EVALUATION OF THE EFFECT OF COMPOUND PPQ-8 COMBINED WITH NITAZOXANIDE AGAINST EXPERIMENTAL TOXOPLASMOSIS

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Abstract

The efficacy of compound PPQ-8 combined with Nitazoxanide against Toxoplasma gondii was examined in a murine model infected with a non-virulent (Me49) strain. Thirty female Swiss- albino mice (6 weeks old) were inoculated by mouth with 20 cysts and divided into 3 groups. The first group was left untreated, the second group treated with Pyrimethamine and sulfadiazine (PYR/SDZ), while, the third one was treated with PPQ-8 and Nitazoxanide (PPQ-8/NTZ), treatment was started 4 days post-infection and continued for 14 consecutive days. Five mice from each group were sacrificed and the rest were observed for 30 days. Results showed that, the median survival duration of the untreated group was 3.66 weeks, while, that of PYR/SDZ and PPQ-8/NTZ groups were 3.86 and 3.77 weeks, respectively. The number and size of Toxoplasma cysts in brain were significantly decreased among PYR/SDZ and PPQ-8/NTZ-treated groups when compared with infected untreated group. Histopathological examination of brain of control mice showed severe inflammation while, mice treated with PYR/SDZ reported moderate inflammation and mice given PPQ-8/NTZ showed mild inflammation. PPQ-8/NTZ induced a significant increase in serum level of IFN-γ more than PYR/SDZ when compared with infected untreated group. Both PYR/SDZ and PPQ-8/NTZ-treated groups decrease of serum TNF-α level when compared with the untreated group. Conclusively, PPQ-8/NTZ combination shows promising results, hence it could be a potential combination used alternatively with PYR/SDZ for treatment of toxoplasmosis.

Keywords:T. gondii, cyst, INFγ, TNF, PPQ-8, Nitazoxanide

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1. INTRODUCTION

Toxoplasma gondii (T. gondii) is a protozoan parasite, which causes toxoplasmosis, a world-wide disease infecting all types of human cells. Toxoplasmosis is transmitted mainly by ingestion of the oocyst, yet other methods of transmissions including organ transplantation, blood transfusion and congenital from mother to feotus are reported**(Cantey et al. 2021).** Despite the high prevalence of the disease globally, the combination of pyrimethamine and sulphadiazine is the main line of treatment although it has several side effects and limited efficacy in some cases **(Dunay et al. 2018).**
Nitazoxanide is a broad-spectrum anti-parasitic drug used against several affecting human pathogens **(Fox and Saravolatz 2005)** including the apicomplexan ones Cryptosporidium parvum **(Doumbo et al. 1997)** and Toxoplasma gondii **(El-Kowrany et al. 2019)**

Compound PPQ-8 (4-[2-chloroquinolin-3-yl]-6-[2,5-dimethoxyphenyl]-2-oxo-1,2-dihydropyridine-3carbonitrile), previously described in **(Abd Elgawad et al. 2019; El-Shehabi et al. 2021)** is a quinoline-based agent synthesized via combination of both therapeutically active moieties; 2-chloroquinoline as lipophilic and the diverse heterocyclic ring structure (having a known antiprotozoal activity) directly attached to C3 of quinoline ring. Therefore, the PPQ-8 has the activity of the two compounds forming its structure.
Previous work reported that PPQ-8 in acute model of toxoplasmosis showed reduction of the parasite numbers in liver and spleen with improvement of the pathology in the liver and spleen. In addition, tachyzoites recovered were smaller in size, with distortion and irregularities in shape besides surface protrusions, erosions and peeling were reported when examined by scanning electron microscopy **(Abd**

Elgawad et al. 2019). In chronic infection, few degenerated brain cysts were detected but no inflammatory response within the brain was found. In both models acute and chronic, PPQ-8 prolonged the survival time of mice **(Abd Elgawad et al. 2019).**
This work aims to evaluate the efficacy of co-administration of compound PPQ-8 and Nitazoxanide against acute T. gondii infection model.

