Acta Parasitologica Globalis 4 (3): 110-120, 2013
ISSN 2079-2018

DOI: 10.5829/idosi.apg.2013.4.3.7584

Evaluation of Vaccination with *Toxoplasma gondii* Trophozoite Lysate and Influenza Virus Vaccine Against Experimental Murine Toxoplasmasis

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Abstract: The current study aimed to assess the role of *T.gondii* parasite lysate immunization with /without Influenza virus as adjuvant against *T.gondii* challenge infection. Laboratory bred mice were immunized with *T.gondii* lysate with/without Infleunza virus vaccine. Brains were examined for counting brain cysts. Sera were tested for IgM and IgG. INF gamma expression was studied by RT- PCR. Histopathololgical examination was done for spleen and brains from all groups. Results revealed that all vaccinated groups showed decreased brain cyst number. IgM and IgG were shown to be increased. INF gamma expression was up regulated in tested groups. There was regression of pathological manifestations in different groups. In conclusion,*T.gondii*lysate vaccine with Influenza virus vaccine can elicit a broad range of immune responses that are capable of decreasing mortality in mice acutely infected with *T. gondii.* It was found that the vaccine is capable of reducing the levels of brain cysts and induced mixed Th1 and Th2 responses.

Key words: *Toxoplasma gondii*-Trophozoite-IgM and IgG- INF gamma

INTRODUCTION

Toxoplasmosis is a foodborne zoonotic disease with global distribution and is caused by a protozoan *Toxoplasma gondii*. The disease is asymptomatic in immunocompetenthosts, but may be a serious clinical problem with lethal outcome [1]. *T. gondii* is reported to infect about one-third of the world's human population. *T.gondii* is the causative agent of congenital infection and abortion in humans. The opportunistic pathogen *T.gondii* is even associated with encephalitis or systemic infections in the immunocompromised, particularly individuals with HIV/AIDS [2]. Toxoplasmosis induces an innate immune response, with alterations in monocytes and dendritic cells contributing to pathogen dissemination and establishment of persistent infection [3].

The ability of the parasite to subvert intracellular signaling pathways in infected cells to evade immunity had been related to specific parasite molecules [3]. Immunity to *T.gondii* infection is dependent on Interferon-gamma (IFN-g) induced by interleukin (IL)-12production from a variety of cell types. Also, IL-10 is important in induction of the immune response to toxoplasmosis, however, *T. gondii* has mechanisms to interfere with host signaling pathways to subvert innate immunity [4,5].

The main obstacle for construction of efficient vaccines is the inadequate cellular immuneresponses, mediated by CD8+ T lymphocytes [6,7]. Efforts of vaccine development against *T. gondii* have been focused mainly on tachyzoite surface antigen 1 (SAG1)[8]. Adjuvant should be included into *T.gondii* vaccines to enhance its effects [9].

Parasite molecules are responsible for stimulation of the host immune system [4,10,11], together with type of inflammatory cells recruited, number and degree of parasite invasion and degradation by phagocytes [3].

Viable parasites and lysates induce down regulation of MHC class II, but their mechanisms might differ as they affect different IFN-gamma responsive promoters [12]. The parasite proteins contribute to *T. gondii* down-regulation of surface MHC class II, increased expression of MHC class I is associated with enhancement of antigen presentation to CD8 T-cells [13].

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The current study aimed to assess the role of *T.gondii* parasite lysate immunization with /without Influenza virus vaccine as adjuvant in inducing immune response against *T.gondii* challenge infection. The effects were studied by detecting brain cysts, *Toxoplasma* IgM and IgG, INF gamma expression and histopathological examination of brains and spleens of mice in different groups.

MATERIALS AND METHODS

Experimental Schedule: Eighty laboratory bred BALB/c mice were divided into four groups(20 mice/each);group 1 (GI) vaccinated by *Toxoplasma* trophozoite lysate with Influenza virus vaccine; group 2(GII) vaccinated by *Toxoplasma* trophozoite lysate. Group 3 (GIII) was vaccinated with Influenza virus vaccine and group 4 (GIV) the control group. Then challenge infection with *Toxoplasma* trophozoites four weeks after the last immunization. Ten mice from each group were sacrificed 5 and 8 weeks after challenge. The parasite burden was evaluated. The immune response was monitored; IgM and IgG level were evaluated by ELISA, Interferon gamma by RT-realtime PCR, histo pathological examination of brains and spleens from all groups of animals was done.

