4.3. Parasitological studies

4.3.1. Parasite load

T. gondii tachyzoites were detected easily in impression smears from liver, spleen and brain specimens of all infected mice with variable densities. In the infected untreated mice (group I), the mean tachyzoite counts per oil immersion field in the liver, spleen and brain were 6.27, 8.09 and 0.26 respectively (Table 2, Figure 2).

Lower tachyzoite counts were found in all treated subgroups particularly those receiving combined drugs (IIa and IId). The percent reductions in counts in the liver and brain of mice treated with SMZ-TMP and SP-metronidazole were significant as compared to untreated mice, yet reductions in the counts from spleen were not significant.

4.4. Viability test

The test was performed on tachyzoites from peritoneal exudates using trypan blue stain, viable tachyzoites appeared with clear cytoplasm of light blue color while dead tachyzoites had dark blue cytoplasm with unrecognized internal structures. Considerable numbers of the collected tachyzoites from all treated subgroups were viable (Figure 3).

4.5. Morphological study by SEM

Scanning electron microscopy (SEM) was used for study of *T. gondii* tachyzoites collected from the peritoneal exudates on the 7thday post infection. In the control group, tachyzoites were mostly crescent shaped with completely smooth surfaces (Figure 4).

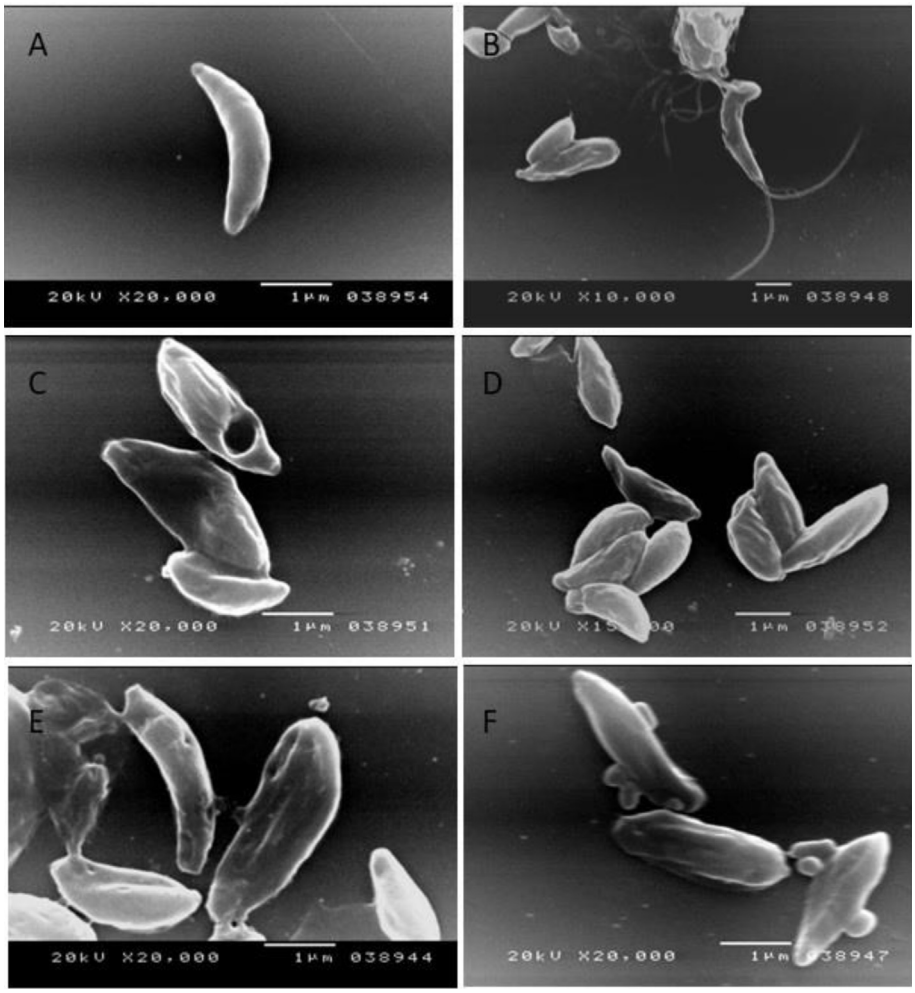

Figure 4. SEM of tachyzoites of infected untreated and treated mice. SEM image of normal untreated tachyzoite showing typical crescent shaped tachyzoite with completely smooth surface (A) (X 20,000). Peeling, erosions and ulcerations were observed in tachyzoites of all treated mice, SEM image of SMZ-TMP treated tachyzoites showing appearance of flagella like structures(B) (X 10,000). SEM image of SMZ-TMP treated tachyzoites showing formation of a hole in the anterior part of the parasite (C) (X 20,000). SEM image of tachyzoites from NTZ treated mice showing minimal changes in the form of irregularities and ridges on the crescent shape surface(D) (X 15,000). The surface of SP treated tachyzoites showed distortion in the apical region of the parasite (E) (X 20,000). SEM image of SP/metronidazole treated tachyzoites showing blebs on the parasite surface (F) (X 20,000).

In all treated subgroups, tachyzoites showed changes in the form of distortion in their crescent shape with reduction in their sizes. The tachyzoite surface showed deep irregular ridges, holes, erosions and ulcerations as well (Figure 4 (B, C, D, E, F)).Changes were more obvious in tachyzoites from SMZ-TMP treated mice which showed flagella like filaments and in the SP-metronidazole which showed blebs on the parasite surfaces.

