Insights on the therapeutic effect of quinolone-based compound, PPQ-8 plus Nitazoxanide, in chronic toxoplasmosis murine model

Original Article

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ABSTRACT

Background: Recent studies showed promising results of the quinolone-based compound (PPQ-8) against schistosomiasis and toxoplasmosis. It is hypothesized that combined treatment by PPQ-8 with an anti-*Toxoplasma* drug may induce a convenient therapeutic effect on chronic toxoplasmosis.
Objective: To evaluate the effect of PPQ-8 combined with nitazoxanide (NTZ) on chronic experimental toxoplasmosis.
Material and Methods: After being infected for three months with ME49 *T. gondii*, forty female Swiss albino mice were randomly split into four equal groups. In comparison to untreated group, mice were treated with pyrimethamine and sulfadiazine (PYR/SDZ), PPQ-8 alone or combined with nitazoxanide (PPQ-8/NTZ). Five mice from each group were sacrificed after the end of treatment (14 d) to evaluate drug efficacy using parasitological (brain cyst count and size), histopathological examination of brain tissues, measurement of brain inducible nitric oxide synthase (iNOS) activity and immunological (IFN-γ and TNF-α) parameters. The remaining 5 mice were monitored for 60 days to calculate survival time for each group.
Results: The number and size of brain cysts were significantly decreased among all treated groups when compared with control infected untreated group. Histopathological studies of brain sections of control mice showed severe inflammation, mice treated with PYR/SDZ had moderate inflammation, those treated with PPQ-8 alone showed mild to moderate inflammation, and those receiving PPQ-8/NTZ had mild inflammation. Treatment with PPQ-8, and PPQ-8/NTZ induced an increase in serum level of IFN-γ, and reduction in the level of TNF-α as well as raised production of brain iNOS level. Treatment with PYR/SDZ-recorded a significant reduction in serum level of IFN-γ, TNF-α and brain iNOS when compared with the infected untreated group.
Conclusion: When combined with NTZ, PPQ-8 showed a promising regimen for treatment of chronic toxoplasmosis due to its synergistic effect. Combined treatment exhibited mild inflammation, increased levels of IFN-γ and iNOS, and decreased TNF-α production.
Keywords: chronic toxoplasmosis; ME49; mice; nitazoxanide; PPQ-8.

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INTRODUCTION

A variety of intermediate hosts are infected by the obligate intracellular parasite *T. gondii*, which is a member of the phylum Apicomplexa. One of the most pervasive parasites in the world, *T. gondii* may infect all warm-blooded creatures, including humans, and it is thought to affect around one-third of the global population[1]. The complicated *T. gondii* life cycle includes three biological stages that can penetrate all types of human cells. These are the sporozoites, found in developed di-sporocystic tetra-sporozoic oocysts; tachyzoites that rapidly multiply in any nucleated cell causing the acute phase of toxoplasmosis; and slowly multiplying bradyzoites in tissue cysts typically found in skeletal muscles, the brain, the eyes, and the heart[2]. In immunocompetent individuals, infection transforms into latency. Development of bradyzoites in tissue cysts is mostly regulated by cellular immunological processes, and they likely remain viable throughout the life of the host[3].

Sulfonamides was the first specific medication used to treat *T. gondii* infection. Later the combination of sulfadiazine and pyrimethamine was evaluated and considered the gold standard against which subsequent regimens are assessed[4]. After that, spiramycine showed significant anti-*T. gondii* action and was specially indicated for treatment of pregnant women as a preventative measure to stop

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maternofetal transmission[5]. Although more options have been created, these three medications were considered as the standard treatment of toxoplasmosis. Unfortunately, all medications used in clinical practice only affect tachyzoites and have no effect on cysts harboring bradyzoites; the parasite's dormant stage[6]. To completely eradicate the parasite, further work is thus required to create novel therapies targeted against bradyzoites. The main goal of combining two drugs is to achieve positive interaction effects. This is attained by evaluating the benefit of combining two drugs as compared to each drug individually. Drug combination is a strategy that could increase treatment efficacy, allow lower doses of each constituent drug, reduce adverse reactions and drug resistance[7].

