S&S

Senses Sci 2020: 7 (3)1068-1104
doi: 10.14616/sands- 2020-3-10681104

Article

Effect of VAM2-2 and VAM2-2 loaded chitosan nanoparticles in treatment of experimental toxoplasmosis: an experimental study

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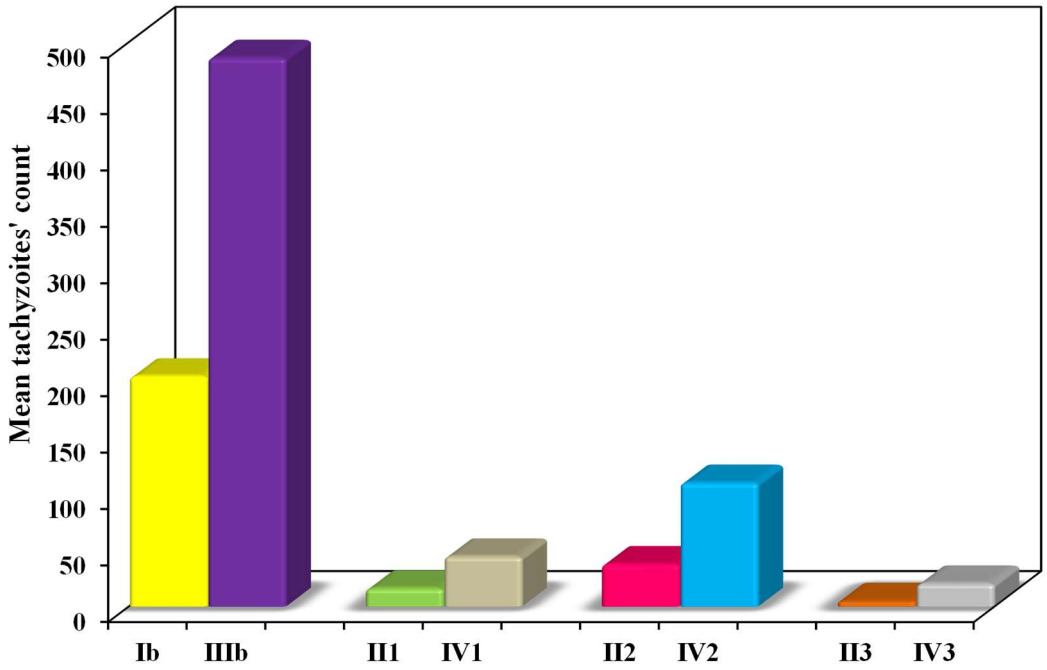
Abstract. *Background*: Toxoplasmosis is a worldwide infection caused by obligate intracellular protozoan parasite, *Toxoplasma gondii*. The standard treatment of toxoplasmosis is a combination of pyrimethamine, sulfadiazine and antifolates. Many side effects were reported with this combination with no alternative drug therapy or effective vaccine. *Methods*: In this study VAM2-2 was designated by TOMOCOMB CARDD approach and a system based on CS NPs was prepared as a potential carrier for VAM2-2 to be evaluated for the first time for their anti-toxoplasma effect in both immunocompetent and immunosuppressed experimental mice. Assessment of drugs effect was achieved through estimating the mortality rate and the survival time of the infected treated mice, studying peritoneal and liver parasite burden, tachyzoites viability and infectivity and ultrastructural tachyzoites changes in comparison with the infected non-treated control animals, were studied. Serum gamma interferon (INFγ) levels were also estimated by enzyme linked immunosorbent assays (ELISA) kits. Drugs safety was biochemically detected by measuring liver enzymes, urea and creatinine in mice sera. *Results*: The obtained results showed that VAM2-2, CS NPs and VAM2-2 loaded CS NPs were effective against acute toxoplasmosis. All treating drugs induced a significant increase in the survival time of the infected treated mice. Treated mice showed a statistically significant reduction in the parasite burden, viability and infectivity of tachyzoites harvested from their peritoneal cavities as compared with the infected non-treated controls. EM examination revealed multiple changes in treated tachyzoites. Serum gamma interferon (INFγ) levels were statistically significantly increased in CS NPs and VAM2-2 loaded CS NPs treated mice in comparison to infected untreated mice and those treated with free VAM2-2. Besides, biochemical results showed

no evidence of toxicity with all therapeutic agents used. VAM2-2 loaded CS NPs gave the best results in all studied parameters.



The effect of VAM2-2, CS NPs and VAM2-2 loaded CS NPs on the tachyzoites' burden in peritoneal fluid of the infected treated immunocompetent and immunosuppressed subgroups of mice compared to the corresponding infected non-treated controls.

Conclusion: all used drugs had promising anti-toxoplasma activities. VAM2-2 loaded CS NPs showed the highest effects in the treatment of acute toxoplasmosis. This will draw the attention to the use of this potential combination as an alternative to the standard therapy in treatment of toxoplasmosis.

Keywords: *Toxoplasma gondii,* VAM2-2, CS NPs, in vivo, experimental study.

Introduction

Toxoplasmosis is a cosmopolitan zoonosis, in addition to be a food and waterborne infection. [1] It is caused by *Toxoplasma gondii* (*T. gondii*); an obligate intracellular coccidian parasite which is a highly successful and remarkable global pathogen due to its ability to infect almost any nucleated cell in any warm-blooded animal.[2] Most infections with *T.gondii* in immune-competent hosts are asymptomatic. Only 10 to 20% of toxoplasmosis cases in immune-competent adults and children are symptomatic.[3, 4] The most common manifestation is cervical lymphadenopathy.[5] It may be accompanied by flu-like symptoms (fever, malaise, sweating, myalgia and sore throat), maculo-papular rash, abdominal pain and hepato-splenomegaly.[6] These symptoms usually resolve within few days. [3, 7] On the other hand, *T. gondii* is a major opportunistic infection in immunodeficiency

conditions, i.e., in AIDS and organ transplant patients. In these patients, reactivation of latent infection or acquisition of parasites from exogenous sources may cause devastating diseases as encephalitis, myocarditis and pneumonitis..[6, 8] Additionally, intrauterine transmission of *T. gondii* from the mother to the foetus during gestation can result in abortion, still birth and severe neonatal and foetal complications including chorioretinitis, cataract, glaucoma, blindness, epilepsy, psychomotor or mental retardation, anemia, jaundice, thrombocytopenia, encephalitis, pneumonitis, lymphadenitis, hepato-splenomegaly.[9, 10]

There is no effective vaccine against toxoplasmosis in humans so, an effective chemotherapy constitutes the only alternative to control the disease. Treatment regimens for infected patients have not essentially changed for years. The recommended drugs for treatment of toxoplasmosis are a combination of pyrimethamine and sulfadiazine or a combination of pyrimethamine with macrolide antibiotics.[11, 12] These combinations are effective in 75 to 89 % of the cases.[3]However, this drug association results in bone marrow suppression requiring discontinuation of treatment or concurrent administration of folic acid. It also exhibits numerous limitations, including poor tolerability (particularly for sulfadiazine) especially in immunocompromised patients and severe side effects. Besides, it cannot be used during pregnancy because of the antifolate effect which has additional deleterious effect during early fetal development.[13]Therefore, there is a critical need for the development and evaluation of new drug or drug combination against *Toxoplasma* with high efficiency and low toxicity. Yet, the time needed to design and develop new drugs represents significant limitations.[14]

In order to reduce costs and time required for formation of synthetic compounds, pharmaceutical companies have to invent new technologies such as the TOMOCOMD–CARDD novel approach (TOpological Molecular COMputational Design–Computer Aided 'Rational' Drug Design) in their quest of new chemical entities (NCE).[15-17] In this approach an in silico virtual world of data, analysis and hypothesis design that reside inside the computer can be used as a successful alternative to the real -world synthesis and screening of compounds in the laboratory. By such methods, the expensive obligation to actual compound synthesis and bioassay is made only after exploring the initial concepts with computational models and screens. In silico screening is now involved in all areas of lead discovery. This theoretical to experimental incorporation procedure is used to find predictive models that allow the rational identification of new antiprotozoan compounds which may meet the dual challenges posed by drug resistant parasites and the rapid progression of protozoal illness.[18]

A new molecular subsystem (a group of novel quinoxalinones) was theoretically selected as a promising lead antiprotozoal drug by TOMOCOMD–CARDD. These derivatives were subsequently synthesized, structurally characterized and experimentally assayed by using in vitro and in vivo strategies. The biological evaluation reported that most of the tested quinoxalinone compounds including multiple VAM2 compounds (7-nitroquinoxalin-3,4 dihydroquinoxalin-1H quinoxalin-2-ones) exhibited a satisfactory antiprotozoan activity against different parasites (*T. cruzi, L. braziliensis, T. vaginalis, P. falciparum* and *T. gondii*)[18] with low unspecific cytotoxicity was observed on the culture cells.[19, 20] They have been also evaluated against *T. gondii* tachyzoites of the RH strain in vitro. Among the ten VAM2 drugs tested by Rivera *et al.* in 2016, several showed a deleterious effect on tachyzoites. However, VAM2-2 which has H atom in the N1, showed the highest toxoplasmicidal activity with no toxic effect on the host cell[14], but little is known about its effects on *T.gondii* infection in vivo.

The exact mechanism of action of VAM2 compounds on parasites is not determined yet. However, the possible mode of action of VAM2-2 as suggested by Rivera N *et al.* in 2016[14] is by its direct effect on cytoskeleton integrity with damage in the pellicle, alternation in the subpellicular cytoskeleton organization and solubilization of structural components such as actin and myosin.[14]Another study has suggested the inhibition of the enzyme trypanothione reductase, a key enzyme of the parasite anti-oxidant defense system, is a possible action mode of the VAM compounds against *T.cruzi*.[20] Also in the same study the authors have reported, through electrochemical studies that nitro derivatives (nitroquinoxaline compounds) could be reduced to form free nitro radicals which act as initiator of oxidative stress between the parasite and the host through the free radical generation which has toxic effect on the parasite.[20]

To reach the stoma of the intracellular apicomplexan parasite like *T.gondii*, a blood-circulating drug has to cross the plasma membrane of an infected cell, the parasitophorous vacuole membrane and the plasma membrane of the parasite.[21] Besides, two membranes of the endothelial cells of capillary vessels, gastro intestinal barrier in oral administration and blood brain barrier in case of brain cystic stage of avirulent strain have to be crossed.[22] Several nanosized delivery systems have been studied in intracellular infections because they are able to deliver the drug to the specific target in the human body. They help the drug to penetrate or overcome the body barriers.[23]

Among polymeric nanoparticles available, chitosan nanoparticles (CS NPs) have received a considerable attention as a potential delivery system for drugs, genes, proteins and peptides by different routes of administration. [24] CS is a modified natural carbohydrate polymer formed by partial N-deacetylation of chitin which is a natural biopolymer derived from crustacean shells such as crabs, shrimps and lobsters. CS is also found in some micro-organisms, yeast and fungi. [25] CS NPs as being a polymeric nanoparticle possess some ideal properties such as being biocompatible, biodegradable, nontoxic and inexpensive. In addition, they possess a positive charge which exhibits absorption enhancing effects making them a promising delivery system for drugs and vaccine [26] In addition to its action as drug delivery system, it has been reported that CS has a significant immunomodulatory activity by encouraging immune cells to secrete a wide variety of pro and anti-inflammatory cytokines as IFN-γ and TNF-$\grave{\alpha}$ and bioactive substances as nitric oxide (NO), reactive oxygen species (RO).[27-30] Furthermore, CS NPs have striking properties such as antibacterial, antitumor, antifungal and antiparasitic effects as well as abilities to heal wounds and stimulate the immune system. [31-36] Recently, CS NPs also, proved to inactivate, inhibit the growth and have cytotoxic effects against *T. gondii* [37]

In view of VAM2-2 promising results against tachyzoites of *T.gondii* in vitro, this work was designed to evaluate its sole efficacy together with the efficacy of its CS NPS loaded form against *T. gondii* tachyzoites in the mouse model in vivo.