Mice: Eightylaboratory bred male BALB/c mice, 8weeks-old. Mice were obtained from Nile Pharmaceutical Company. They were raised in animal house at faculty of Medicine, Menoufiya University in laboratory conditions (25±2°C and 60% relative humidity). Mice were fed on commercial diet *Ad libitum*. All manipulations were carried out according to International Guide to use laboratory animals.

Parasites: Tachyzoites of *T. gondii* were maintained in laboratory and obtained from peritoneal cavity of mice infected three days before. Tachyzoites were washed with phosphate-buffered saline(PBS) and used for challenge infection or stored at -80°C until used [14,15].

Antigen Preparation: Tachyzoites of *T. gondii* obtained from peritoneal exudates from mice infected 3-4 days earlier, then washed, sonicated and centrifuged as previously described. The supernatant from the last centrifugation was used as the source of antigen. Ten mg of protein was determined by a protein assay, modified Bradford method and were used as a standard and stored at -20°Cuntil used [15,16].

Influenza Virus Vaccine: (Influvac 2009/2010, Solvay Biologicals B.V. Netherlands). The vaccine is formed of Influenza virus surface antigens (haemagglutinin and neuraminidase), with potassium chloride, potassium hydrogen phosphate, disodium phosphate dehydrate, sodiumchloride, calcium chloride, magnesium chloride hexahydrate and water in the form of colorless clear liquid. Ten micrograms of the vaccine was given to GI and GIII.

Immunization Protocol: Mice were intramuscularly (i.m.) injected twice with two weeks interval. GI was injected with 20µg of *T. gondii* laysate in PBS with 10 µg of Influenza virus vaccine. GII was injected with 20 µg of *T.gondi* laysate only. GIII was injected with with10 µg of Influenza virus vaccine. GIV was injected with PBS only at similar time points (Control group) [17,18].

***Toxoplasma Gondii* Challenge Infections:** Four weeks after the last immunization, all groups were challenged (i.p.) with 1 x 10^3*T.gondii* tachyzoites obtained from mice peritoneum infected 3 days before [19]. Ten mice were sacrificed 5 and 8 weeks after the challenge.

Quantitation of *Toxoplasma Gondii* Cysts in Mouse Brain:After 5 and 8 weeks, brains of mice were individually homogenized in 3 ml Phosphate Buffered Saline (PBS) and 10 µl of the homogenized brain suspensions was examined in order to calculate the number of *T. gondii* tissue cysts under an optical microscope. This procedure was carried out in triplicate and the mean of three counts was obtained and was then used to calculate the total number of *T. gondii* tissue cysts in each brain sample [20,21].

Tests for *Toxoplasma* Antibodies: *Toxoplasma*-specific IgG and IgM antibodies were searched for in mice sera by the conventional enzyme-linkedimmunosorbentassay (ELISA) according to manufacturer criteria. (CUSABIO, USA. Catalog No CSB-E12828m and CSB-E12829 m, respectively).

IFNgamma mRNA Detection by RT-PCR: For cytokine analysis, the LightCycler is most often used in combination with the dsDNA binding dye SYBR Green I.

The primers used were; Glyceraldehydes 3-phosphate dehydrogenase (GAPDH) house keeping gene.

FW:TCACCACCATGGAGAAGGC; RV: GCTAAGCA GTTGGTGGTGCA and TP: ATGCCCCCA TGTTTGTGATGGGTGT. For IFN-gamma.

FW:TCAAGTGGCATAGATGTGGAAGAA,RV: TGGCTCTGCAGGATTTTCATGandTP: TCACCATCCTTTTGCCAGTTCCTCCAG [22].

Peripheral blood mononuclear cell (PBMC)were prepared from mice by centrifugation of heparinized venous blood on Lymphoprep (Nycomed, Oslo, Norway). Total RNA was isolated using a commercially available reagent (Tripure$^{T.M}$. Roche Diagnostics), Following the manufacturer's instructions. In somecases, total RNA was treated with 10 units of RQ1RNase-free Dnase (Promega, Madison, WI) for 30 min, in order to avoid amplification of contaminating genomic DNA. After the addition of 500 µl of Tripure$^{T.M}$ to inactivate DNase, total RNA was extracted once again. Reverse transcription of mRNA was carried out as follows: 8µl of water containing 500 ng of total RNA were added to 2 µl of oligodT primer (0.5 µg/µl) and incubated at 65 °C for 10 min. Samples were chilled on ice and 10 µl of RT mix containing the following components were added: 4 µl 5XRT buffer 250 mMTris-HCl, pH 8.3, 375 mMKCl, 15 mM$MgCl_2$,2 µl deoxynucleotide triphosphate mix (10 mM each),0.2 µl bovine serum albumin (1mg/ml), 0.6 µl (25 U) human placental ribonuclease inhibitor (RNAguard, Pharmacia Biotech, Sweden), 1 µl (200 U)M-MLV reverse transcriptase (Gibco Life Technologies, Scotland, UK), 0.2 µl H_2O and 2 µl dithiothreitol (100 nM). The samples were then incubated at 37°C for 60min [23,24].