2. PATIENTS AND METHODS

Acute infection in mice was induced using the cystogenic ME49 strain of T. gondii. The strain was kindly provided by the department of medical parasitology, Faculty of Medicine, Alexandria University, and then maintained at our laboratory at department of medical parasitology, Faculty of Medicine, Mansoura University by serial passaging in Swiss strain albino mice according to **Cavalcanti et al. (2011).**

- **ANIMALS**

Female Swiss- albino mice were used (6 weeks old and weighing 18–22 g at the beginning of experiments). Mice were housed and offered drinking water and regular mouse feed ad libitum, unless otherwise specified and maintained under controlled conditions of lighting (12 h light/12 h dark cycle) and temperature (25 ± 2oC). All study procedures were conducted in accordance with the international valid animal ethics guidelines for the care and use of experimental animals and approved by Mansoura Faculty of Medicine Institutional Research Board (approval number: **MDP.19.08.24**), Mansoura University, Mansoura, Egypt.

Tested drugs:

Compound PPQ-8; 4-(2-chloroquinolin-3-yl)-6-(2,5-dimethoxyphenyl)- 2-oxo-1,2-dihydropyridine-3-carbonitrile was designed as the reported method **(Abdel-Fattah et al., 2012)** and previously described **(Abdelgwad et al. 2019; El-Shehabi et al. 2021)** was dissolved in Dimethyl sulfoxide (DMSO) as a vehicle and administered orally at a dose of 20 mg/kg/day.

Nitazoxanide (NTZ) was purchased from (*Al-Andalous for pharmaceutical industries, Egypt)* in the form of a powder and was reconstituted with water to form a suspension. The drug was administered orally at 100 mg/kg/day **(Li et al. 2003).**

Pyrimethamine and sulfadiazine (PYR/SDZ) were purchased from Sigma-Aldrich (*St. Louis, USA*) and diluted in polyethylene glycol (PEG) mol. wt. 200, Sigma-Aldrich (*St. Louis, USA*) and administered orally, in combination, at dosages of 4.4 and 250 mg/kg/day **(Chang et al. 1990).**

Mice infection

Mice used for maintaining avirulent ME49 T. gondii strain were sacrificed eight weeks post infection and brains were removed. For experimental infections, fifteen mice were inoculated by oral gavage with 250 µL brain suspensions assessed to contain 20 cysts as acute infection model **(El-Kowrany et al. 2019).**

- **Experimental design:**

Infected mice were randomly allocated into 3 groups, each with 10 mice at the beginning of the experiment. **Group A:** untreated, **group B:** treated with Pyrimethamine and sulfadiazine (PYR/SDZ), while **group C:** treated with PPQ-8 and nitazoxanide (PPQ-8/NTZ).
Treatment was initiated 4 days post-infection (PI) and maintained for 14 consecutive days.
After the end of treatment, five mice from each group were sacrificed and the rest was observed.

- ***Assessment of drug efficacy***

Mice were monitored daily and the following were reported:

- ***Estimation of mortality rate (MR)***

The mortality rate will be calculated according to the following equation:
MR = (Number of dead mice at the sacrifice time/Number of mice at the beginning of the experiment) X100

- ***Estimation of the survival rate***

To determine the survival rate, 5 mice from each group were observed for 30 days after the end of treatment, at which point the survivors were sacrificed.

- ***Determination of Toxoplasma cyst count and size in brain***

Brains from each group was removed and homogenized in 1 ml of PBS. Cysts were measured by ocular micrometer and counted.