Material and methods

Parasite

Virulent RH HXGPRT (-) strain of *T.gondii* was used in this study and it was maintained in the Medical Parasitology Department, Faculty of Medicine, Alexandria University by serial intraperitoneal (IP) passage of tachyzoites in Swiss albino mice every 5 days.[38]

Drug

Immunosuppressive drug: Cyclophosphamide (Endoxan, Asta Medica AG) was used as an immunosuppressive agent by two IP doses of 70 mg/kg each, with one week interval. [39]

Treating drugs: VAM2-2 was either used alone or was incorporated into CS NPs. VAM2-2 powder was kindly provided by Dr. Vicente J. Arán, Head of the Department of Medicinal Chemistry II of the Medicinal Chemistry Institute (IQM) and Senior Scientist of the Spanish National Research Council (CSIC). VAM2-2 powder was first dissolved in 0.1ml DMSO and then diluted in PBS according to the used dose for mice treatment. [14] CS NPs and VAM2-2 loaded CS NPs were prepared by ionotropic gelation technique based on the interaction between the negative groups of sodium tripolyphosphate (TPP) and the positively charged amino groups of chitosan. [40, 41] A pilot study has been performed in order to adjust the doses and route of administration of the treated drugs. VAM2-2 was administered by five different doses (10, 20, 40, 50 or 100 mg/kg) either IP or orally once daily for four, five or six days starting from the day of infection. The selected minimal effective and safe dose was 40 mg/kg IP once daily for four days starting from the day of infection. While for VAM2-2 loaded CS NPs and CS NPs, the tried doses were 20, 30 and 40 mg/kg IP once daily for four days starting from the day of infection. The selected minimal effective and clinically safe dose was 30 mg/kg for both drugs.

Characterization of NPs [40-43]

Both CS NPs and VAM2-2 loaded CS NPs were characterized. The size and morphological characteristics of NPs were examined by transmission electron microscope (TEM) (JEOL-100 XC). The average hydrodynamic diameter, polydispersity index (PDI) and zeta potential (ζ) of CS NPs and VAM2-2 loaded CS NPs were determined by dynamic laser light scattering (DLS) using a particle size analyzer (Zeta sizer, Malvern Co, UK). The encapsulation efficiency (EE) of VAM2-2 in the CS NPs was calculated by this formula: "EE = [(Dt-Du)/Dt] x 100"

Where Dt is the total amount of drug and Du is the amount of free drug in the supernatant.

Animal grouping and experimental design:

Four- to six-week-old laboratory bred Swiss strain Albino mice, weighing 20–25 g at the beginning of the experiment were used. Following the Egyptian national regulations for laboratory animal experimentation, the experimental protocol was approved by the ethics committee of the Faculty of Medicine, Alexandria University, Egypt and the ethics committee approval number was 0201114. Mice were divided into two main equal groups:

Immunocompetent group

One hundred and twenty mice were used in this group and they were further subdivided into two subgroups:

Subgroup I: Control subgroup

Seventy-five mice were used as controls and they were further subdivided into three subgroups:

Subgroup Ia: Non-infected non-treated immunocompetent mice (15 mice).

Subgroup Ib: Infected non-treated immunocompetent mice (15 mice).

Subgroup Ic: Non- infected treated immunocompetent subgroup (45 mice). This subgroup was further subdivided into three equal subgroups:

Subgroup Ic1: Non-infected VAM2-2-treated immunocompetent mice.

Subgroup Ic2: Non-infected CS NPs-treated immunocompetent mice.

Subgroup Ic3: Non-infected VAM2-2 loaded CS NPs-treated immunocompetent mice.

Subgroup II: Experimental subgroup (Infected treated immunocompetent subgroup)**:** Forty-five mice were used in this subgroup and they were further subdivided into three equal subgroups:

Subgroup II1: Infected VAM2-2-treated immunocompetent mice.

Subgroup II2: Infected CS NPs-treated immunocompetent mice.

Subgroup II3: Infected VAM2-2 loaded CS NPs-treated immunocompetent mice.

Immunosuppressed group:

One hundred and twenty mice were used in this group and they were further subdivided into two subgroups:

Subgroup III: Control subgroup

Seventy-five mice were used as controls and they were further subdivided into three subgroups:

Subgroup IIIa: Non-infected non-treated immunosuppressed mice (15 mice).

Subgroup IIIb: Infected non-treated immunosuppressed mice (15 mice).

Subgroup IIIc: Non- infected treated immunosuppressed mice (45 mice). This subgroup was further subdivided into three equal subgroups:

Subgroup IIIc1: Non-infected VAM2-2-treated immunosuppressed mice.

Subgroup IIIc2: Non-infected CS NPs-treated immunosuppressed mice.

Subgroup IIIc3: Non-infected VAM2-2 loaded CS NPs-treated immunosuppressed mice.

Subgroup IV: Experimental subgroup (Infected treated immunosuppressed subgroup)

Forty-five mice were used in this subgroup and they were further subdivided into three equal subgroups:

Subgroup IV1: Infected VAM2-2-treated immunosuppressed mice.

Subgroup IV2: Infected CS NPs-treated immunosuppressed mice.

Subgroup IV3: Infected VAM2-2 loaded CS NPs-treated immunosuppressed mice.

Infection with T.gondii:

Mice in subgroups Ib, II (1,2,3), IIIb and IV (1,2,3) were IP infected with 5x 10^3 tachyzoites/mouse of RH HXGPRT (-) virulent strain of *T.gondii.* Infection in immunosuppressed mice (subgroups IIIb and IV1,2 and 3) were done 48 hours after the second dose of cyclophosphamide.

Sacrifice of mice:

Six mice from each infected subgroup were sacrificed five days PI. Mice of non-infected treated subgroups (Ic and IIIc) were sacrificed on the fifth day after the first dose of the drugs, whether

VAM2-2 (subgroups Ic1 and IIIc1), CS NPs (subgroups Ic2 and IIIc2) or VAM2-2 loaded CS NPs (subgroups Ic3 and IIIc3). While the non-infected non-treated immunosuppressed mice (subgroup IIIa) were sacrificed 14 days after the first dose of cyclophosamide. The remaining mice from all subgroups were observed daily to determine the survival time.

The effect of the treatment was assessed by:

I. ***Parasitological study:***

1. Estimation of the mortality rate (MR) at the time of sacrifice (fifth day PI) in experimental subgroups of mice as compared with the equivalent controls. [44, 45] The MR% for each subgroup was assessed by the following equation:
   MR = The number of dead mice at the sacrifice date / The number of mice at the beginning of the experiment X100
2. Survival time: [44] The mice of each subgroup that survived after the sacrifice day were left to die spontaneously and the survival time was estimated.
3. Measurement of the parasite burden: It was done at the date of sacrifice of infected mice of subgroups II and IV in comparison to their equivalent control subgroups Ib and IIIb respectively by:

a. Counting of the number of tachyzoites in the peritoneal fluid of each infected mouse using double Neubauer hemocytometer chamber.[46, 47] Then the mean number of tachyzoites in each infected subgroup was estimated.
b. Counting of the number of tachyzoites in ten high power fields of impression smears of liver of each infected mouse, after staining with Giemsa stain. [48, 49] Then the mean number of tachyzoites in each infected subgroup was calculated.

The % reduction (%R) in the parasite burden, whether in peritoneal fluid or liver impression smears, was estimated by the following equation: [50]

$$\%R = (C-W/C) \times 100$$

C: Total parasite burden recovered from the infected control subgroups of mice.
W: Total parasite burden recovered from each infected treated subgroup of mice.

4. Determination of parasite viability by dye exclusion: [51, 52] *T. gondii* tachyzoites collected from the peritoneal fluid of each infected subgroup [Ib, IIIb, II (1,2,3) and IV (1,2,3)] were stained with 0.2% trypan blue stain to determine the parasite viability. The stained smears were examined under the high-power lens of the light microscope (x40). The number of living tachyzoites/100 organisms was counted for each infected mouse and the mean number of living tachyzoites in each subgroup of infected mice was calculated. The % R in parasite viability was estimated as mentioned before.
5. Estimation of animal infectivity: [53, 54] *T. gondii* tachyzoites harvested from the peritoneal fluid of each infected subgroup; Ib, II (1,2,3), IIIb and IV (1,2,3) on the fifthth day PI were IP injected into naïve Swiss Albino mice (ten mice for each subgroup). Five days PI these mice were sacrificed and the effect of the drugs was assessed by estimation of the mortality rate (MR) and the infectivity rate (IR) by the following equation:

IR = The number of infected mice at the sacrifice date / The number of mice at the time of infectivity X100

The mean number of tachyzoites in peritoneal fluid of infected mice and the %R in parasite burden of each subgroup of infected mice were also calculated as mentioned before.

II. Ultrastructural study:

It was done by scanning electron microscopic examination (SEM) [55, 56] and transmission electron microscopic examination (TEM) [56-58] of tachyzoites harvested from the peritoneal fluid of infected treated subgroups of mice (II and IV) in comparison with the infected non-treated control (Ib and IIIb respectively).

III. Immunological and biochemical studies:

Blood samples were collected by cervical incision of each mouse on the day of sacrifice (fifth day PI). Each serum sample was divided into two parts; the first part was used for the immunological study and the second part was used for the biochemical study.

1. ***Immunological study:***

It was done by measuring the IFN-γ in the sera of mice of all subgroups using ELISA kit at a wave length of 450 nm (Chongqing Biospes Co.). The assay was carried out as suggested by the manufacturer.

2. ***Biochemical study:*** **[59, 60]**

The second part of sera from each subgroup was used for the biochemical study to demonstrate the acute effect of VAM2-2, CS NPs and VAM2-2 loaded CS NPs on liver and kidney functions. Liver enzymes; aspartate transaminase (AST) and alanine transaminase (ALT) were measured by double enzymatic reaction method and kidney function tests (serum urea and creatinine) were measured by Jaffe reaction method. In both methods Dimension Xpand Plus Integrated Chemistry System (Siemens) was used. [59, 60]

Statistical analysis of the data **[61]**

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0**.** **(**Armonk, NY: IBM Corp**)** [62] Qualitative data were described using number and percent. The Kolmogorov-Smirnov test was used to verify the normality of distribution of variables. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Significance of the obtained results was judged at the 5% level (p value ≤ 0.05). Chi-square test was used for categorical variables, to compare between different groups. When more than 20% of the cells have expected count less than 5, correction for chi-square was conducted using Fisher's Exact or Monte Carlo correction. While, F-test (ANOVA) was used for normally distributed quantitative variables, to compare between more than two groups, and Post Hoc test (Tukey) was used for pairwise comparisons.

Results

Characterization of nanoparticles (NPs):

1. *TEM analysis:*

   NPs showed spherical to oval shapes with smooth surfaces and they were homogenously distributed as shown in Plate I. VAM2-2 incorporation increases the particle size as compared to blank CS NPs. The blank CS NPs were found to have an average particle size of 123.85 nm, while the VAM2-2loaded CS NPs had an average size of 154.38 nm.