The broad-spectrum NTZ anti-parasitic drug is used against a wide variety of intestinal cestodes, nematodes and protozoa. Moreover, it acts against several anaerobic and microaerophilic bacteria and viruses[8,9]. Recently, NTZ has been tested against acute and chronic toxoplasmosis[10]. Quinolines and their derivatives, such as PPQ-8, have also undergone successful experimental testing against both acute and latent toxoplasmosis[11,12]. Kadri *et al*.[13] evaluated 58 derivatives of 4-piperazinyl quinoline; compounds with a single quinoline ring induced powerful inhibitory action against *T. gondii*. The addition of OH group at position 8, amplified the inhibitory effect by forty times. Moreover, the duplication of quinoline rings greatly increased the activity of molecules. Notably, PPQ-8 is a novel heterocyclic agent synthesized through combination of both therapeutically active molecules: 2-chloroquinoline as lipophilic and the diverse heterocyclic ring that has potent antiprotozoal activity[14]. It has variable and distinct biological effects because of the existence of quinoline nucleus that induces anti-inflammatory, anti-bacterial, anti-viral, anti-fungal, anti-helminthic, and anti-protozoan effects[15]. It was reported that its anti-*Toxoplasma* effect was mediated through apicoplast loss leading to inhibition of tachyzoites growth and function, in addition to interference with topoisomerases enzymes included in parasite DNA replication and repair[13,16].

These encouraging findings prompted us to extend our research to examine the potential efficacy of combining PPQ-8 and NTZ and to document the combined regimen's immunomodulatory effects in mice infected with the ME49 *T. gondii* strain and treated with both medications.

MATERIAL AND METHODS

This experimental case-control study was conducted at the Department of Medical Parasitology, Faculty of Medicine, Mansoura University during the period from September 2019 to March 2021.

Study design: Three groups of mice infected with *T. gondii* ME49 strain were treated with three different regimens PYR/SDZ, PPQ-8 alone and PPQ-8/NTZ and then compared with a fourth infected untreated group. Different parameters used for evaluation of drugs efficacy included measurement of brain cyst count and size, histopathological examination of brain tissues, measurement of brain iNOS activity and immunological assessment.

Parasites: The cystogenic ME49 strain of *T. gondii* was kindly provided by the Department of Medical Parasitology, Faculty of Medicine, Alexandria University. It was maintained at our laboratory by serial passaging in Swiss strain albino mice according to Cavalcanti *et al*.[17].

Tested drugs

1. The PPQ-8 compound [4-(2-chloroquinolin-3-yl)-6-(2,5-dimethoxyphenyl)- 2-oxo-1,2-dihydropyridine-3-carbonitrile] was synthesized as previously reported[18]. A mixture of 2-chloroquinoline-3-carbaldehyde, ethyl cyanoacetate, acetophenone derivative and ammonium acetate, was heated under reflux in ethanol as a solvent, dissolved in dimethyl sulfoxide as a vehicle and administered at a dose of 20 mg/kg/d by oral gavage using a ball tipped feeding needle for 14 successive d[12].
2. Nitazoxanide was purchased from (UTOPIA, Egypt) in the form of a powder (16 gm/bottle), from which 0.6 gm was reconstituted in 30 ml DMSO to yield a final conc of 20 mg/ml. The drug was administered orally at 100 mg/kg/d for 14 successive days[19].
3. Pyrimethamine and sulfadiazine, purchased from Sigma-Aldrich (St. Louis, USA), were diluted in polyethylene glycol (PEG) MW 200 (Sigma-Aldrich, St. Louis, USA), and administered orally at dosages of 4.4 and 250 mg/kg/day respectively, for 14 successive d[20].

Animals: Female Swiss albino mice, 6 w old, weighing 18–22 g, were used. Mice were housed and offered drinking water and regular mouse feed ad libitum, and maintained under controlled conditions of lighting (12 h light/12 h dark cycle) and temperature (25±2°C).

Mice infection: Inoculums were adjusted to 250 µL of brain suspensions containing 10 cysts administered orally and maintained for three months to induce chronic infection.

Experimental groups: Forty infected mice were equally divided into 4 groups; (a): non-treated (control), (b): treated with PYR/SDZ, (c): treated with PPQ-8, and (d): treated with PPQ-8/NTZ. At the end of treatment (14 d), five mice from each group were sacrificed and the remaining mice were observed for an additional 60 d, at which point the survivors were sacrificed and assessed for drug efficacy.

Mortality rate and survival time: Mice were monitored daily to calculate mortality rate and survival time. The mortality rate was calculated according to the following equation: MR = (Number of dead mice at the sacrifice time (after end of treatment)/Number of mice at the beginning of the experiment) X100. For calculation of survival time, mice from the four groups were observed for 60 d after the end of the treatment and the median survival time was reported.