- ***Histopathological studies***

Parts of the brain from all studied groups were fixed in Neutral buffered formalin (10%), dehydrated in ascending grades of ethyl alcohol, cleared in xylol and then embedded in paraffin. Serial sections (5µm thick) were cut using microtome and processed for staining using H&E. Slides were microscopically examined and scored according to Batts and Ludwig (1995) as follow: **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. **Score 3:** severe inflammation, severe gliosis, and frequent giant cells **(Batts and Ludwig 1995).**

- ***Immunological study and cytokines assay***

The serum levels of IFN-γ and TNF-α were measured using ELISA, according to the manufacturer's instructions. Data were presented in pg/mL. The limits of detection were determined from standard curves: IFN-γ, 15 pg/mL; TNF-α, 8 pg/mL **(Gaafar et al. 2014).**

STATISTICAL ANALYSIS:

Data were analyzed using statical package for social sciences (SPSS) software (*SPSS Inc., Chicago, USA*), version 16.0. 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 is equal to or less than 5% ($P \leq 0.05$). 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.

3. RESULTS

One mouse died at the sacrifice time among both groups of PYR/SDZ and PPQ-8/ NTZ, while two mice died from infected untreated group. The reported mortality rate was 20% for both PPQ-8/ NTZ and PYR/SDZ-treated groups and 40% within the infected untreated group with no significant difference among groups.

Thirty days post-treatment, the survival rate was 60% for infected untreated group and increased to 80% in PYR/SDZ and PPQ-8/ NTZ-treated groups. The median survival duration of the control untreated group was 3.66 weeks, while the median survival duration of PYR/SDZ and PPQ-8/NTZ groups were 3.86 and 3.77 weeks (P = 0.825), respectively **(Figure 1).**

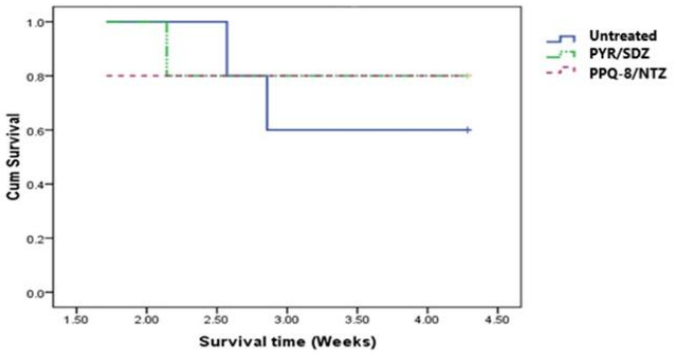

Fig. (1) Kaplan-Meier survival curves for all mice groups. Thirty days post-treatment, the infected untreated group had survival rate of 60% with median survival duration of 3.66 weeks mice in infected and treated with PYR/SDZ and PPQ-8/ NTZ had survival rates of 80%, with median survival duration of 3.86 and 3.77 weeks (P = 0.825), respectively. (Days of survival were counted from the starting of treatment).

Mice group infected and treated with PYR/SDZ showed significant reduction (P <0.001) in the mean number of brain cyst count with percent reduction of 83.3% when compared with untreated group, while infected mice treated with PPQ-8/NTZ showed significant reduction (P < 0.001) in the mean number of brain cysts by 80%, compared with infected untreated mice **(Table 1).** In addition, there was non-significant difference (P = 0.765) in brain cysts count and size of infected mice treated with PPQ-8/NTZ when compared with those infected and treated with PYR/SDZ **(Table 1).** There was statistically significant (P = 0.001) reduction in the size of cysts in the brain homogenate of infected mice treated with PYR/SDZ when compared with infected untreated group with percentage reduction of 41.03% **(Table 1).** Infected mice group treated with PPQ-8/NTZ showed significant reduction (P = 0.001) by 49.7%, when compared with infected untreated group **(Table 1).** In addition, there was significant difference (P = 0.04) in brain cysts size of infected mice treated with PPQ-8/NTZ when compared with those infected and treated with PYR/SDZ **(Table 1).**

Table (1): Effect of treatment with PYR/SDZ and PPQ-8/NTZ on cyst count and diameter in brain homogenates from Toxoplasma gondii -infected mice