The PCR reaction was carried out in a 20-µl final volume containing: H_2O up to 20 µl, 2 µl DNA Master Probes 10X, 5 µl 25 mM MgCl2, 3 µl of 6 pmol/µl forward and reverse primers (final concentration 300 nM), 1 µl of 4 pmol/µlTaqMan probe (final concentration 200 nM), 0.3µl anti-Taq DNA polymerase antibody (Platinum *Taq* antibody, Gibco Life Technologies), 1 µlc DNA or standard dilution. After an initial denaturation step at 95°C for 30 s, temperature cycling was initiated. Each cycle consisted of 95°C for 1 second and 60°C for 20 s, the fluorescence was read at the end of this second step. A total of 45cycles were performed [24].

Histopathological Study: Samples were taken from the spleen and brain of mice in different groups and fixed in 10% formol saline and paraffin bees wax tissue blocks were prepared and stained by hematoxylin and eosin stains for histopathological examination [25].

SDS-PAGE: Whole tachyzoites lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition on gradient polyacrylamide gel. Standards markers of molecular weights (MWs) were used (Sigma Chemical Co. USA). Gels were run at 25 mA/gel and stained [26].

Statistical Methods: Data was collected, tabulated and statistically analyzed using SPSS statistical program version 13. Comparison was done between two groups with non parametric data using Mann-Whitney test and comparison between paired groups with non parametric data with Wilcoxon's Signed test. Also comparison between more than three groups with non parametric data with Krsukal Wallis test was done. Significance was considered at 5% level [27].

RESULTS

Data in Table 1 show that *Toxoplasma* brain cysts detected in different groups were significantly reduced in all groups as compared to GIV. The least number of brain cysts was reported in GI and GII at 5 and 8 w.p.i. as compared to other groups.

Serum anti *Toxoplasma* IgM antibodies was significantly (P<0.001) higher in all groups as compared to GIV. Anti *Toxoplasma* IgM antibodies in mice sera were significantly higher in GI and GII at 5 and 8 w.p.i. (Table 2).

Serum anti *Toxoplasma* IgG was significantly higher in GI as compared to other groups at the tested days(P<0.001). There was significant increase in serum IgG from 5 to 8 w.p.i. in all groups (Table 3).

Table 1: Number of *Toxoplasma* brain cysts in different groups after 5 and 8 weeks of infection

					Krsukal Wallis test	
	GI	GII	GIII	GIV		
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	X^2	*P*.value
After 5weeks of challenge	3.0±2.4	12.7±4.1	40.8±9.2	120±49.8	33.1	<0.0001***
After 8 weeks of challenge	34.5±21.5	97.9±47.7	111.5±47.1	285.0±149.3	18.35	<0.0001***
Wilcoxon Signed test	2.8	2.8	2.7	2.8		
P.value	<0.01**	<0.01**	<0.01**	<0.05*		

*significant **Very highly significant Picogram/ML

Table 2: Anti-*Toxoplasma*IgM in different groups at5 and 8 weeks post infection.

	GI	GII	GIII	GIV	*Krsukal Wallis test*	
	Mean±SD	Mean±SD	Mean±SD	Mean±SD		
Serum IgM	(PG/ML)	(PG/ML)	(PG/ML)	(PG/ML)	X^2	*P*.value
After 5 weeks of challenge	334.3±253.0	254.4±95.6	98.6±61.8	38.5±37.8	17.68	<0.001**
After 8 weeks of challenge	711.8±74.4	365.5±72.3	132.9±34.4	74.4±23.0	35.87	<0.001**
Wilcoxon Signed test	1.98	1.48	1.98	1.94		
P.value	<0.001**	<0.001**	>0.05^{NS}	>0.05^{NS}		

^{Ns}non significant *significant **highly significant

Table 3: Anti-*Toxoplasma* IgG in different groups at 5 and 8 weeks post infection.

