Vitamin D3: Emerging Role in Murine Toxoplasmosis

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Key words:
Toxoplasmosis, Mice, Vitamin D3, Nitazoxanide .

Background and aim of the study: Toxoplasmosis is one of the neglected infections which has a global distribution. Effective treatment against toxoplasmosis is hard to accomplish. Accordingly, we tried to investigate the effect of using vitamin D_3 alone and in combination with nitazoxanide in *Toxoplasma gondii* infected mice.

Materials and Methods: The study was conducted on 100 Swiss albino mice which were infected with the avirulent (ME49) *Toxoplasma gondii* strain. Mice were equally divided into 5 groups: (GI): Infected untreated control group; (GII): Infected and treated by spiramycin; (GIII): Infected and treated by vitamin D_3; (GIV): Infected and treated by nitazoxanide and (GV): Infected and received a (vitamin D_3 –nitazoxanide) combination. The therapeutic impacts of drugs were assessed using; parasitological, histopathological and immunological approaches.

Results: The study showed that the vitamin D_3- nitazoxanide combination induced a significant reduction in the parasitic load in the brain sections of the infected mice. We have also reported a delicate balance in the overall immune response against the parasite after using the drug combination. It was noted that vitamin D_3, used alone, did not significantly reduce the parasitic load despite the notable improvement in the degree of inflammatory infiltration.

Conclusion: The present study revealed that the vitamin D_3–nitazoxanide combination was highly potent in restricting the parasitic load. It controlled the infection without the harmful immunopathological impacts. Thus, vitamin D_3 could be a valuable candidate, either as a sole agent or as an adjunct to other anti-parasitic therapies, in areas where toxoplasmosis is endemic.

INTRODUCTION

Toxoplasma gondii (*T.gondii*) is an intracellular apicomplexan parasite with a broad diversity of intermediate hosts, including humans **[1].** The parasite affects approximately 30 to 50% of the world population **[2].** Although toxoplasmosis is generally asymptomatic in immunocompetent individuals, it causes severe sequelae in immunocompromised hosts. Active infection during pregnancy may cause abortion, congenital anomalies or perinatal death **[3].**

Till now, there is no available single gold standard treatment for toxoplasmosis. Pyrimethamine, sulfonamides or their combinations have been frequently used in the severe form of the disease. However, they were commonly associated with numerous undesirable side effects **[4].** Spiramycin is a potent bacteriostatic macrolide that has been used in the treatment of different parasitic infections, like; toxoplasmosis and cryptosporidiosis **[5, 6]**. It produces a high tissue concentration in the placenta without crossing the placental barrier. Accordingly, it is the drug of choice against *T.gondii* infection during pregnancy. Yet, the emergence of resistant strains of the parasite would potentially limit the therapeutic efficacy of the drug **[7].**

With the increasing number of high-risk individuals, continuous efforts are necessary for generating novel treatment options against *T.gondii* infection **[8].** Recent research studies have focused on developing 1, $25(OH)_2$ D_3 as a potential therapeutic agent. In addition to its role in regulating calcium (Ca) and

phosphorus metabolism, vitamin D_3 is an immune modulator **[9,10]**. Kearns et al. **[11]** has demonstrated its therapeutic immunoregulatory effects in resolving various infectious diseases. The hormonal form of vitamin D, 1, 25-dihydroxyvitamin D_3, is known for its immunosuppressive, anti-proliferative and pro-apoptotic activities. An inhibitory effect of vitamin D_3 on the immune system by targeting T helper 1(Th-1) lymphocytes has been described **[12]**.

Nitazoxanide (NTZ) [2-acetyloxy-N-(5-nitro-2-thiazolyl) benzamide] is a broad-spectrum anti-parasitic compound. It acts against a wide range of cestodes, nematodes and protozoa with notable effects on apicomplexan parasites, particularly, *Cryptosporidium parvum* **[13].** NTZ inhibits pyruvate-ferredoxin oxidoreductase (PFOR) enzyme which is necessary for anaerobic metabolism, without the formation of free radicals or deterioration of the host cells **[14].** Nitazoxanide has been applied in different experimental models for the treatment of toxoplasmosis. **Galvan-Ramirez et al. [15]** reported that nitazoxanide drug reduced *T.gondii* infection more efficiently than pyrimethamine in cultured rat astrocytes and without causing any toxicity to the host cells.