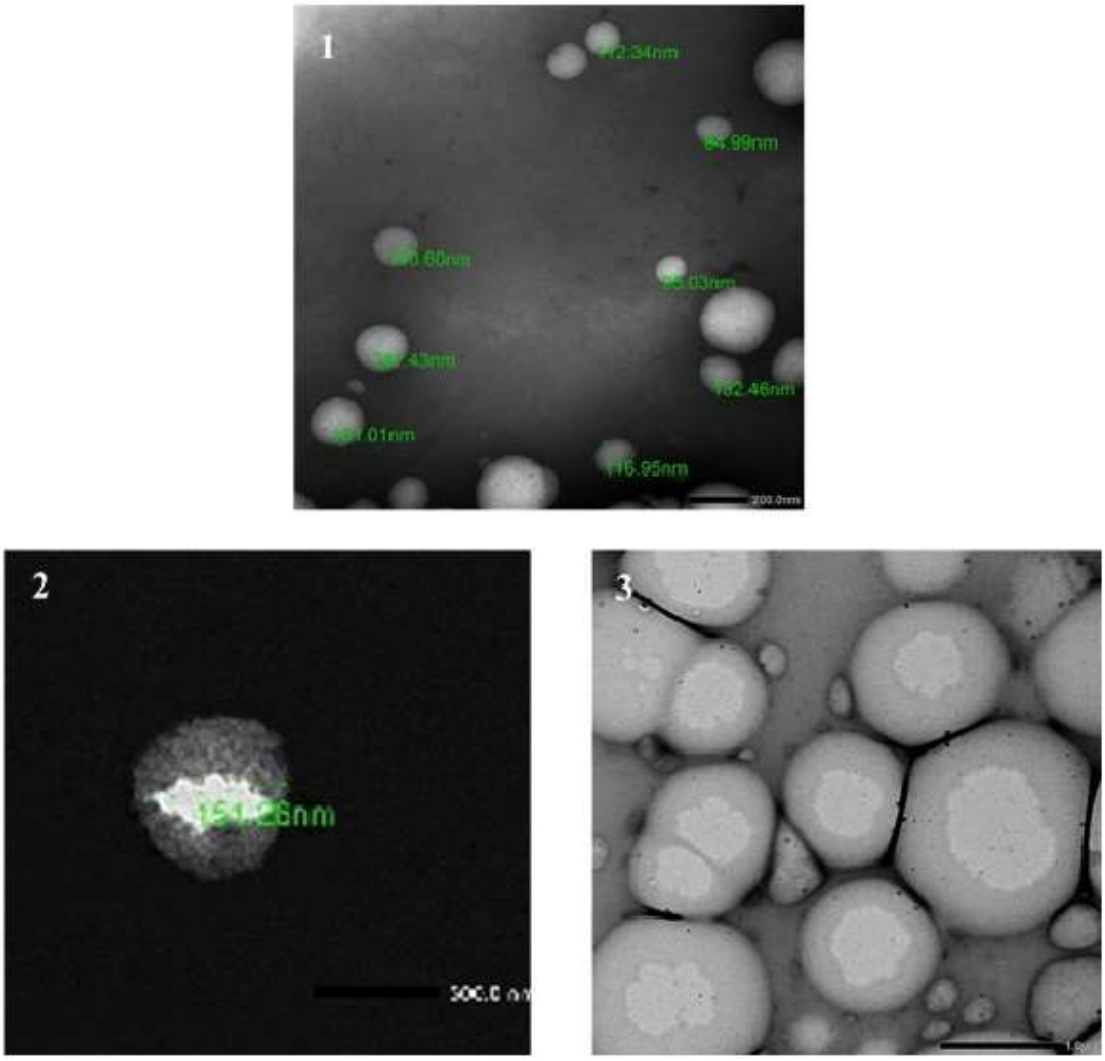


Plate I: TEM of CN and VAM2-2 loaded CN NPS

Fig. 1: TEM of blank CN NPs (x 20000)

Fig. 2 & 3: TEM of VAM2-2 loaded CS NPs (x 40000)

2. *Particle size analysis:*
   The mean hydrodynamic diameter of the prepared CS NPs was 294.8 nm, the PDI was 0.372 and the zeta potential was +18.5 mV. While, the mean hydrodynamic diameter of the VAM2-2 loaded CS NPs was 391.6 nm, the PDI was 0.388 and the zeta potential was +17 mV.
3. *Encapsulation efficiency (EE) of CS NPs:*
   The absorbance intensity of free (unloaded) VAM2-2 in the supernatant was 1.9 at λ_{max} 425 nm which equivalent to 0.0125 mg/ml as calculated from the standard curve. The EE was estimated as 98% (EE = 1-0.0125/1 x 100 = 98%).

Results of parasitological study

1. ***Mortality rate (MR)****:* In the immunocompetent subgroups whether infected or not, no mice died till the sacrifice time (fifth day PI), the MR was 0%, except in the infected non-treated subgroup (Ib), one mouse died before the day of sacrifice (the MR was 6.7%). There was a statistically non-significant difference in the MR between the non-infected non-treated subgroup (Ia) and the other immunocompetent subgroups (P value > 0.05). Furthermore, there was a statistically non-significant difference in the MR among the controls or experimental subgroups. Regarding, the immunosuppressed subgroups; the MR was 0% in the non-infected subgroups whether non-treated or treated. While, three mice died before the day of sacrifice in the infected non-treated subgroup (IIIb), (The MR was 20%). In the infected treated subgroups, the MR was 6.7% (one mouse died before the sacrifice day) in each of subgroup IV1(infected treated with VAM2-2) and subgroup IV3(infected treated with VAM2-2 loaded CS NPs). While, the MR was 13.3 % in subgroup IV2 (infected treated with CS NPs) as two mice died before the sacrifice day. There was a statistically non-significant difference in the MR between subgroup IIIa and the other immunosuppressed subgroups (P value > 0.05). Moreover, there was a statistically non-significant difference in the MR among the control or experimental subgroups.
2. ***Survival time*****:** Non-infected non-treated control subgroups Ia and IIIa showed a mean survival time of 52.33 ± 4.72 and 29.33 ± 1.51 days respectively. While in the infected non-treated mice, the mean survival time was 7.50 ± 0.55 and 4.50 ± 0.55 days in subgroups Ib and IIIb respectively. Mice treated with VAM2-2, CS NPS or VAM2-2 loaded CS NPS showed a statistically significant increase in the survival time when compared to the corresponding infected non-treated controls with a mean of 19 ± 0.89, 13.83 ± 0.75 and 22.67 ± 0.82 days in immunocompetent subgroups II1, II2 and II3 respectively and 13.50 ± 1.05, 9.67 ± 0.82 and 18.17 ± 0.75 days in immunosuppressed subgroups IV1, IV2 and IV3 respectively. There was a statistically significant increase in the mean values of survival time in subgroup III3 in comparison with subgroup II2 only. While, there was a statistically significant increase in the mean values of survival time in subgroups IV1 and IV3 in comparison to subgroup IV2 and in subgroup IV3 comparing with subgroup IV1 as well (**Tables 1 and 2**).
3. ***Parasite burdens:*** A statistically significant decrease in the mean number of tachyzoites in the peritoneal fluid and in the liver impression smears was noticed in all infected treated immunocompetent (II1, II2 and II3) and immunosuppressed subgroups (IV1, IV2 and IV3)

compared with the corresponding infected non-treated subgroups (Ib and IIIb respectively). The % R was 92, 81.5 and 97.7in the peritoneal fluid and 87.8, 76.7 and 93.3 in the liver impression smears in the immunocompetent subgroups (II1, II2 and II3 respectively). While, in the immunosuppressed subgroups (IV1, IV2 and IV3), the % R was 90.9, 77.4 and 95.8 in the peritoneal fluid and 83.3, 73.3 and 91.1 in the liver impression smears respectively. The highest % R was obtained in VAM2-2 loaded CS NPs treated subgroups (II3 and IV3). In immunocompetent mice, there was a statistically significant decrease in the mean tachyzoites number in the peritoneal fluid and in the liver impression smears of VAM2-2 loaded CS NPS treated subgroup (II3) as compared to CS NPS treated subgroup (II2). While, a statistically non-significant decrease in subgroup II3 in comparison with VAM2-2 treated subgroup (II1) and a statistically non-significant increase was recorded in subgroup II2 when compared to subgroup II1. Similarly, in immunosuppressed mice, there was a statistically significant reduction in the parasite burden of VAM2-2 loaded CS NPs treated subgroup (IV3) as compared to either VAM2-2 treated (IV1) or CS NPS treated (IV2) subgroups. In contrary, there was a statistically significant increase in the parasite burden in subgroup IV2 when compared to subgroup IV1 (**Tables 3 and 4**).

Table (3): The effect of VAM2-2, CS NPs and VAM2-2 loaded CS NPs on the tachyzoites' burden in peritoneal fluid of the infected treated immunocompetent and immunosuppressed subgroups of mice compared to the corresponding infected non-treated control

	Infected immunocompetent subgroups				Infected immunosuppressed subgroups			
	Non-treated	**Treated**			**Non-treated**	**Treated**		
	Ib	**II1**	**II2**	**II3**	**IIIb**	**IV1**	**IV2**	**IV3**
X	204.00	16.25	37.75	4.75	486.43	44.50	109.75	20.50
SD.	45.69	0.82	5.81	1.04	7.74	3.62	6.68	1.79
Min.	150.00	15.00	30.50	3.00	478.00	39.00	99.50	18.00
Max.	264.00	17.50	46.00	6.00	500.00	50.00	118.50	22.50
Median	204.00	16.25	36.75	5.00	484.00	44.50	110.50	20.75
% R		92.0	81.5	97.7		90.9	77.4	95.8
p_1		<0.001*	<0.001*	<0.001*		<0.001*	<0.001*	<0.001*
p_2			0.221				<0.001*	
p_3				0.718				<0.001*,
p_4				0.026*				<0.001*

Ib: Infected non-treated immunocompetent subgroup

II1: Infected VAM2-2-treated immunocompetent subgroup

II2: Infected CS NPs-treated immunocompetent subgroup

II3: Infected VAM2-2/CS NPs-treated immunocompetent subgroup

IIIb: Infected non-treated immunosuppressed subgroup

IV1: Infected VAM2-2-treated immunosuppressed subgroup

IV2: Infected CS NPs-treated immunosuppressed subgroup

IV3: Infected VAM2-2/CS NPs-treated immunosuppressed subgroup

X: Mean number of tachyzoites /ml peritoneal fluid ($x10^4$)**, SD.:** Standard deviation, **Min.:** Minimum**, Max.:** Maximum,

%R: % reduction in the tachyzoite count in each infected treated subgroup in relation to the corresponding infected non-treated subgroup

p_1: p value for comparing between **Ib or IIIb** and **the corresponding subgroups**

p_2: p value for comparing between **II1** and **II2** or **IV1** and **IV2**

p_3: p value for comparing between **II1** and **II3** or **IV1** and **IV3**

p_4: p value for comparing between **II2** and **II3** or **IV2** and **IV3**

*: Statistically significant at $p \leq 0.05$

Post Hoc test (Tukey) is used in pairwise comparisons

ANOVA test is used in comparison between more than two groups

Table (4): The effect of VAM2-2, CS NPs and VAM2-2 loaded CS NPs on the tachyzoites' burden in liver impression smears of the infected treated immunocompetent and immunosuppressed subgroups of mice compared to the corresponding infected non-treated control

	Infected immunocompetent subgroups				Infected immunosuppressed subgroups			
	Non-treated	**Treated**			**Non-treated**	**Treated**		
	Ib	**II1**	**II2**	**II3**	**IIIb**	**IV1**	**IV2**	**IV3**
x	3.44	0.42	0.80	0.23	9.00	1.50	2.40	0.80
SD.	0.76	0.08	0.08	0.07	0.52	0.26	0.36	0.14
Min.	2.50	0.30	0.70	0.10	8.20	1.20	2.00	0.60
Max.	4.70	0.50	0.90	0.30	10.00	1.90	3.00	1.00
Median	3.50	0.40	0.80	0.20	9.00	1.45	2.35	0.80
% R		87.8	76.7	93.3		83.3	73.3	91.1
p_1		<0.001*	<0.001*	<0.001*		<0.001*	<0.001*	<0.001*
p_2			0.172				0.002*	
p_3				0.735				0.016*,
p_4				0.020*				<0.001*

Ib: Infected non-treated immunocompetent subgroup

II1: Infected VAM2-2-treated immunocompetent subgroup

II2: Infected CS NPs-treated immunocompetent subgroup

II3: Infected VAM2-2/CS NPs-treated immunocompetent subgroup

IIIb: Infected non-treated immunosuppressed subgroup

IV1: Infected VAM2-2-treated immunosuppressed subgroup

IV2: Infected CS NPs-treated immunosuppressed subgroup

IV3: Infected VAM2-2/CS NPs-treated immunosuppressed subgroup

X: Mean number of tachyzoites in liver impression smears (/HPF)**, SD.:** Standard deviation**, Min.:** Minimum**, Max.:** Maximum, **%R**: %reduction in tachyzoite count in each infected treated subgroup in relation to the corresponding infected non-treated subgroup

p_1: p value for comparing between subgroup **Ib or** subgroup **IIIb** and **the corresponding subgroups**

p_2: p value for comparing between subgroup **II1** and subgroup **II2** or subgroup **IV1** and subgroup **IV2**

p_3: p value for comparing between subgroup **II1** and subgroup **II3** or subgroup **IV1** and subgroup **IV3**

p_4: p value for comparing between subgroup **II2** and subgroup **II3** or subgroup **IV2** and subgroup **IV3**