Animal groups (N = 5)	***Toxoplasma gondii* cyst in brain homogenates (10 µL)**	
	Cyst count (% of reduction)	**Cyst diameter (µm) (% of reduction)**
Infected untreated	6.0 ± 1.41	40.36 ± 1.25
Infected and treated with PYR/SDZ	1.0±0.7 (83.3%)	23.80 ± 3.56 (41.03%)
Infected and treated with PPQ8/NTZ	1.2 ± 0.84 (80%)	20.30± 2.28 (49.7%)
P-Value	<0.001* P1<0.001* P2<0.001* P3=0.765	<0.001* P1 <0.001* P2<0.001* P3=0.04*

Values were expressed as means ± SD.
Values between parentheses refer to the percentage of reduction compared with infected non-treated group.
P1: difference between untreated & treated with PYR/SDZ.
P2: difference between untreated & treated with PPQ8/NTZ.
P3: difference between treated with PYR/SDZ, & treated with PPQ8/NTZ.
* Indicates significance at $P \leq 0.05$.

In infected untreated mice the mean serum level of IFN-γ was 10.09±0.15 pg/ml. In comparison, PYR/SDZ induced significant increase (P1<0.001) in the serum IFN-γ level with mean 12.25±0.21 pg/ml, also PPQ-8/NTZ significantly more increase (P1<0.001) in the production of serum IFN-γ with a mean of 13.44±0.26 pg/ml. **(Table 2).** In infected untreated mice showed a serum TNF-α with a mean 50.24±0.21 pg/ml. in group treated with PYR/SDZ there was significant reduction in serum level of TNF-α with mean of 30.35±0.12 pg/ml **(Table 2).** Mice treated with PPQ-8/NTZ showed significant but less reduction (P1<0.001, 29.9%) in the production of serum TNF-α with a mean of 35.21±0.19 pg/ml **(Table 2).**

Table (2): Effect of treatment with PYR/SDZ and PPQ-8/NTZ on levels of IFN-γ and TNF-α in serum from Toxoplasma gondii-infected mice

Animal groups (N = 5)	IFN-γ (pg/ml)	TNF-α (pg/ml)
Infected untreated	10.09±0.15	50.24±0.21
Infected and treated with PYR/SDZ	12.25±0.21 (21.4%)	30.35±0.12 (39.59%)
Infected and treated with PPQ8/NTZ	13.44±0.26 (33.2%)	35.21±0.19 (29.9%)
P-Value	P<0.001* P1<0.001* P2<0.001* P3<0.001*	P<0.001* P1<0.001* P2<0.001* P3<0.001*

Values were expressed as means ± SD.
Values between parentheses refer to the percentage of reduction compared with infected non-treated group.
P1: difference between untreated & treated with PYR/SDZ.
P2: difference between untreated & treated with PPQ8/NTZ.
P3: difference between PYR/SDZ, & treated with PPQ8/NTZ.
* Indicates significance at P ≤ 0.05.

Generally, Toxoplasma lesions in the brains of mice were characterized by meningitis and encephalitis with glial nodules, vascular cuffing by lymphocytes and focal mononucleated cell infiltrates besides diffuse infiltrates of mononuclear cells were also found.

Infected untreated mice had score 3 that showed severe inflammation, necrosis and appearance of dense lymphoplasmacytic inflammatory cellular infiltrate, many giant cells with dense reactive gliosis **(Fig. 2A, B).** Mice treated with PYR/SDZ had score 1 and 2 with moderate lymphocytic inflammatory cellular infiltrate and mild reactive gliosis with few giant cells. some degenerated neurons were seen **(Fig. 2C, D).** Brain sections of mice treated with PPQ-8/NTZ had score 1 with mild lymphocytic inflammatory cellular infiltrate together with some degenerated neurons, mild reactive gliosis and scattered giant cells degenerated Toxoplasma cysts with surrounding mild reactive gliosis **(Fig. 2E, F).**