Mean cyst count and size in brain: Mice were sacrificed, and brains were removed and each one was homogenized in 1 ml of sterile phosphate-buffered saline, pH 7.2. Homogenates were examined by bright-field microscopy at X40 magnification. Cysts were measured using ocular micrometer and were counted from 4 separate 20 µL aliquots, then counts were used to estimate the total number of cysts per brain[21].

Histopathological study: One half of brain from all studied subgroups was fixed in neutral buffered formalin (10%) and processed for paraffin embedding and staining with H&E then examined for histopathological lesions. Scoring was as follows: score 0: no inflammation, no necrosis, no gliosis; score 1: mild inflammation, mild gliosis, and scattered giant cells; score 2: moderate inflammation, moderate gliosis, and few giant cells; and score 3: severe inflammation, severe gliosis, and frequent giant cells[22].

Immunological study: Blood samples (1 ml) were obtained from each mouse. Mouse IFN-γ ELISA kit [IFNG] (CAT#ab100689, ABCAM, Cambridge, UK) and mouse TNF-α matched antibody pair set [ABP-S-07101] (CAT#: ab100747, ABCAM, Cambridge, UK) were used for assessment of IFN-γ and TNF-α according to the manufacturer's instructions[23,24]. Data were presented in pg/ml. The limits of detection were determined from a standard curves (TNF-α, 8 pg/ml; IFN-γ, 15 pg/ml)[25].

Activity of iNOS: We followed the manufacturer's instruction for the colorimetric iNOS activity assay kit (cat#: ab211083; Abcam, Waltham, MA, USA) to measure the brain NOS activity[26]. In brief, ~100 mg of brain tissue from each mouse were pooled, then homogenized in 200 µl of ice-cold assay buffer, followed by centrifugation for 10 min; 60 µl of the supernatant containing 200-400 ug protein was used. The optical density was detected at 530-540 nm. The activity of iNOS was expressed as nmol nitrite/mg protein.

Statistical analysis: Data were analyzed using statistical package for social sciences (SPSS) software (SPSS Inc., Chicago, USA), version 16.0. Quantitative data were described as mean and standard deviation (SD) after testing for normality by Shapiro-wilk test. One way analysis of variance (ANOVA) test with least significant difference (LSD) post hoc multiple comparisons was used for comparison between groups. Qualitative data were described as numbers and percentages. Monte Carlo test was used for comparison between groups. The results were considered significant when the probability of error equaled or was less than 5% (P<0.05).

Ethical consideration: The use and handling of animals in the study complied with guidelines of committee of research ethics. The study protocol was approved by the institutional review board committee, code number: 19.08.24.

RESULTS

Mortality rate of chronic animal model: One mouse died from the PYR/SDZ, PPQ-8 and the PPQ-8/ NTZ groups, while two mice died from control untreated group at the scarification time with mortality rates of 10%, 10%, 10%, and 20% respectively, and without any significant difference between these groups.

Survival time of chronic animal model: Sixty days post-treatment, the survival rate was 60% for infected untreated group and increased to 80% in PYR/SDZ, PPQ-8 and PPQ-8/NTZ treated groups. The median survival duration of the control untreated group was 49.6 d, while the median survival duration of PYR/SDZ, PPQ-8 and PPQ-8/NTZ groups increased to 57.96, 58.03 and 58.94 d respectively, without significant correlation (Table 1).

Brain cyst count and cyst size: The number of *Toxoplasma* cysts in brain specimens showed significant (P<0.001) reduction among PYR/SDZ, PPQ-8 and PPQ-8/NTZ-treated groups when compared with control untreated group by 51.13%, 50% and 64.20%, respectively. Additionally, the size of the brain

Table 1. Kaplan-Meier overall survival among the studied groups.

	Median survival time	SE	95% CI	Statistical analysis
Control	49.6	0.815	38.423-60.781	**Log rank (Mantel-Cox) test:** 1.025, *P:* 0.599
PYR/SDZ	57.96	0.256	54.495-61.509	
PPQ-8	58.03	0.165	55.945-60.512	
PPQ-8/ NTZ	58.94	0.128	57.246-60.753	
Overall survival	55.531	0.327	51.044-60.018	

SE: Standard error; **CI:** confidence interval.