*: Statistically significant at $p \leq 0.05$

Post Hoc test (Tukey) is used in pairwise comparisons

ANOVA test is used in comparison between more than two groups

4. ***Parasite viability by dye exclusion test:*** Using 0.2 % trypan blue stain, the viable tachyzoites appeared light blue in colour with clear cytoplasm. While the dead tachyzoites showed dark blue cytoplasm with unrecognized internal structures (Plate II). A statistically significant decrease in the viability of the tachyzoites harvested from the peritoneal fluid of all infected treated immunocompetent subgroups and immunosuppressed subgroups when compared with the corresponding infected non-treated subgroups. Regarding the immunocompetent subgroups, % R was 65.5, 50.1 and 79.2 in subgroups II1, II2, and II3 respectively. While, in the immunosuppressed subgroups, the % R in subgroups IV1, IV2 and IV3 was 62.2, 27 and 69.2 respectively. In both immunocompetent and immunosuppressed subgroups, when comparing the number of viable tachyzoites in infected subgroups treated by VAM2-2 loaded CS NPS (subgroups II3 and IV3) with those treated by VAM2-2 (subgroups II1 and IV1) or CS NPs (subgroups II2 and IV2), there was a statistically significant decrease in VAM2-2 loaded CS NPs treated subgroups when compared to either VAM2-2 treated or CS NPs treated subgroups. On the other hand, the mean number of viable tachyzoites was statistically significantly increased in CS NPs treated subgroups in comparison with VAM2-2 treated subgroups (**Table 5**).
5. ***Animal infectivity:*** Naive mice that have been sub-inoculated with tachyzoites from the infected-treated subgroups showed IR of 30, 50 and 20% in immunocompetent subgroups II1x, II2x and II3x and IR of 40, 70 and 30% in immunosuppressed subgroups IV1x, IV2x and IV3x respectively compared to 100% in their corresponding controls (Ibx and IIIbx subgroups respectively). There was a statistically significant reduction in the mean number of tachyzoites in the peritoneal fluid of naive mice infected with tachyzoites obtained from all treated subgroups as compared with the infected non-treated control with %R of 97.7, 86.4 and 99.2 in immunocompetent subgroups II1x, II2x and II3x respectively and %R of 95.9, 77.2 and 97.8 in immunosuppressed subgroups IV1x, IV2x and IV3x respectively. A statistically significant reduction in the mean number of tachyzoites was obtained in the VAM2-2 loaded CS NPS treated subgroups compared to VAM2-2 or CS NPs treated subgroups in both immunocompetent and immunosuppressed animals. On

the other hand, there was a statistically significant increase in the parasites burden of CS NPs treated subgroups in comparison with VAM2-2 treated subgroups (**Table 6**).

Results of electron microscopic study (EMS)

1. ***SEM:***

The ultrastructure of *T. gondii* tachyzoites of the infected non-treated mice appeared nearly similar in both immunocompetent (Ib) and immunosuppressed subgroups (IIIb) by SEM. Tachyzoites were generally crescent shape with completely smooth regular surfaces (Plates III, IV and V1). Most tachyzoites of subgroup II1 (immunocompetent VAM2-2 treated) showed irregular ridges on their surfaces (Plate III 2 &3). Some tachyzoites showed surface protrusions (Plate III 2),
other showed distortion in their crescent shape with reduction in the size (Plate III 3). The changes in tachyzoites of subgroup IV1(immunosuppressed VAM2-2 treated) were less obvious. The surfaces of most of them were nearly smooth, however, multiple dimples (Plate III 4) and erosions (Plate III 5) were observed in some tachyzoites. In subgroup II2 (immunocompetent CS NPs treated), the surfaces of some tachyzoites were irregular with multiple ridges (Plate IV 2 &3) with distortion in the shape of some tachyzoites (Plate IV 3). Less surface changes were observed in subgroup IV2 (immunosuppressed CS NPs treated) and their crescent shape was preserved, although dimples (Plate IV 4) and protrusions (Plate IV 5) were noticed in some tachyzoites. On the other hand, severe morphological changes were noticed in tachyzoites of subgroups II3 (immunocompetent VAM2-2 loaded CS NPs treated). Most of them appeared shrunken and obviously distorted. Moreover, the tachyzoite surfaces showed irregularities with numerous ridges (Plate V 3, 4 & 5). Some tachyzoites showed surface erosions (Plate V 2), other showed surface compressions (Plate V 4). Dimples and leakage of the internal content from one end was noticed in some tachyzoites (Plate V 4). Distortion in the apical region was noticed in some tachyzoites as well (Plate V 2 & 4). On the other hand, some tachyzoites of subgroup IV3 (immunosuppressed VAM2-2 loaded CS NPs treated) still preserved their crescent shape (Plate V 5), while other were distorted, shrunken and oval in shape (Plate V 6 & 7). The surfaces of some tachyzoites were irregular with erosions (Plate V 5), while little irregularities were present in other tachyzoites with surface protrusions (Plate V 6 &7).

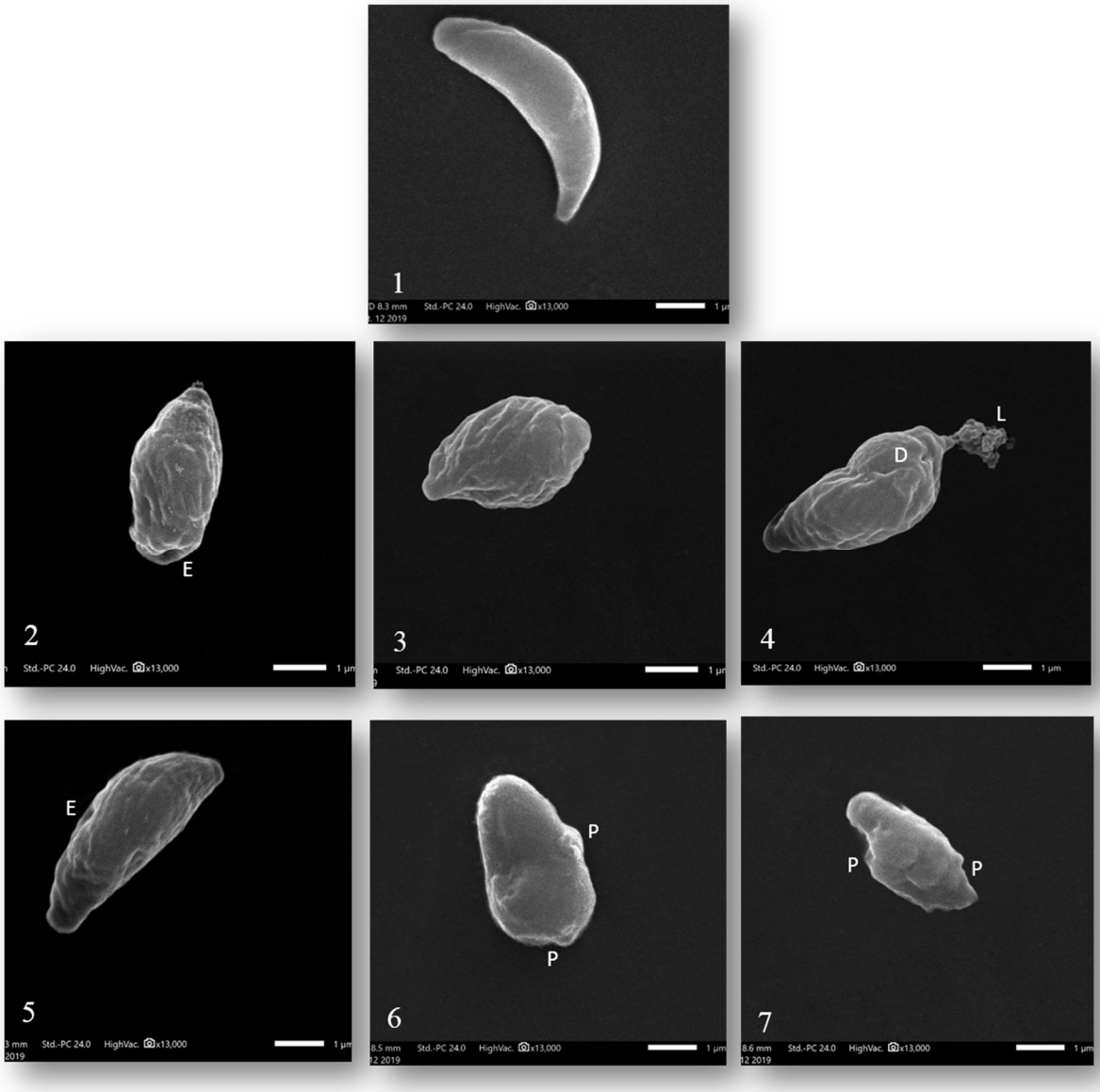


Plate V: SEM of *T. gondii* tachyzoites of infected non treated and VAM2-2 loaded chitosan nanoparticles (CS NPS) infected treated mice

Fig. 1: SEM of normal non-treated tachyzoite showing crescent shape tachyzoite having completely smooth regular surface (x13000).

Fig. 2: SEM of VAM 2-2 CN treated tachyzoite of immunocompetent infected mice demonstrating distortion of its crescent shape and in the apical region, irregularities in its surface with multiple ridges and erosion (x13000).

Fig. 3: SEM of VAM 2-2 CN treated tachyzoite of immunocompetent infected mice showing distortion of its crescent shape, reduction in its size, irregularities on its surface with multiple ridges (x13000).

Fig. 4: SEM of VAM 2-2 CN treated tachyzoite of immunocompetent infected mice revealing distortion of its crescent shape, irregularities in its surface with multiple ridges, compressions, dimples (D) and leakage of the internal content from one end (L) (x13000).

Fig. 5: SEM of VAM 2-2 CN treated tachyzoite of immunosuppressed infected mice showing its crescent shape, rough irregular surface with erosions (E) (x13000).

Fig. 6: SEM of VAM 2-2 CN treated tachyzoite of immunosuppressed infected mice illustrating shrunken oval shaped tachyzoite with smooth most parts of its surface and protrusion (P) (x13000).

Fig. 7: SEM of VAM 2-2 CN treated tachyzoite of immunosuppressed infected mice showing shrunken distorted oval shaped tachyzoite with irregular surface and protrusions (P) (x13000).