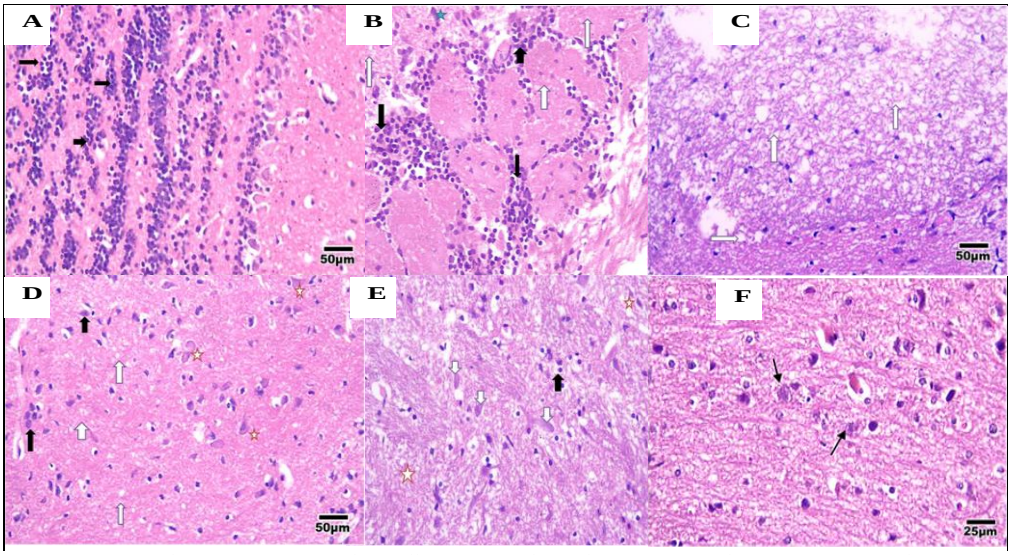

Fig. (2) Histopathological study of brain sections (H&E) from different groups of mice for Acute model of Toxoplasma gondii infection. (A) Brain tissue of infected untreated group revealed dense lymph-plasmacytic inflammatory cellular aggregates (black arrows) (x200). (B) Brain tissue of infected untreated group revealed dense lymphoplasmacytic inflammatory cellular infiltrate (black arrows) together with dense reactive gliosis (white arrows) (x200). (C) Brain tissue of infected group treated with PYR/SDZ revealed moderate reactive gliosis (white arrows) (x200). (D) Brain tissue of infected group treated with PYR/SDZ revealed moderate lymphocytic inflammatory cellular infiltrate (black arrows) together with moderate reactive gliosis (white arrows) with some degenerated neurons (white stars) (x200). (E) Brain tissue of infected group treated with PPQ-8/NTZ revealed mild lymphocytic inflammatory cellular infiltrate (black arrows) together with some degenerated neurons (white arrows) & mild reactive gliosis (white stars) (x200). (F) Brain tissue of infected group treated with PPQ-8/NTZ revealed degenerated Toxoplasma cyst (black arrow) with surrounding mild reactive gliosis (x400).

Table (3): Inflammatory score of brain tissue from Toxoplasma gondii-infected mice and treated with PYR/SDZ and PPQ-8/NTZ

Animal groups (N = 5)	Inflammatory score
Infected untreated	2.34±0.21
Infected and treated with PYR/SDZ	0.958±0.02
Infected and treated with PPQ8/NTZ	0.848±0.01
P-Value	P<0.001* P1<0.001* P2<0.001* P3=0.177

Values were expressed as means ± SD.
Values between parentheses refer to the percentage of reduction compared with infected non-treated group.
P1: difference between untreated & treated with PYR/SDZ.
P2: difference between untreated & treated with PPQ8/NTZ.
P3: difference between PYR/SDZ, & treated with PPQ8/NTZ.
* Indicates significance at $P \leq 0.05$.