Various studies have analyzed the role of vitamin D in different intracellular protozoan infections like; *Leishmania Mexicana* and malaria **[16, 17].** Yet, there's quite a lack of reports which investigated its role in *T.gondii* infection. Hence, the present work aimed to evaluate the therapeutic effects of vitamin D_3 as a sole agent and after its combination with nitazoxanide in *T. gondii* infected mice.

MATERIALS AND METHODS

The avirulent (ME49) *T.gondii* strain:

Regular maintenance of the strain was achieved through oral administration of the Swiss albino mice with diluted brain suspension of previously infected mice containing approximately (25 cysts in 0.1 ml). The process was repeated every 45-60 days **[18]**.

Mice grouping and experimental design:

The study was conducted at the post-graduate research laboratory at the Medical Parasitology Department, Faculty of Medicine, Zagazig University. It was carried out on 100 laboratory-bred Swiss albino male mice, aged 7 - 8 weeks, weighing 20-25 g. All mice were housed in well-ventilated cages and were regularly offered standard pellet food and water **[19]**. Examination of mice faeces was performed to exclude any parasitic infections **[20]**. The selected mice were equally divided into the following groups:

(GI): Infected untreated control group.

(GII): Infected and treated by spiramycin.

(GIII): Infected and treated by vitamin D_3.

(GIV): Infected and treated by nitazoxanide.

(GV): Infected and received a combination of vitamin D_3 and nitazoxanide.

Drugs:

Spiramycin (Rovac®, Delta Pharma, Egypt) was administered at a dose of 400 mg/kg/day **[21]**. The drug was given at a fixed hour daily for seven days.

Nitazoxanide (Al- Andalous for pharmaceutical industries, Egypt) was given orally at a dose of 150 mg/kg/day, for 14 days, based on previous studies on experimental cryptosporidiosis **[22]**.

Both spiramycin and nitazoxanide were administered starting from the 45th day post-infection, at the onset of chronic toxoplasmosis, according to the drug table of Paget and Barnes **[23]**.

Vitamin D_3 solution (Product No: 740292, Sigma–Aldrich, Cairo, Egypt) was administered intraperitoneally to each mouse at a dose of 0.5µg/kg/ 2days. The treatment began three days before the infection (two doses) and continued for 14 days (seven doses). The timing of commencing Vitamin D_3 treatment as well as the duration of therapy were conducted according to the protocols described by Rajapakse et al. **[24,43]**.

Upon completion of the treatment, all animals were sacrificed after the last dose of therapy by cervical dislocation.

Experimental infection:

Infection of mice was initiated by oral administration of 25 cysts in 0.1 ml of diluted brain suspension using a stomach tube. The infection was obtained from the brain tissues of another mouse, which was infected 45-60 days earlier **[18]**. The mouse was sacrificed and the brain was removed and homogenized in 1-ml saline under sterile conditions. A glass

homogenizer was used to release tissue cysts. One drop (25 µl) of the brain homogenate was spread on a slide and microscopically examined. Tissue cysts were then counted in four drops and multiplied by 10 to estimate the number of cysts in one ml of the brain suspension **[25]**. The infective dose was determined to be 25 cysts in 0.1 ml of the brain suspension. Tissue cysts in brain homogenate of *T. gondii* infected mice are shown in Figure (1). The therapeutic effects of the used drugs were assessed using; parasitological, histopathological and immunological measures:

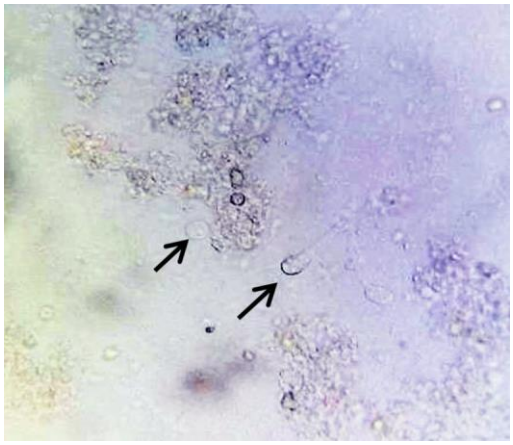

Figure 1: *Toxoplasma gondii* tissue cysts in brain suspension. Light microscopic examination of tissue cysts obtained in brain suspension of *T.gondii* infected mice (black arrows, X400)

1- Parasitological assessment:

Parasite load:

T.gondii tissue cysts containing bradyzoites were counted in Giemsa stained smears from the brain sections of all study groups. In each mouse, the brain tissues were examined using oil immersion. The mean value of 10 different fields was calculated for each group **[26]**.