2. *TEM*

T. gondii tachyzoites of the infected non-treated mice appeared similar in both immunocompetent (Ib) and immunosuppressed subgroups (IIIb) by TEM. Tachyzoites were crescent shape with apparently normal apical complex. The cytoplasmic and the nuclear membranes were intact. Small lipid bodies were observed in the cytoplasm (Plates VI, VII and VIII 1 & 2). Plate VI demonstrates TEM changes in tachyzoites of the infected subgroup of mice treated with VAM 2-2 whether immunocompetent (II1) or immunosuppressed (IV1). Some tachyzoites preserved their crescent shape (Plate VI 2), while other tachyzoites were distorted with deformed apical complex (Plate VI 3& 4). Multiple wide cytoplasmic vacuoles (Plate VI 2 & 4) or even extensive vacuolation of the cytoplasm (Plate VI 3) were observed. Most tachyzoites showed irregular disrupted plasma membrane (Plate VII 2, 3 &4), sometimes with protrusion (Plate VI 4). The nucleus appeared deformed in some tachyzoites (Plate VI 4). Tachyzoites of subgroup IV1illustrated widening of cytoplasmic vacuoles (Plate VI 5& 6) or multiple coalesced vacuoles (Plate VI 7). Furthermore, there were protrusion (Plate VI 5) and irregularities with separation of the plasma and nuclear membranes (Plate VI 6&7). Enlargement in the dense granules was noticed in some tachyzoites (Plate VI 6 &7). Other tachyzoites showed altered structure of the dense granule (Plate VI 7). While, tachyzoites of subgroup II2 (immunocompetent CS NPs treated) preserved their crescent shape with apparently normal endoplasmic reticulum and nucleus with intact nuclear membrane (Plate VIII 2 &3). However, irregular disrupted plasma membrane and wide cytoplasmic vacuole were observed in them (Plate VII 2 &3). On the other hand, some tachyzoites of subgroup IV2(immunosuppressed CS NPs treated) showed irregular plasma membrane with depression, in addition to irregular and separated nuclear membrane with apparently normal endoplasmic reticulum (Plate VII 4). Whereas, tachyzoites of subgroup II3(immunocompetent VAM2-2 loaded CS NPs treated) showed marked distortion in their crescent shape with severely deformed apical complex and extensive cytoplasmic vacuolation. In addition, irregularities, disruption and separation of the plasma membrane with leakage of the internal content were demonstrated. Furthermore, the nucleus appeared shrunken with irregular nuclear membrane. Some tachyzoites showed complete destruction of the internal structures giving ghost-like appearance (Plate VIII 2, 3 &4). The changes observed in subgroup IV3 (immunosuppressed VAM2-2 loaded CS NPs treated) were less marked. However, disrupted plasma membrane with leakage of the internal content (Plate VIII 5), irregular plasma membrane with protrusion (Plate VIII 6) and wide cytoplasmic vacuole (Plate VIII 5 & 6) were noticed. Irregularities and separation of the nuclear membrane were also detected (Plate VIII 6). In

addition, some tachyzoites were shrunken and showed distortion in their crescent shape with wide cytoplasmic vacuoles and multiple dense granules (Plate VIII 7).

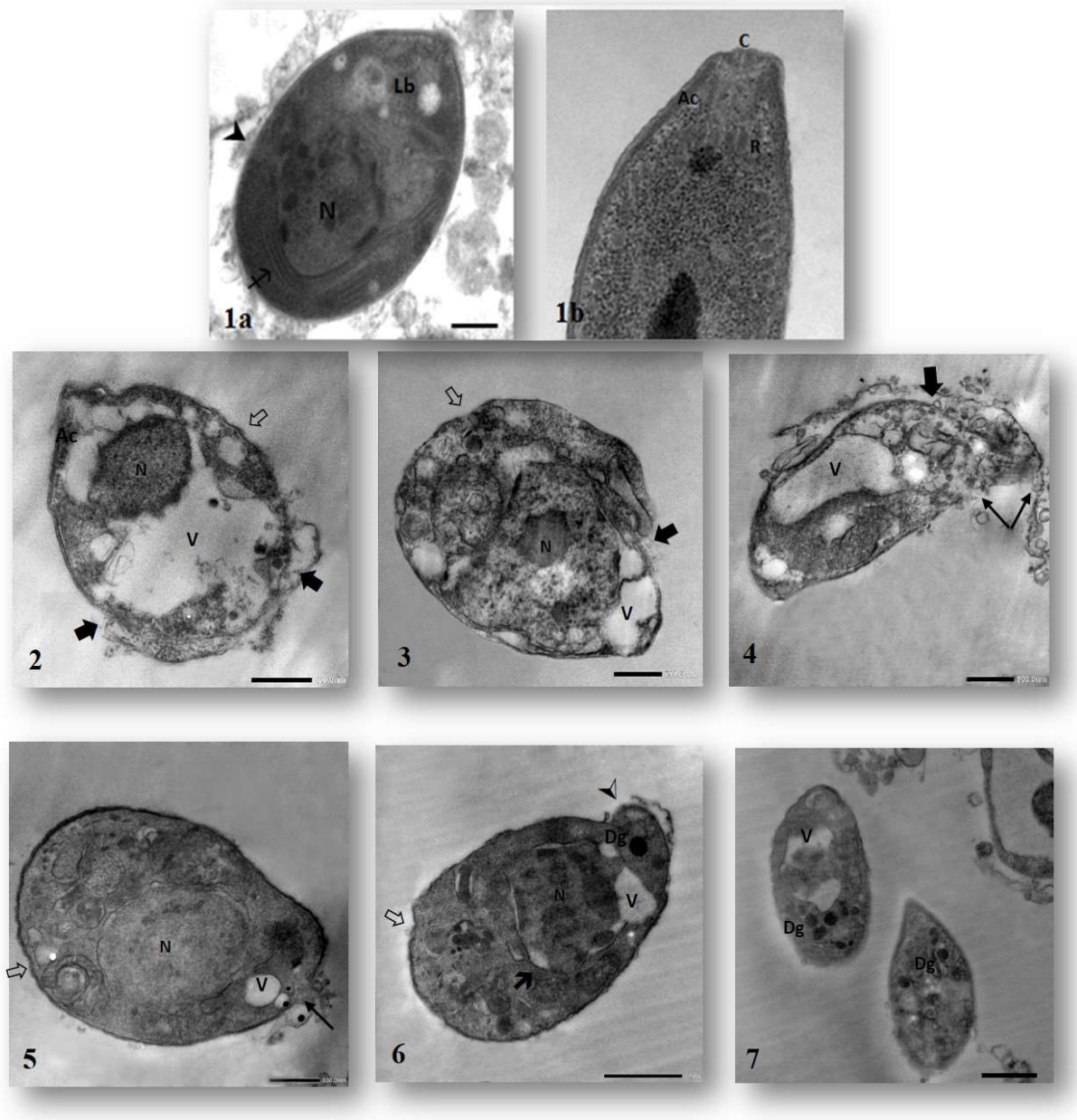


Plate VIII: TEM of *T. gondii* tachyzoites of infected non-treated and VAM2-2 loaded CN NPS **treated mice.**

Figs 1a: Longitudinal section of normal non-treated crescent shaped tachyzoite having intact regular plasma membrane (➤), lipid body (Lb), endoplasmic reticulum (➶) and nucleus (N) with intact nuclear membrane (x10000).

Fig. 1b: Longitudinal section of normal non-treated tachyzoite showing rhoptries (R) and conoid (c) apical complex (AC) (x 7500)

Fig. 2: Longitudinal section of VAM 2-2 CN treated tachyzoite of immunocompetent infected mice clarifying distortion in its crescent shape, extensive cytoplasmic vacuolation (v), deformed apical complex (Ac),

irregular (⇨) and separated (➡) plasma membrane with leakage of the internal content, and irregular nuclear membrane (➔) (x10000).

Fig. 3: Longitudinal section of VAM 2-2 CN treated tachyzoite of immunocompetent infected mice demonstrating multiple cytoplasmic vacuoles (v), irregular (⇨) and separated (➡) plasma membrane and shrunken nucleus (x10000).

Fig. 4: Longitudinal section of VAM 2-2 CN treated tachyzoite of immunosuppressed infected mice showing ghost like appearance of its cytoplasm, deformed apical complex (Ac), extensive cytoplasmic vacuolation (v) and disrupted (→) and separated (➡) plasma membrane with leakage of the internal content (x10000).

Fig. 5: Longitudinal section of VAM 2-2 CN treated tachyzoite of immunosuppressed infected mice revealing irregular (⇨), disrupted (→) plasma membrane with leakage of the internal content and wide cytoplasmic vacuole (v) (x10000).

Fig. 6: Longitudinal section of VAM 2-2 CN treated tachyzoite of immunosuppressed infected mice showing irregular plasma membrane (⇨) with protrusion (➢), wide cytoplasmic vacuole (v), enlarged dense granule (Dg), irregularities and separation of the nuclear membrane (➔) (x10000).

Fig. 7: Longitudinal section of VAM 2-2 CN treated tachyzoites of immunosuppressed infected mice clarifying distortion in their crescent shape, diminution in their size, wide cytoplasmic vacuoles (v) and multiple dense granules (Dg) (x7500).

Results of immunological and biochemical studies

1. *Results of immunological study*

As regards the non-infected subgroups whether immunocompetent or immunosuppressed, a statistically non-significant decrease in the mean values of serum IFN-γ was observed in the VAM2-2 treated subgroups (Ic1 and IIIc1) in comparison with subgroups Ia and IIIa respectively (non-infected non-treated subgroups). On the other hand, a statistically significant increase was recorded in CS NPs treated subgroups (Ic2 and IIIc2) and in VAM2-2 loaded CS NPs treated subgroups (Ic3 and IIIc3) in comparison with either subgroups Ia and IIIa or Ic1and IIIc1 respectively. While, the mean values in subgroups Ic3 and IIIc3 were statistically non-significant increase in comparison with subgroup Ic2 and IIIc2 respectively. Concerning the infected subgroups, a statistically significant increase in the mean value of serum IFN-γ was recorded in subgroups Ib and IIIb (infected non-treated) as compared to subgroups Ia and IIIa respectively. In the infected treated subgroups, there was a statistically non-significant difference in the infected VAM2-2 treated subgroups (II1 and IV1) in comparison with subgroups Ib and IIIb respectively. While in the infected CS NPs treated subgroups (II2 and IV2) and in the infected VAM2-2 loaded CS NPs treated subgroups (II3 and IV3), the recorded increase was statistically significant in comparison with either subgroups Ib and IIIb or II1 and IV1 respectively. Whereas, the mean values in subgroups II3 and IV3 were statistically non-significant increase in comparison with subgroups II2 and IV2 respectively (**Tables 7 and 8**).

2. *Results of biochemical study*

a. ***Measurement of liver transaminases***

As regards non-infected subgroups, the changes in the mean values of AST and ALT in all treated subgroups either immunocompetent (Ic1, Ic2 and Ic3) or immunosuppressed (IIIc1, IIIc2 and IIIc3) were statistically non-significant in comparison with the corresponding non-treated control subgroups (Ia and IIIa respectively). Similarly, the difference in the mean values between the three treated subgroups whether immunocompetent or immunosuppressed was statistically non-significant (**Tables 8 and 9**).

Regarding the infected subgroups, the mean values of liver enzymes (AST and ALT) in the infected non-treated immunocompetent subgroup Ib and the infected nontreated immunosuppressed subgroup IIIb were significantly higher than the levels detected in the non-infected non-treated immunocompetent subgroup Ia and the non-infected immunosuppressed non-treated IIIa respectively. While in the infected treated subgroups either immunocompetent (II1, II2, and II3) or immunosuppressed (IV1, IV2, and IV3), a statistically significant decrease in mean values of AST and ALT were recorded in comparison with their corresponding infected non-treated control (Ib & IIIb respectively). On the other hand, there was a statistically significant increase in the mean values of both AST and ALT in CS NPs treated subgroups (II2 and IV2) in comparison with either VAM2-2 treated subgroups (II1 and IV1) or VAM2-2 loaded CS NPs treated subgroups (II3 and IV3). While, there was a statistically significant decrease in VAM2-2 loaded CS NPs treated subgroups in comparison with VAM2-2 treated subgroups whether immunocompetent or immunosuppressed. (**Table 9 and 10**)

b. ***Measurement of serum urea and creatinine***

Concerning the levels of serum urea and creatinine in the non-infected subgroups, the recorded changes in the values of all treated non infected subgroups were statistically non-significant in both immunocompetent (Ic1, Ic2 and Ic3) and immunosuppressed (IIIc1, IIIc2 and IIIc3) subgroups in comparison with their corresponding non-treated control (Ia and IIIa respectively). Moreover, the difference in the mean values between the three treated subgroups whether immunocompetent or immunosuppressed was statistically non-significant too (**Tables 10 and 11**).