	GI	GII	GIII	GIV	*Krsukal Wallis test*	
	Mean±SD	Mean±SD	Mean±SD	Mean±SD		
Serum IgG	(PG/ML)	(PG/ML)	(PG/ML)	(PG/ML)	X^2	*P*.value
After 5 weeks of challenge	703.9±199.9	379.2±87.2	314.5±26.9	199.9±35.3	26.78	<0.001**
After 8 weeks of challenge	3357.6±501.9	2876.2±212.5	2092.0±288.9	1732.4±147.9	25.27	<0.001**
Wilcoxon Signed test	2.8	2.8	2.8	2.8		
P.value	<0.01*	<0.01*	<0.01*	<0.05*		

*significant **highly significant Picogram/ML

Table 4: Interferron gamma gene expression by reverse transcriptase polymerase chain reaction in different groups after 5 weeks of infection in relation to house keeping gene GADPH.

					Krsukal Wallis test	
	GI	GII	GIV	GV	X^2	*P*.value
Relative INF gamma copies/ GADPH copies Ratio	61.6±4.9	30.8±11.1	23.3±7.6	4.9±2.1	33.56	<0.001**

**highly significant

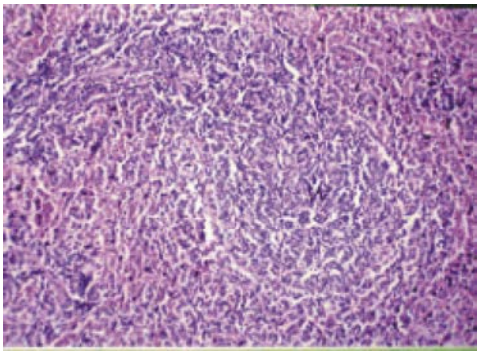

Fig. 1: Shows spleen of mice in GI with lymphoid hyperplasia in white pulp(w) (5 weeks post infection H&E X40).

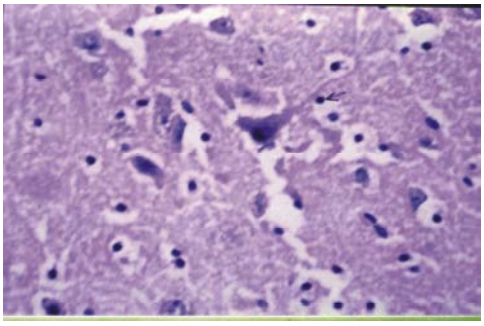

Fig. 2: Shows brain of mice in GI with diffuse gliosis (↑) in cerebrum hyperplasia (5 weeks post infection H&E X160).

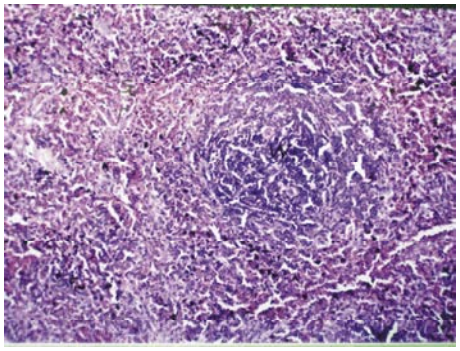

Fig. 3: Shows spleen of mice in GI with hemosiderosis in red bulb(s) (8 weeks post infection H&E X40).

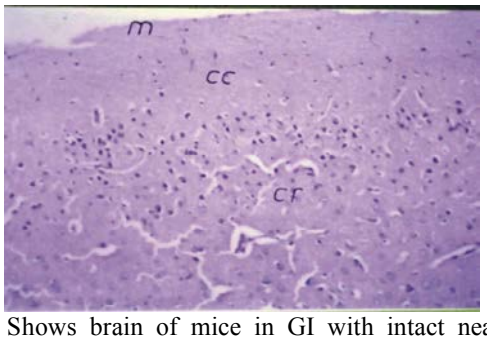

Fig. 4: Shows brain of mice in GI with intact nearly normal histological structure of meninges (m), cerebral cortex (cc) and cerebrum (cr) (8 weeks post infection H&E X40).

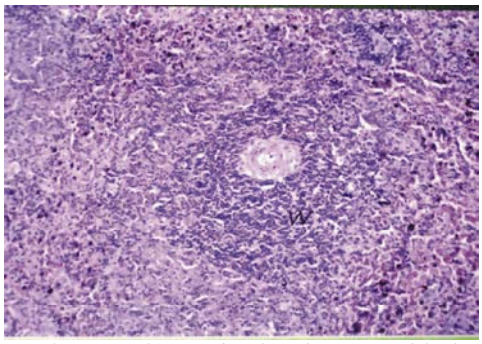

Fig. 5: Shows spleen of mice in GII with lymphoid hyperplasia in white bulb(w) with hemosiderosis (5 weeks post infection H&E X40).