Parasite reduction percentage (%R):

The reduction in the parasite burden was calculated using the following equation:

$$\%R = \frac{100(C - E)}{C}$$

% R: % reductions, C: control group and E: experimental groups of mice [27].

2- Histopathological assessment:

Tissue samples from brain tissues were fixed in 10% formal saline solution, dehydrated in ascending grades of ethyl alcohol, then, they were cleared in xylol, and kept as paraffin blocks. Sections of 4 µm thickness were cut and stained with haematoxylin and eosin (H&E) **[28]**. All slides were then examined microscopically for detection of *T.gondii* tissue cysts. In the brain tissues, the total numbers of focal or diffuse inflammatory foci were counted and inflammatory infiltrations in the meninges were analyzed. The inflammatory score was represented as arbitrary units as follow; 0–2, mild; 2–4, moderate; 4–6, severe; and above 6, very severe **[29]**.

3. Immunological assessment:

Estimation of interferon-gamma (IFN-γ) levels by ELISA:

Serum samples were obtained from all infected and control groups at the end of the study (60 days post-infection) **[30]**. Interferon-gamma (IFN-γ) levels in serum samples were estimated using Mouse IFN- γ ELISA Set (BD Biosciences Pharmingen, Cat. No. 555138, San Diego, CA 92121, USA). The kit was used according to the manufacturer instructions. A photometric measurement at 450 nm was carried out to estimate the absorbance concentration of the samples.

Statistical methods:

Data were analyzed using Statistical Package for the Social Sciences "SPSS version 22". ANOVA F-test was performed to calculate the difference between quantitative variables among different study groups **[31]**. Quantitative data were described using mean and standard deviation (SD). P value<0.05 indicated significant results **[32]**.

RESULTS:

1. Parasitological results

Parasitic count

Toxoplasma gondii tissue cysts were detected in impression smears from the brain tissues of all infected groups with variable densities. The mean values of the parasitic burden obtained in different study groups are demonstrated in Figure (2). Our results revealed a statistically significant difference in the mean cyst counts among the studied groups (P<0.001, Table 1). In the infected untreated group (GI), the mean count of tissue cysts per oil impression fields was (11.03±3.5) (Table 1, Figure 2). In (GV), after

combining vitamin D_3 and nitazoxanide, the reduction percentage reached (78.24%) with a highly significant difference as compared to the infected untreated group (GI) (P<0.001, Table 1). It was noted that, vitamin D_3, used alone in (GIII), did not significantly reduce the parasitic load. The reduction percentage in cyst counts was only (27.47%) (Table 1).

2- Histopathological results:

The brain sections of the infected untreated group (GI) showed multiple *T. gondii* cysts (Figure 3a), along with several pathological sequelae, like severe meningitis with marked mononuclear cellular infiltrations (Figure 3 b, c, respectively). Resolution of the pathological changes was reported in all of the treated groups. In (GIII), after administration of vitamin D_3, a remarkable reduction in the degree of cellular inflammation was denoted (Figure 3d). Nitazoxanide induced a notable improvement in the degree of meningitis (Figure 3e). Degeneration of tissue cysts was demonstrated in (GV) (Figure 3f).

Forty (40%) of *T.gondii* infected untreated mice (GI) exhibited severe inflammatory cellular infiltration. However, the severity percentage didn't exceed (15%) among different treated groups (Figure 4). There was no significant difference in the degree of inflammatory infiltration changes among the treated groups (P > 0.05, Table 2).

3- Immunological results:

(IFN-γ) measurement:

Serum levels of IFN-γ were assessed as an indicator of the immunomodulatory effect of the different drugs used during the study (Figure 5). The infected untreated group (GI) showed a serum IFN-γ level with a mean value of (125.2± 39.9) pg/ml. The nitazoxanide-treated group (GIV) exhibited the highest level of IFN-γ production (280.7±88.8) with statistically significant difference, compared to (GI) (P < 0.001, Table 3). On the other hand, the lowest serum levels of the cytokine (93.23± 25.6, P< 0.05), were obtained in the vitamin D_3-treated group (GIII), as shown in Table (3).