Eventually, as regards the infected subgroups, the mean values of serum urea in the infected non-treated immunocompetent subgroup Ib and the infected non-treated immunosuppressed subgroup IIIb were significantly higher than the levels recorded in the non-infected non-treated immunocompetent subgroup Ia and the non-infected non-treated immunosuppressed IIIa respectively. While, a statistically significant decrease in mean values of serum urea was recorded in the infected treated subgroups either immunocompetent (II1, II2 and II3) or immunosuppressed (IV1, IV2 and IV3) in comparison with their corresponding infected non-treated control (Ib and IIIb respectively). Nevertheless, there was a statistically significant increase in the mean values of serum urea in CS NPs treated subgroups (II2 and IV2) in comparison with either VAM2-2 treated subgroups (II1 and IV1 respectively) or VAM2-2 loaded CS NPs treated subgroups (II3 and IV3 respectively). While, there was a statistically significant decrease in VAM2-2 loaded CS NPs treated subgroups in comparison with VAM2-2 treated subgroups whether immunocompetent or immunosuppressed. Meanwhile, a statistically non-significant difference in the mean values of serum creatinine was

observed in both immunocompetent and immunosuppressed infected treated subgroups in comparison with their corresponding infected non-treated control. Similarly, the difference between the three treated subgroups whether immunocompetent or immunosuppressed was statistically non-significant (**Table 11 and 12**).

Discussion

The intracellular parasite *T.gondii* is recognized as an important apicomplexan protozoan that causes significant morbidity and mortality, particularly in immuno-compromised and congenitally infected individuals worldwide. [63, 64] with increasing numbers of high risk individuals and the absence of a proper vaccine, continued efforts are necessary for the development of effective and safe novel treatment options against *T.gondii*.

To the best of our knowledge, the current study is the first to evaluate VAM2-2 as a therapeutic agent against experimental toxoplasmosis. As VAM2-2 was not evaluated in vivo against *T.gondii*, so its ability to penetrate the body and the parasite barriers was not determined. So, CS NPs was used in the present study as carrier of VAM2-2. Therapeutic agents loaded on CS NPs were found to be more stable, permeable and bioactive.[65, 66] Chitosan was proved to be a potent activator of macrophage and natural killer cells. Moreover, it was documented that chitosan solution enhanced both humoral and cell mediated immune responses.[67] Furthermore, Cs proved to have cytotoxic effects against *T.gondii*[37] besides, it can specifically bind to macrophages receptors where *Toxoplasma* reside and by this way increase the uptake of the loaded drug. Moreover, CS is an acid resistive material leading to its resistant to instant lysosomal digestion within the macrophages, consequently, release the loaded drug in a sustained manner at the target site.[68] Thus, the present study designed to investigate the anti-toxoplasma activity induced by quinoxalinone derivatives, VAM2-2 whether free or after loading on CS NPs.

In the present study, CS NPs and VAM2-2 loaded CS NPs were prepared by ionotropic gelation technique based on the interaction between the negative groups of sodium tripolyphosphate (TPP) and the positively charged amino groups of CS. [41] The advantage of the ionotropic gelation method is being generally used because it is a simple, easy and fast to be carried out without applying harmful organic solvents, heat or vigorous agitation.[42]

The characterization of CS NPs and VAM2-2 loaded CS NPs in the present study was done by TEM analysis, particle size analysis, zeta potential and determination of encapsulation efficiency. Characterization results indicated ideal properties for systemic drug delivery, where particle size increased upon drug loading which attributed to the incorporation of the drug inside the CS NPs. In addition, the particles appeared regular distinct spherical to oval in shape and homogeneously distributed. These finding were in agreement with those reported by other researchers.[69, 70] Moreover, the PDI was less than 0.5, which indicates homogeneous nature of the formulation and favorable particle size distribution.[71] The results of the present study also demonstrated respective zeta potentials of the prepared particles which denoted binding of the negatively charged VAM2-2 to the positively charged CS NPS. Furthermore, the EE was 98%. The resulted positive charge of zeta potential and PDI less than 0.5 with high EE were in line with previous studies done by Ali M *et al.*, in 2013[72] , Piras *et al.* in 2015 [42] and Hagras *et al.*, in 2019[71].

Cyclophosphamide was used as an immunosuppressive drug to induce immunosuppression in mice due to its specific immunosuppressive activity on both humoral and cell mediated immunities.[73] It was administered in two doses prior to infection, which was reported to increase its immunosuppressive efficiency.[74] In addition, repeated drug administration, as used in the present study, induces better immunosuppression and avoids recovery of the immune response.[75]

In the present study, the dose and the route of administration of VAM2-2, CS NPs and VAM2-2 loaded CS NPs were selected after a pilot study and the selected dose was 40 mg / kg IP for four days starting from the day of infection which was the minimal effective and safe dose that induced reduction in parasite load. While for VAM2-2 loaded CS NPs and CS NPs, the minimal effective and safe dose chosen was 30 mg/kg for both. They were given as VAM2-2 by IP route with the same duration of treatment to be compatible with that of VAM2-2.

The anti-toxoplasma effects of VAM2-2, CS NPs and VAM2-2 loaded CS NPs were evaluated in experimentally infected mice with tachyzoites of RH strain of *T. gondii* through parasitological, electron microscopic, immunological and biochemical studies.

Concerning the MR, there was a statistically non-significant difference in MR between the non-infected non-treated subgroups (Ia or IIIa) and the corresponding non-infected or infected treated subgroups whether immunocompetent or immunosuppressed. This could be explained by low dose of infection (5 x 10^3 tachyzoites) and the short time of assessment of the MR, on the sacrifice day (the fifth day PI). Only one and three mice died before the day of sacrifice among the infected non-treated immunocompetent and immunosuppressed mice respectively. This might be referred to the virulent nature of RH strain of *T.gondii,* plus the immunosuppressive effect of cyclophosphamide in immunosuppressed subgroups. Similarly, Martins-Duarte *et al.* in 2015[76] reported 0% mortality in the first seven days PI among ciprofloxacin infected treated mice and untreated control infected with 5 x 10^3 tachyzoites. Furthermore, in a study conducted by El Zawawy *et al.* in 2015,[77] the MR was 10% and 5% in infected mice with 10^4 tachyzoites/mouse treated with triclosan and triclosan- loaded liposomal nanoparticles respectively compared to 0% in infected non treated mice and this MR was statistically non-significant.

Regarding the survival time, VAM2-2, CS NPs, and VAM2-2-loaded CS NPs were able to induce a statistically significant increase in the mean survival time of the immunocompetent and immunosupressed infected mice as compared to the infected untreated control. However, the least mean survival time was revealed in CS NPs treated subgroups. Mice treated with VAM2-2-loaded CS NPs exhibited the longest mean survival time whether immunocompetent or immunosuppressed (subgroups II3 and IV3 respectively) with a mean of 22.67 ± 0.82 and 18.17 ± 0.75 respectively. This longer survival time could be referred to the synergistic effect of both VAM2-2 and CS NPs against tachyzoites of *T.gondii* which could be explained as co-operation between the mechanism of action of both drugs. Loading of spiramycin on CS NPs also prolonged the survival time in treated mice in studies carried out by Etewa *et al.* in 2018 [70] and Hagras *et al.* in 2019 [71]

In the context of parasite load, this study revealed a statistically significant reduction in the mean tachyzoites count in the peritoneal fluid and liver impression smears among all treated mice compared to the infected untreated controls. Regarding the use of VAM2-2 alone, the % R in the peritoneal fluid and liver impression smears was 92 and 87.8 for subgroup II1 and 90.9 and 83.3 for subgroup IV1 respectively. In the in vitro study conducted by Rivera *et al*. in 2016 [14], VAM2-2 was

able to induce tachyzoites growth inhibition up to 90% at a concentration of 23.4 μM. Intravacuolar parasite count was also reduced (after 24 hours of treatment by VAM2-2) to about two tachyzoites in most PVs compared to the control that have about 16 intravacuolar parasites in each PV. In addition, VAM2-2 reduced the invasion rate of the tachyzoites to 4% at concentration of 23.4 μM. The authors stated that, VAM2-2 had the ability to induce alternation in the morphology of tachyzoites and blocked their invasion capabilities which in turn reduce the intracellular proliferation. This obvious decrease in tachyzoites proliferation probably resulted from alterations in the subpellicular cytoskeleton integrity by VAM2-2 which could also affect the actin-myosin system, that plays a role in the late stage of division process of tachyzoites.[14, 78] Similarly, Martins-Alho in 2014[18] declared 99.37% growth inhibitory effect of VAM2-2 on *T.vaginalis* at a concentration of 2.34 μM. On the other hand, quinoxaline derivatives proved their efficiency against other protozoan parasites as *Plasmodium yoelii yoelii* , *Trypanosoma cruzi* and *leishmania peruviana*.[19, 79, 80]

Concerning CS NPs infected treated subgroups, The % R in the peritoneal fluid and liver impression smears were 81.5 and 76.7 for subgroup II2 and 77.4 and 73.3 for subgroup IV2 respectively. The anti-toxoplasma effect of CS NPs could be explained by the interaction between positive charged amino group of CS and negatively charged outer cell membrane components of tachyzoites including phospholipids and proteins. This augments the permeability of the cell wall and induce the leakage of cellular contents leading to cell inhibition or killing. In addition, penetration of CS molecule into the nuclei and binding with parasite DNA possibly inhibit the formation of mRNA, synthesis of various proteins and DNA transcriptation.[37] Moreover, growth inhibition through blockage of nutrient flow has been suggested by several researchers. [41] Teimouri in 2018[37] reported that CS NPs were able to cause significant reduction in the parasite burden in the peritoneal fluid of infected treated mice compared to control group with tachyzoites growth inhibition rates of 86%, 84% and 79% in mice receiving LMW, MMW and HMW CS NPs respectively. Furthermore, Gaafar *et al.* in 2014[69] reported reduction in tachyzoite count in liver and spleen impression smears after treatment with CS NPs whether given before or after the infection. This reduction was higher with increasing the dose of CS NPs. In addition, variable % R, from 23.94 to 85.08%, was reported in previous studies.[70, 71, 81] This reduction explained by the sticky properties of CS which increased the retention and contact time to the tachyzoites.[81]

VAM2-2-loaded CS NPs showed the highest tachyzoites % R in the peritoneal fluid and liver impression smears as compared to the other treating drugs with values of 97.7 and 93.3 for subgroups II3 and 95.8 and 91.1 for subgroup IV3 respectively. The reduction in the mean number of tachyzoites in subgroup II3 was statistically significant in comparison with subgroup II2, while it was statistically non-significant as compared to subgroup II1. In addition, there was a statistically significant reduction in subgroup IV3 in comparison with both subgroups IV1 and IV2. CS NPs encapsulation of VAM2-2 increased the drug penetration through the parasite membranes, enhanced its bioavailability and prolonged the retention time which intensify the anti-toxoplasma effect of VAM2-2. In addition, these two compounds (VAM2-2 and CS NPs), which have different mechanisms of action against *T.gondii* can co-operate to enhance the final anti-toxoplasma effect. The ability of CS NPs to potentiate the effect of other drugs particularly the standard anti-toxoplasma drug, spiramycin, is reported by other studies.[70, 71, 81]

In the current study, there was a statistically significant decrease in the mean number of viable tachyzoites in immunocompetent and immunosuppressed infected treated subgroups as compared

to the corresponding infected control subgroups. In VAM2-2 treated mice, the % R in parasite viability was 65.5 for subgroup II1and 62.2 for subgroup IV1. In the in vitro study carried out by Rivera *et al*. in 2016 [14] VAM2-2 showed clear and marked deleterious effect on tachyzoites viability with limited toxic effect on the human epithelial type 2 culture cells (HEp-2). VAM2-2 induced gradual toxic effects on the viability of tachyzoites in a dose dependent manner. The authors stated that, VAM2-2 had the property to diffuse through the parasite membranes leading to swelling and lysis, hence reduce parasite viability. In addition, Rivera-Borroto *et al*, in 2009 [82] demonstrated that VAM2-2 at the concentration of 100 μg/mL displayed marked trichomonacidal activities of 99.37% and 100% at 24 hours and 48 hours respectively with mild toxic effect on host cells.