Toxoplasma cysts revealed a significant (P<0.001) reduction in their diameters by 23.95% in the group treated with PYR/SDZ, 39.6% in PPQ-treated group and 45.31% in the group treated with PPQ-8/NTZ, compared with the infected untreated group (Table 2).

Histopathological study of the brain: Sections from the brain tissues of infected untreated mice revealed severe inflammation, severe gliosis, frequent giant cells and well-defined cysts which were found mainly in white matter, and few cysts were also detected in the junction between grey and white matters. Inflammatory cellular infiltrates (mainly lymphocytes, histiocytes and giant cells) were found in the perivascular spaces as well as underlying the meninges. Most of the brain vessels were dilated, congested, and distended with inflammatory cells (Fig. 1a, b, h).

Most brain tissues of mice administered PYR/SDZ showed mild to moderate inflammation, moderate gliosis, few giant cells and degenerated cysts (Fig. 1c, d, h). Most brain sections of mice treated with PPQ-8 showed moderate to mild gliosis, few inflammatory cells and scattered giant cells. There were many degenerated cysts that showed marked reduction in both number and diameter (Fig. 1e, h), while PPQ-8 /NTZ showed some improvement of the brain pathology with few inflammatory cells, degenerated cysts, mild gliosis, and inflammation (Fig. 1f, g).

Immunological study and inducible nitric oxide synthase (iNOS) activity: Treated group with PPQ-8 and PPQ-8/NTZ showed statistically significant (P<0.05, and <0.001 respectively) increase in serum level of IFN-γ (43.40±1.67 pg/ml, 50.4±1.68 pg/ml respectively); while PYR/SDZ-treated group showed significant decrease (12.60±0.55 pg/ml) when compared with the control group (39.20±3.35 pg/ml) with significant difference (P=0.0001) within the three treated groups. Treatment with PYR/SDZ induced a significant (P<0.001) reduction in TNF-α production (21.66±2.41 pg/ml). Also mice treated with PPQ-8 and PPQ-8/NTZ recorded significant (P=0.001, and <0.001, respectively) reduction of TNF-α (26.34±2.19

Table 2. Effect of PPQ-8, PPQ-8/NTZ and PYR/SDZ on cysts count and diameter in brain homogenates from *T. gondii*-infected mice.

Animal groups (N=5)	***T. gondii* cyst in brain homogenates (10 µl)**	
	Brain cyst count	**Brain cyst size (µm)**
Infected untreated	17.6 ± 1.14	38.4 ± 0.89
PYR/SDZ	8.6 ± 0.89 (51.13%)	29.20 ± 1.92 (23.95%)
PPQ-8	8.8 ± 2.77 (50%)	23.2 ± 5.54 (39.6%)
	(P = 0.00018)	(P = 0.0003)
PPQ-8/NTZ	6.3 ± 0.71 (64.20%)	21.0 ± 1.22 (45.31%)
***P* value**	P<0.001*, $P1$<0.001*, $P2$<0.001*, $P3$ =0.01*	P<0.001*, $P1$<0.001*, $P2$<0.001*, $P3$ =0.001*

Values are expressed as mean ± SD. Values between parentheses refer to percentage of reduction compared with infected non treated group. ***P:*** Significant difference between infected non-treated and PPQ-8-treated groups. ***P1:*** Significant difference between infected non-treated and PYR/SDZ-treated groups. ***P2*:** Significant difference between infected non-treated and PPQ-8/NTZ-treated groups. ***P3*:** Significant difference between PYR/SDZ-treated and PPQ-8/NTZ-treated groups.