4. DISCUSSION

Toxoplasma gondii infects all nucleated cells in human body. In acute infection, the parasite reaches different organs through blood and lymphatic circulation, while in chronic stage, cystic lesion is a common form seen in different organs as brain, eye and skeletal muscles **(Dubey and Jones 2008).** The unique pathogenesis of T. gondii presents challenges for drug therapy.

Treatment of toxoplasmosis in human is still hard to conduct. 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 are associated with numerous and severe side effects **(Dunay et al. 2018).** Consequently, the search for alternative therapeutic medicines is of great priority.

The current available treatment for toxoplasmosis uses the combination of sulfadiazine and pyrimethamine but it is not suitable for use in all cases and it has severe side effects, so non-toxic and more effective new chemotherapeutic alternatives are urgently needed.

The results presented here clearly demonstrate the anti-Toxoplasma activity of the combination of PPQ-8 and NTZ in murine infection induced with a mouse-non-virulent (type-2) parasite.

In this study, treatment using the PYR/SDZ and PPQ-8/ NTZ increases the survival duration when compared with infected untreated. In addition, the reported mortality rate was 20% for both PPQ-8/ NTZ and PYR/SDZ-treated groups and 40% within the infected untreated group.

Previous work reported that MR when using NTZ alone with the same dose used in this study is 24% **(El-Korany et al. 2019)**, so addition of PPQ-8 reduces the mortality rate by a mechanism which needs to be elucidated.

Herein, the use of PPQ-8 combined with NTZ was efficient in reducing cyst count compared to PPQ-8 and NTZ alone **(Abd Elgawad et al. 2019; El-Kowrany et al. 2019).** This significant synergistic effect although still lower than PYR/SDZ combination but could be used in control of toxoplasmosis in a reduced dosage with few side effects.

Histopathological studies showed improvement of brain pathology, presence of degenerated cysts and mild reactive gliosis with reduced inflammatory score when using PPQ-8/NTZ. These findings were expected due to reduction in brain tissue parasitism and cyst count.

Previous work, NTZ is partially absorbed from the gastrointestinal tract and then rapidly hydrolyzed into the active metabolite; tizoxanide**(Stockis et al. 1996),** which has poor permeability to blood brain barrier **(Ruiz-Olmedo et al. 2017),** so we may expect the effect on brain pathology is probably due to PPQ-8.

In our previous work, we reported that PPQ-8 in a dose of 20 mg/kg reduced the number of cysts to 40.65%, probably due to direct effect on the parasite as the majority of the detected cysts have degenerated contents **(Abd Elgawad et al. 2019).** Although there is no complete clearance of the tissue cyst and but the remaining cysts in our study were smaller in size, suggesting that PPQ-8 effectively penetrates the blood brain barrier (BBB) and it is able to retard the parasite growth and development.

The ability of PPQ-8 to permeate the blood brain barrier (BBB) is expected based on the calculated lipophilicity (clogP) of PPQ-8, which equals to 3.25, so, PPQ-8 is relatively lipophilic and is more likely to permeate the BBB and the cell membrane **(Banks 2009; Yang and Hinner 2015).**

In the study using PPQ-8/NTZ caused significant increase in the serum level of IFN-γ and TNF-α more than PYR/SDZ, when compared with infected untreated control.