Regarding CS NPs infected treated subgroups, the % R in parasite viability was 50.1 and 27 for subgroups II2 and IV2 respectively. The reduction of tachyzoite viability by CS NPs could be explained by its anti-toxoplasma effect as mentioned before. Moreover, it was suggested that effective binding and aggregation of the CS molecules blocking the neutral flow and ultimately leading to cell lysis.[41] Similar results were reported by Esboei in 2020[83] and Teimouri *et al*. in 2018 [37] who found reduction in the viability of tachyzoites in vitro under the effect of CS NPs which elevated by increase the dose of the drug and the duration of exposure. Similar effect of CS NPs was reported against *Trichomonas gallinae* trophozoites[32] and *Giardia intestinalis* cyst in vitro [34] and *Cryptosporidia parvum* oocysts in vitro and in vivo[84]. The authors referred the effect of CS NPs on *Cryptosporidia* oocysts to its ability to adsorb to the surface of oocysts wall causing its disruption through the formation of pits or dimples which could lead to leakage of the oocyst's components. In addition, the wall of *Cryptosporidia* oocysts has negative charge that increase the ability of CS (positively charged) and oocyst to stick together and in turn increase the CS NPs anti-cryptosporidial activity. [84] This can also be applied on *T. gondii.*

VAM2-2 loaded CS NPs induced the highest decrease in the mean number of viable tachyzoites with % R of 79.2 for subgroup II3 and 69.2 for subgroup IV3. The reduction was statistically significant in comparison to the corresponding infected treated subgroups. Loading of VAM2-2 on CS NPS increased the drug penetration into the tachyzoites, by their small size, which led to intensify its effect on tachyzoites viability. Similar results of reduction of tachyzoites viability were recorded in previous studies when anti-toxolasmic drugs were loaded on different nanoparticles. [77, 85]

As regards animal infectivity, tachyzoites collected from infected non-treated control subgroups Ib and IIIb were highly infectious to naive mice (100% IR), while there was a dramatic drop in the infectivity power of tachyzoites gathered from the infected treated mice. There was a statistically significant decrease in the mean number of tachyzoites in the peritoneal fluid of both immunocompetent (II1x, II2x and II3x) and immunosuppressed (IV1x ,IV2x and IV3x) subgroups in comparison to their corresponding controls (subgroups Ibx and IIIbx respectively). In VAM2-2 treated mice, the IR was 30% and 40% with % R of 97.7 and 95.9 in subgroups II1x and IV1x respectively. The decrease in the parasite infectivity and parasite burden referred to the damaging effect of VAM2-2 on subpellicular cytoskeleton of tachyzoites. Lack of functional subpellicular cytoskeleton make the organism incapable of invading new host cells, in addition to its inability to nuclear division or budding.[86]

Concerning mice infected by the tachyzoites harvested from the infected CS NPs treated subgroups, the IR was 50% and 70% with % R of 86.4 in subgroup II2x and 77.2 in subgroup IV2x

respectively. Teimouri *et al*. in 2018 [37] reported that no tachyzoites or cysts were detected in peritoneal exudate (after five days) or in the brain (after two months) of mice I.P inoculated with in vitro CS NPs treated tachyzoites which indicated that CS NPs had a considerable inhibitory potency to the infection. In the present study, the mean number of tachyzoites in subgroup II2x and IV2x was statistically significant higher compared to subgroup II1x and IV1x respectively. These results indicated the more potent anti-toxoplasma activity of VAM2-2 than CS NPs.

Regarding mice infected by the tachyzoites taken from the infected VAM2-2 loaded CS NPs treated subgroups, the IR was 20% and 30% with % R of 99.2 for subgroup II3x and 97.8 for subgroup IV3x. The reduction in the mean number of tachyzoites in subgroup II3x was statistically significant when compared to subgroup II2x. While, there was a statistically non-significant reduction in subgroup II3x in comparison to subgroup II1x. Meanwhile, there was a statistically significant reduction in subgroup IV3x in comparison with subgroup IV1x and IV2x. These results indicated that CS NPs encapsulation of VAM2-2 caused a reduction in the parasite infectivity and tachyzoites burden in the peritoneal fluid, which emphasized its role in enhancing the anti-toxoplasma effect of VAM2-2 besides decreasing its dose. Similar reduction in tachyzoites infectivity were recorded by El Zawawy *et al*. in 2015 [77] upon using liposomal encapsulated TS which induced reduction in tachyzoites infectivity rate (10%) in comparison to TS alone (60%). The % R in parasite burden was 97.5% and 83.6% respectively which might be related to delayed death phenomena reported by Burkhardt *et al*. in 2007.[87]

From the results of the different parasitological parameters in the present study it was noticed that the anti-toxoplasma effects of the tested drugs VAM2-2, CS NPs and VAM2-2 loaded CS NPs were more pronounced in the immunocompetent subgroups than in the immunosuppressed subgroups. This might be explained by the combined effect of the drugs and the intact immune cells on the tachyzoites.

Ultrastructurally, the SEM of *T. gondii* tachyzoites in the infected treated mice with VAM2-2 (subgroups II1& IV1) showed distortion in the crescent shape and various surface irregularities in the form of (irregular ridges, dimples,erosions and/or protrusions). TEM of tachyzoites in these subgroups showed deformed apical complex, enlargement or alternation in dense granules and multiple wide or coalesced cytoplasmic vacuoles. Some tachyzoites also lost the integrity of the cytoplasmic membrane and the nuclear membrane, sometimes with nuclear deformity. Distortion in the shape of tachyzoites induced by VAM2-2 could be due to alteration in the subpellicular cytoskeleton by the drug as suggested by Rivera et al in 2016[14] as the shape and the dynamic properities of tachyzoites depends mainly on the cortical cytoskeleton and subpellicular micotubules.[86] Deformity in the apical region is an indication of the efficiency of VAM2-2 on tachyzoites invasion capability as the organelles in the apical region are essential for entry of the organism into the host cells and in turn causing their multiplication and proliferation.[88]

Similar changes in tachyzoites were reported by Rivera *et al*. in 2016 [14] by SEM and TEM after in vitro treatment with VAM2-2. They reported that with higher concentrations of VAM2-2, a gradual and irreversible destruction of the parasite appeared. This referred to direct effect of VAM2-2 on cytoskeleton integrity causing alteration in the subpellicular cytoskeleton organization and damage of pellicle or even the intracellular organelles as dense granules. The latter act as export pathway of tachyzoites, similar to that take place by rhoptry secretion, but its effect appear usually later after invasion of the host cell and during intracellular replication which contributes to cell cycle arrest.[89]

In addition, VAM2-2 has the ability to target actin filaments causing its solubilization which lead to damage of cytoskeleton also.[14] Similarly, treatment of tachyzoites with quinazalinone and quinazoline by El Tombary *et al.,* in 1999[90] showed smooth homogenous surface with loss of their ridges. In addition to redundancy in their membrane and disorganized conoid. Similar changes were also reported by Hammouda *et al.,* in 1992[91] after treatment of tachyzoites with pyrimethamine or spiramycin. They became rounded or triangular with loss of their conoids. These referred by the authors to interference of the drugs with DNA synthesis or interference with the folic acid cycle.

Regarding the infected treated mice with CS NPS (subgroups II2& IV2), SEM showed multiple ridges on the tachyzoites surface with dimples and protrusions. TEM showed slight distortion in the crescent shapes of tachyzoites with an apparently normal structure of the nucleus, while the apical complex appeared deformed. In addition, there were irregularities and disruption in the plasma membrane with vacuolations in their cytoplasm. Similar ultrastructure changes in *T.gondii* tachyzoites by CS NPs treatment were reported in several studies.[37, 71] Teimouri *et al*, in 2018 [37] detected irregular surface protrusions with multiple deep ridges by SEM in tachyzoites surface treated with LMW CS NPs. They contributed these changes to the electrostatic interaction between the positively charged CS molecules and the negatively charged outer membrane components of the tachyzoites. In addition to penetration of CS molecules into the nuclei and binding with the parasite DNA. These led to changes in shape of tachyzoites, cell wall integrity and permeability. Tachyzoites recovered from the treated mice in the study conducted by Hagras *et al.* in 2019 showed distortion in their crescent shape, loss of smooth surface and loss in the conoid in addition to dimples, ridges, papules, protrusions, erosions, deep furrows and/or ulcerations.[71] CS NPs also induced ultrastructure deformities in *Cryptosporidium parvum* oocysts in the study carried out by Ahmed *et al.* in 2019, in the form of disruption in wall with pits or dimples, formation of multiple vacuoles and leakage of the oocyst components. This led to oocysts enlargement then the oocysts wall cracked under pressure with expulsion of all their contents.[84]

The ultrastructural changes of tachyzoites were more noticed in the present study in those treated with VAM2-2 loaded CS NPS (subgroups II3 and IV3). The tachyzoites appeared, by SEM, shrunken and obviously distorted with deformed apical region. Moreover, the tachyzoite surfaces showed numerous deep ridges, dimples, protrusions, compressions and erosions. Some tachyzoites showed leakage of the internal contents. In addition, by TEM, they showed completely disintegrated plasma membrane with leakage of the internal contents also. There were irregularities and separation of the nuclear membranes. Complete destruction of the internal structures with extensive cytoplasmic vacuolations and ghost-like appearance of some tachyzoites were also noticed. These severe ultrastructural changes noticed in VAM2-2 loaded CS NPS subgroups could be attributed to the combined effect of VAM2-2 and CS NPS on the tachyzoites. The efficiency of CS NPs in enhancing the effect of different drugs on the ultrastructure of tachyzoites has been proven by Hagras *et al.,* in 2019 [71, 92], Etewa *et al.* in 2018[70] and Gaafar *et al.* in 2014[69].

The effect of the treating drugs; VAM2-2, CSNPs and VAM2-2 loaded CS NPS on the ultrastructure of tachyzoites was more obvious in the immunocompetent subgroups. This might be explained by the combined effect of the drugs and the intact active host immune system on the tachyzoites. These ultrastructure changes could explain the loss of invasion and intracellular

proliferation of the treated tachyzoites which subsequentially caused decrease in parasite burden. Furthermore, it could explain the decrease in viability and infectivity of the treated tachyzoites.

To study the effect of VAM2-2, CS NPs and VAM2-2 loaded CS NPS on the immune system in the present work, the level of IFN-γ in sera of all mice subgroups was determined on the fifth day PI. IFN-γ was chosen to measure in the present study as it is crucial for host defense against *T.gondii* and it is critical for control of the acute infection. It has It has an important role in recruiting macrophages to sites where antigens are present. It also has a direct inhibitory action on the growth of intracellular organisms as *T.gondii*.[93] Suzuki *et al.* in 1988[94] injected RH strain infected mice with monoclonal antibody to IFN-γ and revealed inhibition of macrophages activation, which kill tachyzoites, with death of injected mice in comparison to non-injected mice which survived and developed chronic *Toxoplasma* infection . Similarly, Hunter *et al.,* in 1994[95] reported that, treatment with anti IFN-γ-antibody abolished the increase in the NK cell activity and resulted in earlier mortality of infected mice. These suggested that IFN-γ is an important mediator of host resistance against acute *T.gondii* infection. In addition, it is essential to control chronic infection.[96]

Concerning the immunocompetent subgroups, the mean value of serum IFN-γ in the infected non-treated subgroup (Ib) was significantly higher than the level recorded in non-infected non-treated subgroup (Ia). This may be attributed to the production of IFN- γ by NK and T cells as a result of pathogen recognition by Toll- like receptors on macrophages (TLRs).[97] Similarly, Hamad *et al.* in 2018[98] reported increase in serum circulating IFN-γ during infection with 10^3 tachyzoites of RH strain of *T.gondii* when compared to the normal controls.

Since, it was the first time to evaluate the effect of VAM2-2 in vivo against *T.gondii* infection, no data were available about its effect on IFN-γ. The level of IFN-γ in non-infected VAM2-2 treated subgroups (Ic1) was nearly equal to that of the non-infected non-treated subgroup (Ia) indicating that VAM2-2 almost has no effect on the immune system of the mice and on IFN-γ production. On the other hand, there was non-significant difference in serum IFN-γ levels between infected VAM2-2 treated subgroup II1 and subgroup Ib. The elevation in IFN-γ level in this case could be referred mainly to *Toxoplasma* infection.