Table (1): The parasite count and reduction percentage in the brain tissues of different study groups .

Brain /%R	GI (n=20)	GII (n=20)	GIII (n=20)	GIV (n=20)	GV (n=20)	F	P value
Mean ±SD	11.03±3.5	4.0 ± 1.2$^{\$}$	8.0 ± 2.3	5.6 ± 1.8$^{\$}$	2.4 ±0.8$^{\$\$}$	50.53	**<0.001****
%R		63.74%	27.47%	49.23%	78.24%		

GI: Infected untreated control group; GII: Infected-spiramycin treated group; GIII: Infected-vitamin D_3 treated group; GIV: Infected-nitazoxanide treated group; GV: Infected and received combination of nitazoxanide and vitamin D_3; % R: percentage of reduction; SD: standard deviation; n: number of mice in each group.
F: F test (ANOVA); **P<0.001(statistically highly significant)
$: significant with GI; $$: highly significant with GI

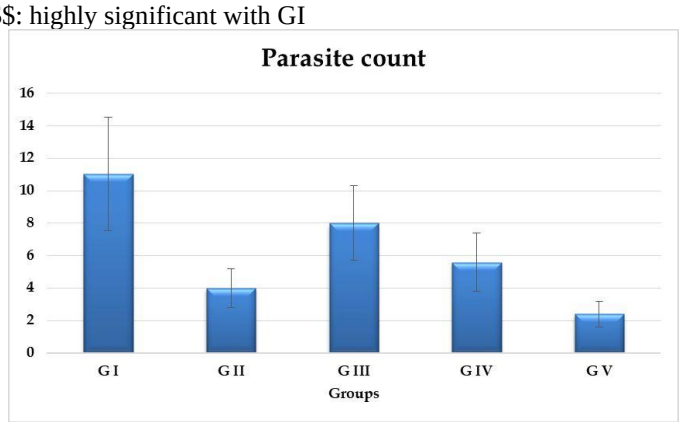

Figure 2: Parasite count in the brain sections of different groups. Mean *T.gondii* cysts count in the brain sections of different groups; GI: Infected untreated control group; GII: Infected-spiramycin treated group; GIII: Infected-vitamin D_3 treated group; GIV: Infected-nitazoxanide treated group; GV: Infected and received combination of vitamin D_3 and nitazoxanide.