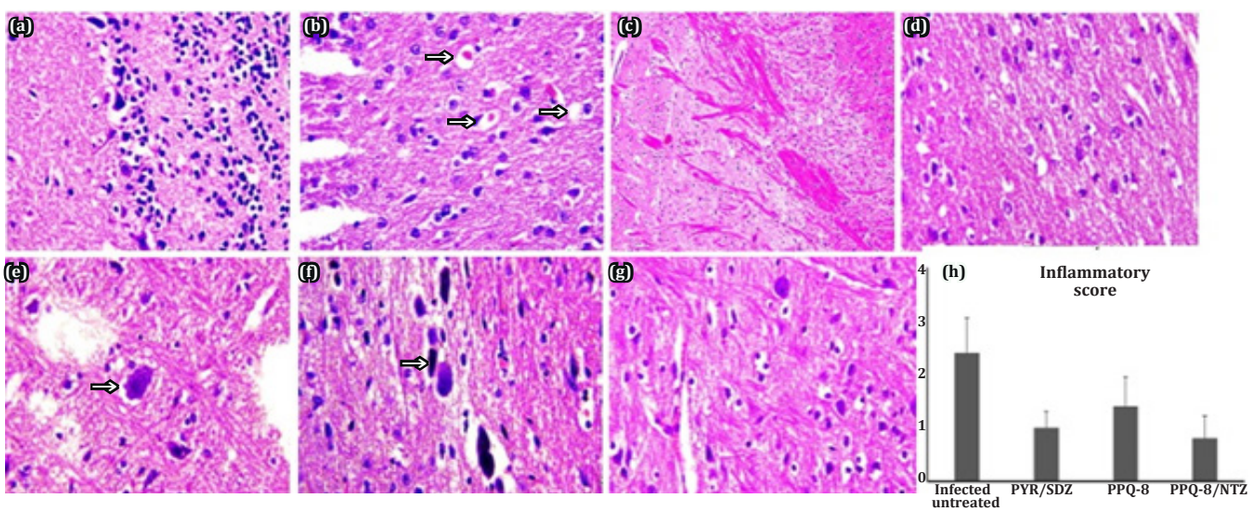

Fig. 1. Histopathological study of brain sections (H&E ×400) from different groups of mice of chronic models of *T. gondii* infection. Infected untreated mice revealed **(a)** multiple degenerated neurons together with extensive lymphocytic infiltrate, **(b)** multiple eosinophilic granular bodies (white arrows), **(c)** dense reactive gliosis. Infected treated mice with PYR/SDZ revealed **(d)** normal brain tissue cellularity with mild gliosis, **(e)** *Toxoplasma* cyst (white arrow). Infected treated mice with PPQ-8/NTZ revealed **(f)** normal brain tissue cellularity with mild gliosis and ovoid *Toxoplasma* cyst (white arrow), **(g)** mild lymphocytic infiltrate together with mild reactive gliosis. **(h)** Scoring of brain tissue from infected untreated, infected and treated with PYR/SDZ and infected and treated with PPQ-8/NTZ.

pg/ml and 20.54±2.12 pg/ml respectively) when compared with the untreated group (51.06±0.89 pg/ml) (Table 3).

The production of iNOS was reported in brain tissue from the four tested groups: Compared with the infected control mice, PPQ-8 and PPQ-8/NTZ-treated mice showed a significantly higher production of iNOS (1.32±0.29 nmol/mg and 2.90±0.23 nmol/mg) with respective P values (<0.01, <0.001). However, significantly lower iNOS production was observed in the PYR/SDZ-treated (0.26±0.11 nmol/mg, P<0.01) when compared with the infected untreated group (0.72±0.26 nmol/mg) with significant difference within the three treated groups (Table 3).

Table 3. Immunological studies and iNOS activity among the studied groups.

	Infected untreated	PYR/SDZ	PPQ-8	PPQ-8/NTZ	Statistical analysis	
					Four groups	Within groups
IFN-γ (pg/ml)	39.2 ± 3.35	12.6 ± 0.55	43.4 ± 1.67	50.4 ± 1.67	P = 0.0001	P1 = 001, P2 = 0.03, P3 = 0.0001
TNF-α (pg/ml)	51.06 ± 0.89	21.66 ± 2.41	26.34 ± 2.19	20.54 ± 2.12	P < 0.001	P1 < 0.001, P2 = 0.001, P3 < 0.001
iNOS (nmol/mg)	0.72 ± 0.26	0.26 ± 0.11	1.32 ± 0.29	2.90 ± 0.23	P < 0.0001	P1 = 0.001, P2 = 0.001, P3 < 0.001

Values are expressed as mean ± SD. ***P*1:** Significant difference between infected non-treated and PYR/SDZ-treated groups. ***P*2:** Significant difference between infected non-treated and PPQ-8-treated groups. ***P*3:**: Significant difference between infected non-treated and PPQ-8/NTZ-treated groups.