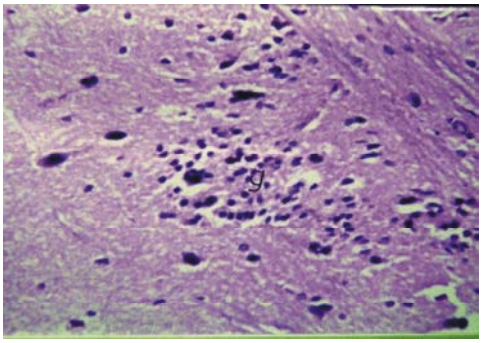

Fig. 6: Shows brain of mice in GII with focal gliosis in cerebrum (g) (5 weeks post infection H&E X80).

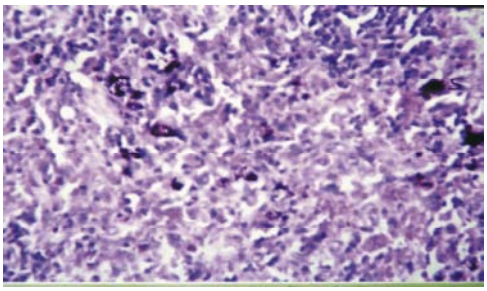

Fig. 7: Shows spleen of mice in GII with hemosiderosis in red bulb (s). (8 weeks post infection H&E X160)

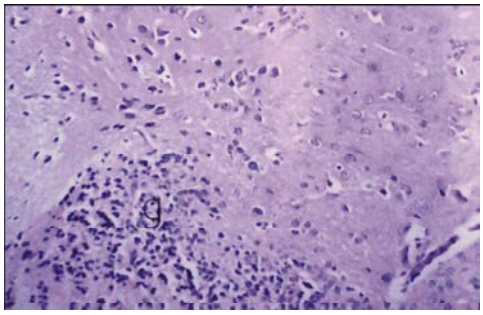

Fig. 8: Shows brain of mice in GII with gliosis (g) in cerebrum (8 weeks post infection H&E X40).

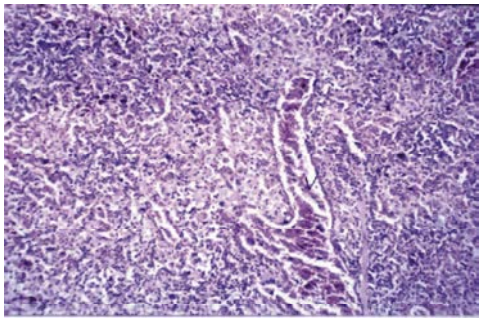

Fig. 9: Shows spleen of mice in GIII with congestion of blood vessels (v) (5 weeks post infection H&E X40).

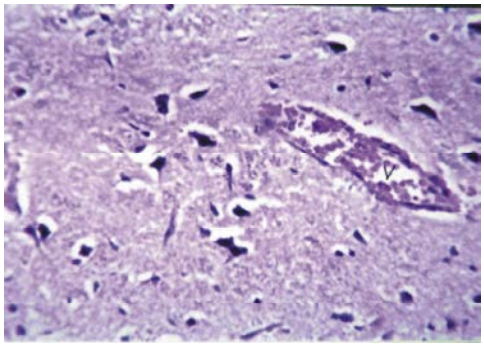

Fig. 10: Shows brain of mice in GIII with congestion of cerebral blood vessels (v) (5 weeks post infection H&E X80).

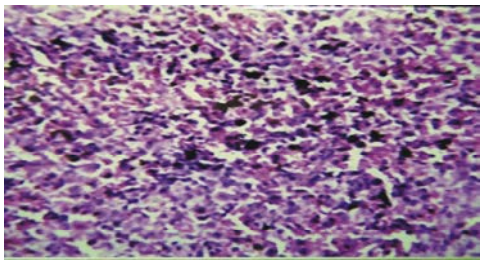

Fig. 11: Shows spleen of mice in GIII with hemosiderosis in red pulps (s). (8 weeks post infection H&E X80).