5. Discussion

Treatment of human *T. gondii* infection is still arduous to conduct [29].An ideal drug for the treatment of toxoplasmosis would show parasiticidal properties against the different parasitic stages and effective penetration and concentration in the organs. Many therapeutic drugs are used but, they may be associated with numerous and severe side effects. Consequently, the search for alternative therapeutic medicines is a great priority. The present work studied the therapeutic effects of SMZ-TMP, NTZ, SP alone and SP-metronidazole against the virulent *T. gondii* strain in a murine model.

Concerning the survival time and mortality rate, the infected untreated mice started to die on the 6th day post infection, no mice survived beyond the 8th day. These findings were in agreement with the results of Grujićet al. (2005) [30], Wang et al.(2013) [31] and Eissa et al.(2015) [32].However, Denkers et al.(1993) [33] reported a maximum survival time of 14 days after subcutaneous inoculation of 2000 tachyzoites of the RH strain.

As for treated mice, all used drugs were able to induce a statistically significant increase of their survival time and decrease of mortality rate. The combined treatment with SMZ-TMP and SP-metronidazole were found remarkably efficient, compared to NTZ and SP alone. In these two subgroups, the longest survival times of 12 and 17 days were observed respectively, but mortality rate on the 7th day was 90% in the infected untreated group. Various results were obtained by different authors who reported survival rates that varied between 100% and 40% after using different doses of TMP-SMZ [34, 35].Differences regarding mice survival and mortality rates may be explained by variation in infection routes, the number of inoculated tachyzoites, the different doses of the used drugs and the mode of administration and duration of treatment.

The parasite load showed a pronounced reduction, in the mean tachyzoites count in the liver, spleen and brain impression smears, among the treated mice as compared to the controls. In the liver impression smears, the percent reduction of tachyziotes count was statistically significant only in mice treated with SMZ-TMP and SP-metronidazole. A low parasite reduction that was not statistically significant, was noted in the spleen, among all mice treated subgroups. In the brain, the highest reduction of parasite burden was detected in mice treated with SP-metronidazole (73%), SP (58%) alone followed by SMZ-TMP (50%); NTZ gave the least parasite reduction (7.7%). These results are consistent with a previous study which reported that SP-metronidazole caused a pharmacokinetic drug-drug interaction that led to 67% higher SP brain uptake [30].Similarly, Chew et al.(2012) [9] reported a significant reduction in the number of brain cysts after treatment with SP-metronidazole in a mouse model of chronic toxoplasmosis.

Considering viability, a higher proportion of viable tachyzoites was found in the peritoneal exudates. This may be attributed to the delayed

death phenomenon described by El-Zawawyet al.(2015) [21]who reported that tachyzoites remain viable for a while after using triclosan and they continue replication but are unable to successfully reinvade other host cells and die shortly.

For additional evaluation of the effect of the used drugs on *T. gondii* tachyzoites, an ultrastructural study was performed. Tachyzoites from the control mice appeared elongated, often crescent-shaped with a rounded pole at one end, a more or less pointed extremity at the other end and have smooth surfaces. SEM performed on tachyzoites collected from all treated subgroups showed an array of distortion, protrusion, compression, peeling, erosions, ulcerations, formation of flagella like structure and the presence of unusual unknown bodies attached to the parasite. These changes could explain the loss of the invasive power and reproduction capability of the treated tachyzoites that in turn led to a fall in parasite burden with increase in the survival time of the treated mice. Similar structural changes were reported by El-Zawawy et al. (2015) [21] and Mady et al. (2016) [36].Ultrastructural changes of tachyzoites were more evident in mice treated with SMZ-TMP. This drug in particular induced the appearance of flagella or filaments like structures with other changes in the parasite morphology. These striking changes may be ascribed to the ability of SMZ-TMP to cause over expression of an endogenous SAS6-l protein that forms prominent filaments extending from any part of the tachyzoite [37].Moreover, SMZ-TMP has antifolates mechanism that may stabilize cell membrane and interfere with microfilament function and blocking the actin gel (required for gliding motility) changing the resistance of the cell membrane and inhibiting parasite entry into the host cells [18].Antifolates mechanisms can also inhibit parasite cell division, DNA/RNA production and protein synthesis. Gaafar et al.(2014) [20] suggested that the changes in the shape of the organisms may be secondary to changes resulting from interference of the drugs with parasite DNA synthesis or interference with folic acid cycle.

With respect to the above mentioned results, this study revealed that SP-metronidazole, gave the best effect on both the survival time and parasite load in liver, spleen and brain. The efficacy of this combination may be attributed to parasite protein synthesis inhibition by SP together with high tissue penetration capacity of metronidazole [38]. There have been no reports dealing with the effectiveness of SP-metronidazole upon acute toxoplasmosis in experimental animals. SMZ-TMP was the second successful combination; SP came third, while NTZ was the least effective in parasite load reduction within the examined tissues which may be due to the low dose used. El-Kowranya et al (2019) reported that NTZ had significant treatment effect on acute and chronic *Toxoplasma gondii* (ME49 strain) [39]. However, there are no previously published reports regarding the effect of NTZ against the RH strain of *T. gondii* in a murine model. Further studies with different doses are needed to elucidate any treatment efficiency. In conclusion, results emphasized that all used drugs succeeded to prolong the survival time of the mice as compared to controls. SP-metronidazole exhibited the best effect; this combination is nontoxic to human and may represent a good starting point of a potentially effective novel treatment to human toxoplasmosis.

Declarations

Author contribution statement

A. Allam: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

A. Shehab and H. Fara: Analyzed and interpreted the data; Wrote the paper.

N. Mogahed, N. El-Latif and Y. Elsayed: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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