There was a statistically significant increase in the mean value of serum IFN-γ in non-infected and infected CS NPs treated subgroups (Ic2 and II2) and VAM2-2 CS NPs-treated subgroups (Ic3 and II3) in comparison to the corresponding non-treated subgroups (Ia and Ib) or VAM2-2 treated subgroups (Ic1and II1). This could be attributed to the immunomodulatory effect of CS NPs inducing a strong cell-mediated immune response. Many studies had reported that chitosan has a significant immunomodulatory activity, by encouraging immune cells to secrete a wide variety of pro- and anti-inflammatory cytokines.[27-30] In infected mice, the combined effect of infection and CS NPs in stimulating the immune system led to more elevation in the IFN-γ level. These results are in agreement with that of Hamad *et al.* in 2020 [81] and 2018[98] who reported a statistically significant increase in IFN-γ level in sera of infected mice treated with CS or CS NPs in comparison with non-infected treated mice, as a result of enhancement of immunity. Similarly, Gaafar *et al.* in 2014 [69] reported increased serum level of IFN-γ in infected mice treated with CS NPs for four days PI. They attributed their results to the effect of CS NPs in stimulating the immune system leading to increased production of IFN-γ.

In the present work, despite that there was non-significant difference between infected VAM2-2 CS NPs-treated subgroup (II3) and infected CS NPs-treated subgroup (II2) regarding the level of IFN-γ, mice in subgroup II3 had recorded the highest % reduction in the tachyzoites burden and tachyzoites viability which could be contributed to the synergistic effect of VAM2-2 and CS NPS on the parasite with increased IFN-γ production by the activated intact immune cells under the effect of CS NPS. Loading of anti-toxoplasma drugs on CS NPs; spiramycin, Ag NPs or spiramycin and Ag NPs induced more pronounced elevation in IFN-γ level in comparison with free drugs in previous studies.[69, 81, 98]

As regards the immunosuppressed subgroups, the mean value of serum IFN-γ in the non-infected non-treated subgroup (IIIa) and the non-infected VAM2-2 treated subgroup (IIIc1) was very low. This could be due to the immunosuppression of mice induced by administration of cyclophosphamide which consequently decrease the IFN-γ level. Zoheir *et al.* in 2015[99] reported down regulation of the IFN- γ genes by cyclophosphamide administration. However, IFN-γ was significantly increased in the non-infected CS NPs treated subgroup (IIIc2) and in non-infected VAM2-2 CS NPs-treated subgroup (IIIc3) in relation to either subgroup IIIa or IIIc1. Thus, it may be suggested that CS NPs could ameliorate the immunosuppressive effect of cyclophosphamide, and hence the level of IFN-γ increased in these subgroups of mice. Similar results were obtained by Mudgal *et al.* in 2019 [100] who reported that CS NPs and gallic acid loaded CS NPs (cGANP) were able to induce a significant increase in the IFN-γ levels in immunosuppressed mice by cyclophosphamide. Also, Zhai *et al.* [101] in 2018 stated that administration of chito-oligosaccharides (COS) (degraded products of chitosan or chitin) to cyclophosphamide immunosuppressed mice had led to markedly restore the reduced indices of spleen and thymus, NK cell activity, macrophages phagocytic index and both T- and B-lymphocytes proliferation and reversed the immunosuppressive effect of cyclophosphamide.

Concerning the immunosuppressed infected non-treated subgroup (IIIb), the mean value of serum IFN-γ was significantly higher than the level documented in non-infected non-treated subgroup (IIIa). This may be attributed to the production of IFN- γ in response to infection as in immunocompetent infected non-treated subgroup (Ib) but with lower extent due to the immunosuppressive effect of cyclophosphamide. This reflected on the tachyzoites count and viability since they were higher in subgroup IIIb than in subgroup Ib. In infected treated subgroups, there was a statistically significant increase in mean value of serum IFN-γ in the infected CS NPs-treated subgroup (IV2) and infected VAM2-2 CS NPs-treated subgroup (IV3) in comparison with the infected non-treated subgroup (IIIb) and the infected VAM2-2 treated subgroup (IV1). In spite of the immunosuppressive effect of cyclophosphamide in subgroup IV2 and IV3, administration of CS NPs could overcome this suppressive effect and induced some degree of stimulation to the immune system which led to elevation of IFN-γ. However, the mean value of IFN-γ level in these subgroups was much lower than those in the corresponding immunocompetent subgroups II2 and II3. Thus, the reduction in tachyzoites count and viability in subgroup IV2 could be attributed mainly to the direct effect of CS NPs on the parasite rather than its effect as an immunostimulant. Similarly, the reduction in tachyzoites count and viability in subgroup IV3 could be attributed to the direct potent action of VAM2-2 and CS NPs on the tachyzoites.

The development of well tolerated and safe chemotherapy against *T.gondii* is a highly valuable goal especially with increasing number of high risk individuals as immunocompromised patients

and pregnant women. In the present work, a biochemical study was performed to assess the toxic effect of VAM2-2, CS NPs and VAM2-2 loaded CS NPs on the liver by measuring liver transaminases; AST and ALT and on the kidney by measuring urea and creatinine to ensure their safety.

Concerning the non-infected treated subgroups in the current study, VAM2-2, CS NPs or VAM2-2 loaded CS NPs did not induce any toxic effects on the liver or the kidneys as shown by the normal levels of liver enzymes, urea and creatinine recorded in comparison to the non-infected non treated subgroup Ia.

All previously conducted invitro study on VAM2-2 stated that the drug had mild toxic effect on culture cells.[14, 82] Rivera *et al.* in 2016 [14] stated that exposure of cells to different doses of VAM2-2 drug for 24 hours did not change the morphology of uninfected host cell and had a little effect on their viability indicating that this drug specifically affected the viability and structural integrity of the parasite.

As regards CS, it is a natural and safe polymer.[102] It is permitted for food applications in some countries[103] and it is approved for usage by USA food and drug administration (FDA).[104]Multiple in vivo studies has documented that chitosan has no toxic effects.[105-109] On the other hand, CS at dose of 50gm/kg diet could significantly decrease the elevated serum AST, ALT levels in rats.[110] Moreover, it could protect rats against high fat diet that induced hepatic steatohepatitis.[111]

The mean value of serum liver enzymes (AST and ALT) and urea in the infected non-treated subgroup (Ib) was significantly higher than the level recorded in non-infected non-treated subgroup (Ia). This could be referred to extensive and progressive damage in the liver by *T.gondii* owing to remarkable proliferation of the organisms.[112] This liver damage leads to metabolic changes causing decrease in hepatic protein synthesis.[112] Furthermore, the increase in the urea level could be attributed to the deleterious effects of *T.gondii* on the kidney which decrease the urea excretion from the body and subsequently increase its serum level.[112] In addition, over stimulation of immune responses by virulent RH strain of *T.gondii* with over induction of inflammatory cytokines (Th1 cytokines) lead to extensive tissue damage which suggest that the resulting pathology is partially immune-mediated.[96] The results of the present study were in agreement with previous studies which reported significant increase in liver enzymes and kidney function test in response to RH strain of *T.gondii*. [98, 113-115]

In the immunocompetent infected treated subgroups, there was a statistically significant decrease in mean value of serum liver enzymes and urea in all infected treated subgroups II1, II2 and II3 in comparison with the infected non-treated subgroup (Ib). This might be explained by the anti-toxoplasma effects of the tested drugs which ameliorate the effect of infection on liver and kidney. In the present work, the capability of VAM2-2, CS NPs and VAM2-2 loaded CS NPs to alleviate *Toxoplasma* infection was proved by decrease tachyzoites burden and viability, thereby the expected pathological effect of *Toxoplasma* on the liver and kidney could be diminished. Consequently, the level of liver enzymes and urea significantly decreased after treatment. This clear up that short term treatment with any of these drugs led to non-significant effect on liver and kidney function tests. A significant decrease in liver enzymes and or renal function tests after treatment of experimental toxoplasmosis by several drugs was reported in previous studies.[113, 115, 116]

In the present work, the level of liver enzymes and urea in VAM2-2 loaded CS NPs subgroup nearly returned to normal level indicating the significant therapeutic effects of VAM2-2 loaded CS NPs against the infection which in turn alleviate its deleterious effects on liver and kidney. This could

be attributed to the combination of VAM2-2 and CS NPs which led to drug dose reduction and consequently increase its safety. Loading of several anti-toxoplasma drugs on CS NPs could also decrease the damage produce by the parasite on the liver as reported by Etewa *et al.* in 2018 [70] and Hamad *et al.*, in 2018[98] when using spiramycin-loaded CS NPs.

In the present study, it was found that the level of liver enzymes (AST and ALT) was increased in the immunosuppressed non-infected non-treated subgroups. This could be contributed to the toxic effect of cyclophosphamide on liver enzymes. Hepatotoxicity associated with cyclophosphamide therapy is infrequent but possible, where liver enzymes' level may be markedly increased but usually resolve after discontinuation of the drug.[117, 118]

Regarding the immunosuppressed infected non-treated subgroup (IIIb), the mean value of serum level of liver enzymes and urea was significantly higher than the level estimated in non-infected non-treated subgroup (IIIa). This may be attributed to flaring up of the infection due to immunosuppression with increase the rate of growth and multiplication of the parasite and subsequently increase its cell damaging effect. This was documented by the reported elevation in the tachyzoites count in all immunosuppressed subgroups. Moreover, liver enzymes increased under the effect of cyclophosphamide as well.

On the other hand, a statistically significant decrease in the level of liver enzymes and urea was found in the all immunosuppressed infected treated subgroups (IV1, IV2 and IV3) in comparison with the infected non-treated subgroup (IIIb). However, the mean value of liver enzymes and urea level in these subgroups was higher than those in the corresponding immunocompetent subgroups (II1, II2 and II3 respectively). The highest level of liver enzymes and urea was noticed in infected CS NPs treated subgroup (IV2) which could be explained by the highest number of tachyzoites in this subgroup in comparison to subgroups IV1 and IV3.

In the present study, the serum level of creatinine was within the normal range in all studied subgroups whether infected or not, immunocompetent or immunosuppressed. The increase in urea level in absence of increase in creatinine level could be referred to an activation of protein metabolism in toxoplasmosis rather than presence of renal lesion.[119] Mordue *et al.* in 2001 [96] stated that significant pathology during acute RH strain infection was usually restricted to the liver and lymphoid tissues. Similarly, Mordue *et al.* in 2001 [96], Al- Kaysi *et al.* in 2010 [115] and Aleksandro *et al.* in 2013 [119] documented non-significant difference in creatinine level between RH strain tachyzoites infected mice and non-infected mice. However, significant increase in creatinine level in response to toxoplasmosis was documented by Garedaghi *et al.* in 2012[120], Al-Jowari *et al.* in 2014[121] and Al-Khamesi *et al.* in 2016 [122]. These differences in creatinine level between our study and the previous studies could be attributed to the difference in the type of infected host or the timing of blood collection from the host for measurement of the serum creatinine level. It was found that when creatinine level was measured early during the course of the infection (on third- or fifth-day PI), normal levels were detected.[115, 119] While, late measurement of creatinine level (starting from seventh day PI) had resulted in high creatinine level.[116]

In conclusion, the present study proved the efficacy of VAM2-2 against experimental acute toxoplasmosis. VAM2-2 led to significant reduction in the parasite burden, parasite viability, and infectivity of *T.gondii* tachyzoites in both immunocompetent and immunosuppressed mice. This was supported by obvious ultrastructural changes in tachyzoites without evident toxicity to liver and

kidney. loading of VAM2-2 on CS NPs was efficient in lowering its dose with more reduction in parasite burden, parasite viability and parasite infectivity, in addition to greater ultrastructural changes in tachyzoites and increase in serum immunological marker (IFN-γ) which clarified the superior effect of this combination. Therefore, VAM2-2 loaded in CS NPs can be considered a promising alternative to the standard therapy for treating toxoplasmosis especially in immunocompromised host and pregnant females in whom its adverse effects cannot be tolerated. Further studies on VAM2-2 and VAM2-2 loaded CS NPs are highly recommended to evaluate their effects against cysts of the avirulent strain of *T.gondii*.

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