DISCUSSION

T. gondii is primarily a neurotropic pathogen with higher affinity for the central nervous system tissues[27]. Hence, the slowly replicating bradyzoites induce disruption of neurons connections with changes to their structures, in addition to a unique intracerebral immune response mediated by different cytokines to limit *T. gondii* reproduction[28,29]. The combined PyR/SDZ treatment which is considered as the gold standard against toxoplasmosis, is unfortunately not effective against bradyzoites, and no regimen has been proven to be effective against chronic toxoplasmosis[5]. In our study, we tested the anti-*Toxoplasma* effect of PPQ-8, a synthetic quinoline, in combination with NTZ. Quinolines compounds were used for treatment of several parasites including helminths and protozoa[30,31]. The PPQ-8 as previously described in two studies[12,32] contains a quinoline group, so it is expected to have anti-malarial and anti-helminthic activities[33,34], and it is expected to have antioxidant and antiparasitic effects owing to its pyridine structure[35,36].

Prior research demonstrated that PPQ-8 was effective in treating both acute and chronic toxoplasmosis in experimental mice[12]. We recorded a substantial reduction in the number and size of brain cysts by 64.20% and 45.31% respectively, while administering PPQ-8 at a dosage of 20 mg/kg in conjunction with 100 mg/kg NTZ. In a former study conducted in our laboratory, a similar dose of PPQ-8 (20 mg/kg) was tested in mice with chronic toxoplasmosis. The results showed a significant (P>0.01) reduction in brain cyst diameter by 48.44%, a significant reduction in cyst count by 40.65%, a significant (P>0.05) extension of the mice's survival time by 80%, as well as less inflammation and gliosis with cyst degeneration when compared with infected untreated group[12]. On the other hand, it was reported that NTZ alone (100 mg/kg), produced a lower reduction in the number of brain cysts by 35.2% (P=0.014)[10]. This greater decrease in tissue cyst count and diameter attained in our study with PPQ/NTZ (64.2%, 45.31%, respectively), may be explained by the effect of both drugs on tachyzoites before switching to bradyzoites. However, the major effect is likely due to the direct action of PPQ-8 on the tissue cysts. Based on the computed lipophilicity, PPQ-8 is a moderately lipophilic drug and is more likely to penetrate the blood-brain barrier (BBB) and cyst wall without rupturing it[37,38] to destroy the parasite either directly or indirectly through apicoplast disruption[11,39].

It was reported that NTZ inhibited pyruvate-ferredoxin oxidoreductase (PFOR), an enzyme essential for anaerobic energy metabolism[40]. The lower anti-*Toxoplasma* effect of NTZ, may be attributed to its effect on tachyzoites. It was proved that NTZ was more effective against metabolically active tachyzoites and immature bradyzoites than against mature bradyzoites within the cyst[41,42]. However, the immunomodulatory effect of both drugs cannot be ignored. Mukherjee and Pal[43] postulated that the increase in the survival time of infected animals from 40% to 80% and the reduction of the mortality rate from 20% to 10% besides the reduction of brain pathology were due to destruction of more parasites and the anti-inflammatory effect of the drug regimen used.

The immunomodulatory action of NTZ was supported in a former study[9] that explained the wide-range effect of NTZ on several pathogens. In the same manner, quinolines including PPQ-8 affect several pathogenic organisms and this could entail different mechanisms of action besides the immunostimulatory

effect supported by research work[44,45]. From our previous work, we have some evidence that PPQ-8 could as reported work through effecting the apicoplast[11,39]. So to further investigate other mechanisms of action, we assessed the proinflammatory mediators TNF-α and INF-γ as representatives for immunological effect and iNOS to measure antioxidant effect. Mice treated with PYR/SDZ had the lowest level of serum IFN-γ. This was previously recorded by Rossignol *et al*.[9] who reported that IFN-γ showed the lowest level in acute and chronic toxoplasmosis, when compared with NTZ- treated or infected untreated mice. It is understood that PYR/SDZ fights *Toxoplasma* by inhibiting the metabolic enzymes, such as dihydrofolate reductase and dihydropteroate synthetase, preventing folic acid production[46]. In such a way, PYR/SDZ interfer directly with the parasite viability without stimulation of the immune system. Hence the possibility of reactivation of latent infection is more common.