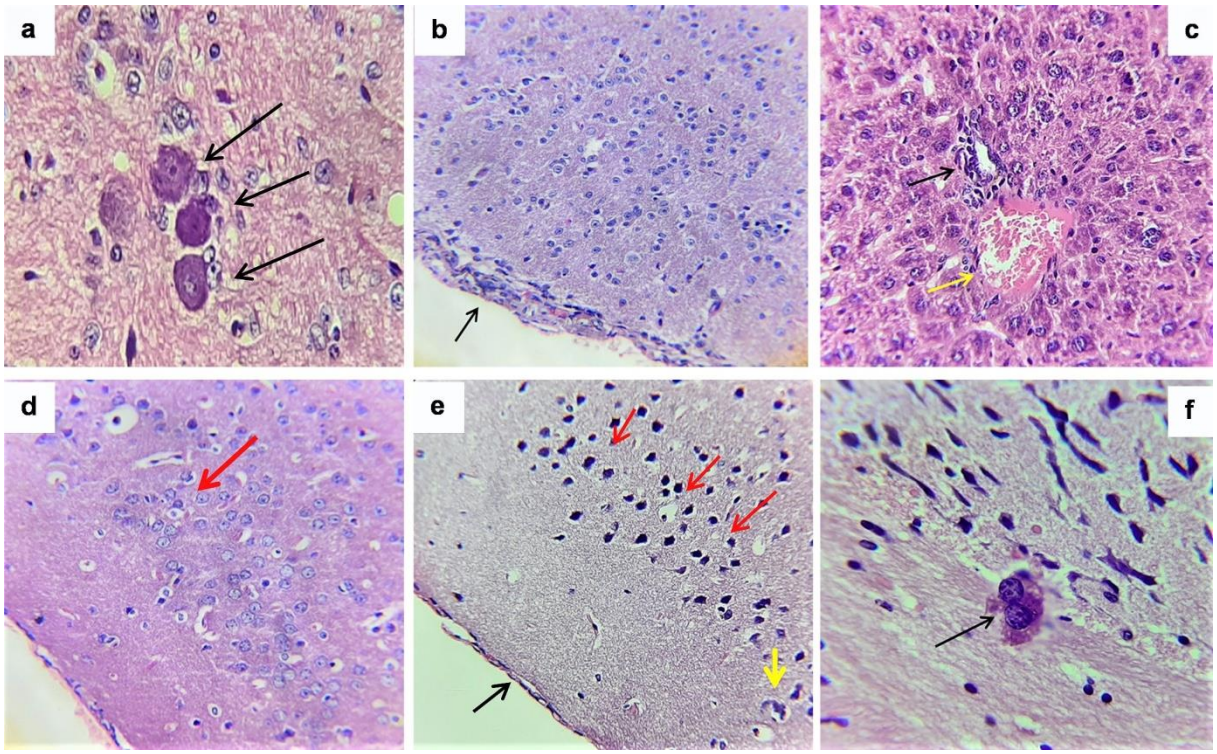

Figure 3: Histopathological findings in the brain sections of different infected mice. Brain sections of different groups: (a,b,c) Infected untreated control group (GI), showing (a) Multiple T. gondii cysts (black arrows) (b) Severe inflammation of the meninges (black arrow) (c) Severe mononucelar cellular infilartion (black arrow) and dilated vessel containing inflammatory cells (yellow arrow). (d) Brain section in the infected-vitamin D_3 treatd group (GIII) showing mild infiltration of mononuclear cells (red arrow). (e) Brain section of the infected–nitazoxanide treated group (GIV) showing moderate cellular infiltrations (red arrows), mild meningitis (black arrow), with vascular congestion (yellow arrow) (f) Sections in the brain of the mice infected and received (vitamin D_3- nitazoxanide) combination (GV), showing degeneration of existing *T. gondii* cyst (black arrow) [H&E stain, X 400].

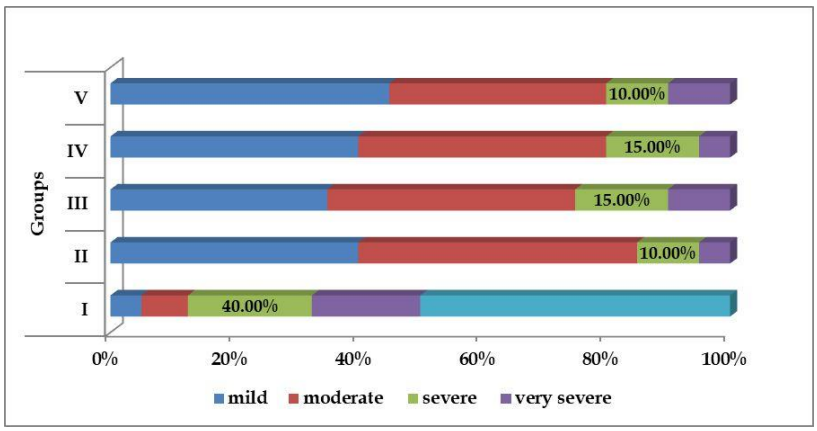

Figure 4: Inflammatory infiltration changes in the brain sections of different study groups. Distribution of cellular inflammatory infiltrations among different groups: GI: Infected untreated control group; GII: Infected-spiramycin treated group; GIII: Infected-vitamin D_3 treated group; GIV: Infected-nitazoxanide treated group; GV: Infected and received (vitamin D_3 -nitazoxanide) combination.

Table (2): Inflammatory infiltration changes among experimentally treated groups.

Inflammatory infiltration degrees	GII (n=20)		GIII (n=20)		GIV (n=20)		GV (n=20)		χ^2	DF	P value
	N	%	N	%	N	%	N	%			
Mild	8	40.00%	7	35.00%	8	40.00%	9	45.00%	1.57	9	0.99 **NS**
Moderate	9	45.00%	8	40.00%	8	40.00%	7	35.00%			
Severe	2	10.00%	3	15.00%	3	15.00%	2	10.00%			
Very severe	1	5.00%	2	10.00%	1	5.00%	2	10.00%			

GI: Infected untreated control group; GII: Infected-spiramycin treated group; GIII: Infected-vitamin D_3 treated group; GIV: Infected-nitazoxanide treated group; GV: Infected and received combination of nitazoxanide and vitamin D_3; n: number of mice in each group.
$\chi 2$: Chi square test; DF: Degree of freedom; NS: P>0.05 (statistically non-significant)