Infection with T. gondii elicit mainly cellular immune response via macrophages, dendritic cells and T lymphocytes (Th1), which secrete pro-inflammatory cytokine such as interferon-gamma (IFN-γ), tumor necrosis factor (TNF-α) and IL-6 **(Dupont et al., 2012).**

IFN-γ triggers several anti-parasitic mechanisms in macrophages and natural killer cells, including the production of nitric oxide, reactive oxygen intermediates and the induction of tryptophan starvation **(Däubener et al. 1996; Lang et al. 2007).** This immune response leads to control of infection by inducing the conversion of the fast-replicating tachyzoite to the slow-replicating bradyzoite located in tissue cysts **(Bohne et al. 1999).** However, this immune response is not able to completely clear Toxoplasma infection and the tissue cysts remain largely quiescent for the life of the host, but in immunocompromised patients, they can reactivate and cause life-threatening diseases **(Suzuki et al. 2010).**

The immunomodulatory effect of NTZ will maintain the chronic state of infection which will give a chance for PPQ-8 to work since we have found in our previous work that PPQ-8 worked better on chronic state of infection **(Abd Elgawad et al. 2019).**

The ability of PYR/SDZ to fight T. gondii infection is attributed to a direct anti-parasitic effect without adequate stimulation of the immune system, this may lead to rupture of the tissue cysts reactivate the disease **(Wei et al., 2015).**

The ability of PPQ-8/NTZ to produce less reduction than PYR/SDZ is important since TNF-α plays an important role specifically in protecting against T. gondii infection, some studies have documented the important role of TNF-α in the resistance to acute and chronic Toxoplasma infection **(Deckert-Schlüter et al., 1998; Yap et al., 1998)** reduction of TNF-α level by anti-TNF agents, etanercept, leads to reactivation of latent toxoplasmosis infection by ME49 strain as well as increase the risk of acute toxoplasmosis **(El-Sayed et al., 2016).** In addition, blocking this cytokine may increase risk of infectious complications **(Toussirot et al. 2007).**

Herein, the combination of PPQ-8/NTZ has better effect than using PPQ-8 alone. The anti-protozoan effect of NTZ could be due to direct effect on Toxoplasma since NTZ depolarizes the mitochondrial membrane along with the inhibition of quinone oxidoreductase NQO1, nitroreductase-1 and protein disulphide isomerase. NTZ also inhibits the glutathione-S-transferase (a major detoxifying enzyme) and modulates a gene (avr-14 gene) encoding for the alpha type subunit of glutamate-gated chloride ion channel present in the nematodes **(Dupouy-Camet 2004).**

Although the mechanism of action of PPQ-8 is still to be clarified but it is probably due to direct effect on the parasite since previous work reported that the majority of the brain cysts have degenerated contents **(Abd Elgawad et al. 2019)** and retarded tachyzoites.

In conclusion, the potential combination of PPQ-8/NTZ points to the need to extend these studies on large scale aiming for a new treatment of human toxoplasmosis. The administration of PPQ-8 could be considered as a possibility to develop new treatment combination for toxoplasmosis, leading to fast recovery and less relapses of the disease. Also, such combination could be a potential substitute of PYR / SDZ and is an opportunity to decrease PPQ-8/NTZ doses and their related side effects.

DECLARATIONS

- **ETHICAL APPROVAL**

All study procedures were conducted in accordance with the international valid animal ethics guidelines for the care and use of experimental animals and approved by Mansoura Faculty of Medicine Institutional Research Board (approval number: MDP.19.08.24), Mansoura University, Mansoura, Egypt.

- **COMPETING INTERESTS**

The authors declare no competing interest.

- **AUTHORS' CONTRIBUTIONS**

Amira Taman: Initiates the idea, put the plan for work, guide the experimental work, wrote the manuscript.
Ahmed Gomaa: Conducted the experimental work.
Ayat El blihy: Participated in the experimental work.
Basem Mansour: Participated in the experimental work.
Mona Younis: Participated in the experimental work (Histopathology)
Goman El Ganyny: Revised, edited the manuscript.
All authors reviewed and approve the final version of the manuscript.

- **FUNDING:** NA

- **Availability of data and materials**

The data and parasitological materials are available in the Department of Medical Parasitology, Faculty of Medicine, Mansoura University.

Consent to participate

All authors have declared their participation in this research.

Consent for publication

All authors have read and approved the final version of this paper for publication.

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