On the other hand, mice treated with PPQ-8/NTZ had the highest serum level of IFN-γ. These findings demonstrated that the immunostimulatory impact of PPQ-8/NTZ on toxoplasmosis may be endorsed. Given that the immunomodulatory function of NTZ was previously hypothesized, we anticipate that PPQ-8 may be the reason for this synergistic impact on IFN-γ. In fact, IFN-γ primarily supports the host's defense against acute and chronic toxoplasmosis. Activated macrophages, T lymphocytes, NK cells, and T cytotoxic cells all generate IFN-γ when *T. gondii* enters the host[47]. Notably, IFN-γ plays a role in the conversion of rapidly dividing tachyzoites to the bradyzoites[48], in addition to maintenance of a chronic toxoplasmosis condition by suppressing conversion of bradyzoites to active tachyzoites[49]. It also has antiparasitic capabilities and aids in the generation of nitric oxide and harmful oxygen radicals, and its decreased production is associated with a higher risk of acute infection and mouse mortality from excessive parasite multiplication[50]. Our results showed that levels of TNF-α decreased significantly in all treated groups when compared with untreated group. It is worth mentioning that TNF-α is a proinflammatory cytokine produced mainly by macrophages early after an infection and is involved in the innate immune response against intracellular pathogens[51]. In toxoplasmosis, IFN-γ stimulates production of TNF-α by macrophages, neutrophils, T cells and dendritic cells. On the contrary, fatty acids of *T. gondii* such as myristic and palmitic acids, diminish TNF-α production in macrophages as a strategy by the parasite to evade the host immune system[52].

Moreover, an experimental study using a neutralizing TNF monoclonal antibody led to reactivation of toxoplasmic encephalitis (TE) in chronically infected C57BL/6 mice[53]. Additionally, TNF-α is identified as essential in controlling both acute and chronic TE in mice, but it is not required for the control of the parasite in other organs[54]. Hence, the reduced levels of TNF-α obtained after treatment with PPQ-8 and PPQ-8/NTZ is beneficial since it leads to limitation of TE, restriction of parasite growth through tryptophan starvation, plus activation of parasite killing functions of microglia through iNOS-dependent and -independent mechanisms[55]. The lower levels of TNF-α in mice treated with PPQ-8 and PPQ-8/NTZ were probably due to the reported anti-inflammatory effect of quinolines and nitazoxanide[43,56].

Our results showed that iNOS level in brain tissue increased in groups treated with PPQ-8 and PPQ-8/NTZ and reduced after treatment with PYR/SDZ. Similar results were reported by Rossignol *et al*.[9] using immunohistochemistry. The iNOS is mainly promoted by IFN-γ and it stimulates NO production that has immunoregulatory and antiparasitic effects. As previously reported, during the late stage of infection, iNOS production was essential for the prevention of the proliferation of tachyzoites in the brain and the development of TE in addition to reduction of the *Toxoplasma* immunopathology because of NO production[57,58]. In addition, it was previously shown that inflammatory monocytes expressing iNOS can protect against lethal oral toxoplasmosis[59]. In another study, low or no iNOS expression contributed to uncontrolled parasite multiplication that in addition to inflammatory and necrotic lesions in the CNS induced the mortality of TNFRp55$^{(-/-)}$ and iNOS$^{(-/-)}$ mice[60]. Accordingly, the elevated levels of iNOS in PPQ-8 and PPQ-8/NTZ-treated group are probably due to increased production of IFN-γ and TNF-α. In addition, NTZ enhances the production of iNOS in brain tissue[61]. The lower level of iNOS production observed in the PYR/SDZ-treated group could be due to the lower production level of IFN-γ reported in our study.

The ability of PPQ-8/NTZ to cross the BBB and reach CNS tissue opens the way to development of selective agents against *Toxoplasma* particularly in mildly immunosuppressed patients, who are at risk for reactivation of acute toxoplasmosis. Moreover, the recent proof of anticancer activity of PPQ-8, due to its Pim-1 Kinase inhibition activity[62], and the potential anticancer effect of NTZ[63,64] should be considered as a recent combination for treatment of cancer patients with toxoplasmosis. In addition, because of the high susceptibility of cancer patients to toxoplasmosis, using PPQ-8/NTZ regimen will be a double-edged weapon to fight both cancer and toxoplasmosis with few side effects.

However, further *in vivo* studies focusing on using PPQ-8 with variable doses are needed to define the optimum efficacy. Besides, identification of the PPQ-8 targets is essential to elucidate how PPQ-8 acts against *T. gondii*.

It can be concluded that the synergistic effect of PPQ-8 with NTZ showed the anti-*T. gondii* potential of

this combination compared with the standard regimen PYR/SDZ. The PPQ-8 enhances the anti-toxoplasmosis effect of NTZ through direct anti-*Toxoplasma* effect and enhancement of the immune system.

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