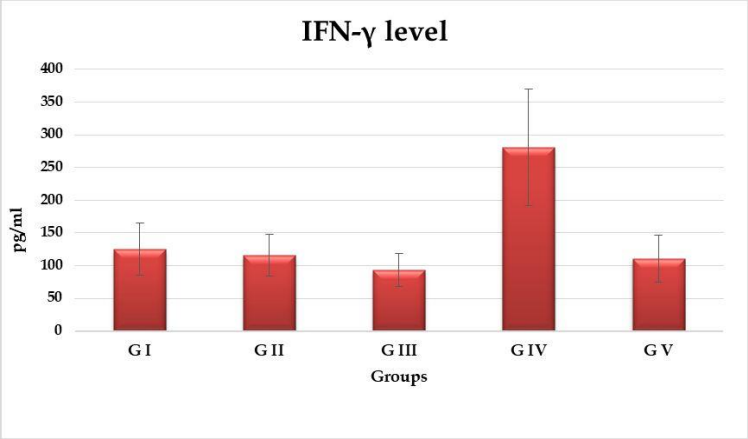

Figure 5: IFN-γ serum Levels in different groups. Mean values of IFN-γ serum Level (pg/ml) in different study groups: GI: Infected untreated control group; GII: Infected and treated by spiramycin; GIII: Infected and treated by vitamin D_3; GIV: Infected and treated by nitazoxanide; GV: Infected and received (vitamin D_3 -nitazoxanide) combination.

Table (3): IFN-γ Level (pg/ml) in serum samples of different study groups.

IFN-γ	GI (n=20)	GII (n=20)	GIII (n=20)	GIV (n=20)	GV (n=20)	F	P
Mean±SD	125.2± 39.9	115.6±32.2	93.23± 25.6$^{\$}$	280.7±88.8$^{\$\$}$	110.45± 36.3	47.14	**<0.001****

GI: Infected untreated control group; GII: Infected-spiramycin treated group; GIII: Infected-vitamin D3 treated group; GIV: Infected-nitazoxanide treated group; GV: Infected and received combination of nitazoxanide and vitamin D_3; SD: standard deviation; IFN-γ: Interferon-gamma; n: number of mice in each group.
F: F test (ANOVA); **P<0.001(statistically highly significant)
$: significant with GI; $$: highly significant with GI

DISCUSSION

Over the past 20 years, the treatment scheme of human toxoplasmosis remained unchanged **[33]**. Most of the currently used therapeutic drugs are associated with numerous and severe side effects **[34]**. Furthermore, owing to the growing resistance of the parasite, the current anti-Toxoplasma drugs have become less effective due to the evolving genetic mutations **[35]**. In line with this, the search for alternative drugs or drug combinations demands a challenging effort **[4]**.

The antimicrobial effects of vitamin D_3 are due to its over-expression of different antimicrobial peptides such as beta-defensin2 and cathelicidin **[36]**. Moreover, vitamin D_3 is a good inducer of nitric oxide (NO) production **[37]**, which in turn, has potent antimicrobial and anti-parasitic properties **[38]**.

Nitazoxanide (NTZ) was used in the treatment of different parasitic infections. It has shown excellent in vitro activities against a wide variety of protozoa and helminths **[39]**. Several recently conducted studies have managed to evaluate the

effect of the drug in experimental *T.gondii* infection **[34, 40, 41]**.

Our study has demonstrated a significant difference (P< 0.001) in the mean *T.gondii* cysts count among different groups. A notable reduction in the parasitic load was demonstrated in (GII), after spiramycin intake (Table 1). The anti-parasitic potential of spiramycin against *T.gondii* was supported by Grujić et al. **[42]** who reported the ability of the drug to effectively reduce cyst numbers in both acute and chronic murine toxoplasmosis. The current study also denoted that despite the reduction in the parasitic load in the vitamin D_3,-treated group (GIII), the difference was not statistically significant (P>0.05) when compared with (GI) (Table 1). Likewise, Rajapakse et al. **[43]** have proved the role of vitamin D_3 in reducing the parasitic burden in *T.gondii* infected mice by inhibiting the intracellular proliferation of the parasite. Conversely, Ghaffarifar et al. **[44]** have reported that using vitamin D_3 did not have any considerable effect on the inhibition of tachyzoites proliferation in mice experimentally infected with the virulent (RH) *T.gondii* strain.

According to our results, the combination between vitamin D_3 and nitazoxanide in (GV) has significantly reduced the parasitic count (P<0.001, Table 1). Different combination trials have been experimentally introduced for the treatment of toxoplasmosis. Etewa et al. **[45]** presented a combination between spiramycin and methotrexate. Yet, they reported an increase in the cysts number in the brain tissues of the experimentally treated mice. Omar et al. **[46]** denoted that the combination between spiramycin and the anti-inflammatory drug, aminoguanidine, significantly reduced the parasitic burden in T.gondii infected mice.

Histopathological findings in the present work, have matched the parasitological results. The infected untreated group (GI) which exhibited the highest parasitic load (Table 1), had also shown several pathological alterations, like; severe meningeal inflammation and marked mononuclear cellular infiltrations (Figure 3 b, c, respectively). The study has also reported a notable reduction in the degree of cellular inflammatory infiltrations in the vitamin-D_3 treated group (GIII) (Figure 3d). These results come in accordance with Rajapakse et al. **[24]** and Rajapakse et al. **[43]** who reported improvement of pathological changes along with a reduction in the parasite burden after vitamin D_3 treatment in T.gondii infected mice.

A marked reduction in the degree of meningitis was noticed after administration of nitazoxanide in (GIV) (Figure 3e). The promising effect of nitazoxanide in reducing the degree of inflammation in brain tissues of *T. gondii* infected mice was endorsed by El-Kowrany et al. **[40]**. The combination between vitamin D_3 and nitazoxanide in (GV) has induced a noted reduction in the severity of pathological changes as well as degeneration of existing tissue cysts (Figure 3f).

In our study, we obtained the highest significant level of IFN-γ production (280.7±88.8, P<0.001) in the nitazoxanide treated group (GIV) (Table 3). This finding could be attributed to the fact that nitazoxanide is a strong inducer of the Th1 immune response and in turn, IFN-γ release, which controls tachyzoites replication, as confirmed by Munoz et al.**[47]**. In contrast, the lowest level of the cytokine was detected in the vitamin D_3-treated group (GIII) with a mean value of (93.23± 25.6) (Table 3). Rajapakse et al. **[24]** explained that the relatively low serum IFN-γ level in vitamin D_3 treated mice was due to its inhibitory effect on Th1 immune cells and consequently impairing IFN-γ production. They also confirmed that despite the immune-suppressive effect of the vitamin, no increase in parasite load was observed, which indicated that vitamin D_3 inhibits T.gondii proliferation at a cellular level.

According to the obtained results, a balanced level of IFN-γ was demonstrated in (GV) with a mean value of (110.45± 36.3). The detected IFN-γ measurement was not as low as the level obtained in the vitamin D_3 treated group (93.23± 25.6), neither as high as that exhibited in the nitazoxanide treated mice (280.7±88.8, P<0.001) (Table 3). Hence, our research verified that combining both nitazoxanide and vitamin D_3 has created a delicate modulation in the overall immune response against toxoplasmosis.

CONCLUSIONS:

Results of the current research showed that using vitamin D_3 alone did not significantly reduce the parasitic load in the brain sections of *T.gondii* infected mice. However, after its combination with nitazoxanide, a significant reduction was obtained. We have also shown that the drug

combination has induced a notable balance in the immune response against toxoplasmosis. Therefore, we recommend using vitamin D_3-nitazoxanide drug combination as a promising alternative treatment option for T.gondii infection.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of interest: The authors report no conflicts of interest regarding the publication of this manuscript.

Ethical considerations

The current research was approved by the Ethical Committee of Zagazig University and was conducted according to the international regulations and guidelines of animal experiments.

HIGHLIGHTS:

- Till now, there is no available single gold standard treatment for toxoplasmosis.
- Vitamin D_3 has potent antimicrobial and anti-parasitic properties.
- Using vitamin D3 alone in the treatment of toxoplasmosis had no significant impact on the parasitic burden.
- Vitamin D_3-nitazoxanide drug combination induced a significant balance in the immune response against toxoplasmosis.
- Vitamin D_3-nitazoxanide drug combination would be a promising alternative treatment option for *Toxoplasma gondii* infection..

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