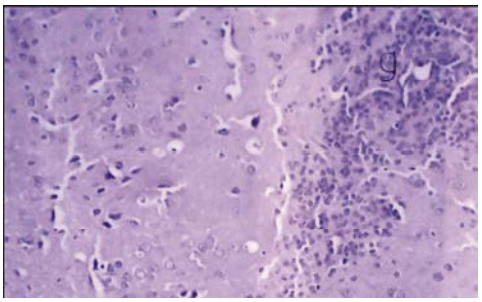

Fig. 12: Shows brain of mice in GIII with gliosis (g) in cerebrum (8 weeks post infection H&E X40).

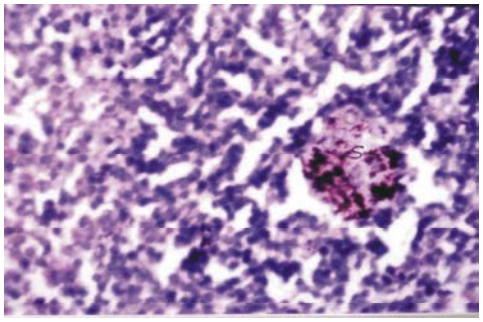

Fig. 13: Shows spleen of mice in GIV with hemosiderosis (s) in red pulps (5 weeks post infection H&E X160)

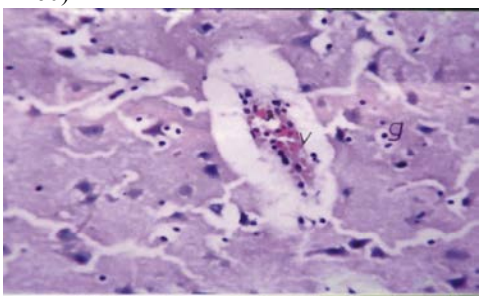

Fig. 14: Shows brain of mice in GIV with congestion of blood vessels(v) and diffuse gliosis (g) in cerebrum (5 weeks post infection H&E X80)

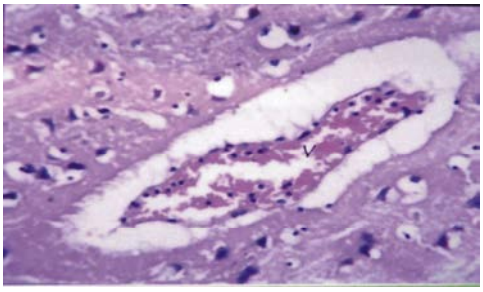

Fig. 15: Shows brain of mice in GIV with congestion in cerebral blood vessels(v) (5 weeks post infection H&E X80).

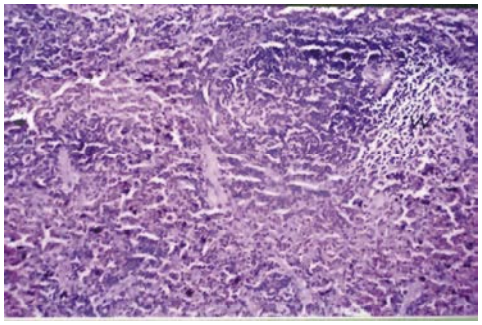

Fig. 16: Shows spleen of mice in GIV with necrosis in lymphoid cells (w) in white pulps (8 weeks post infection H&E X40).

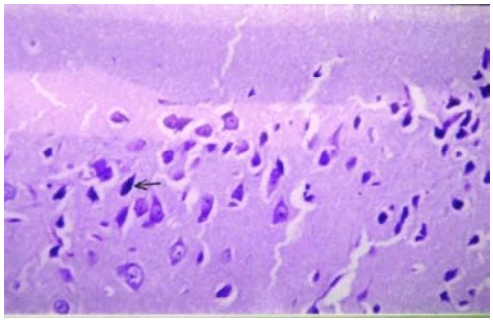

Fig. 17: Shows brain of mice in GIV with neuronal degeneration in cerebrum (8 weeks post infection H&E X80).

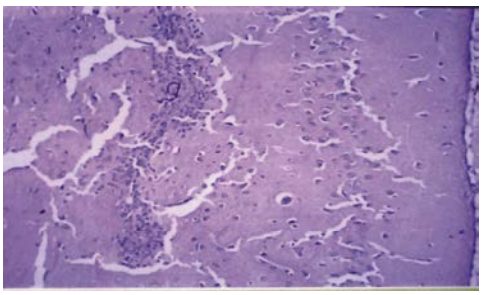

Fig. 18: Shows brain of mice in GV with *Toxoplasma* brain cyst, edema in meninges(m) and gliosis(g) in cerebrum (8 weeks post infection H&E X40).

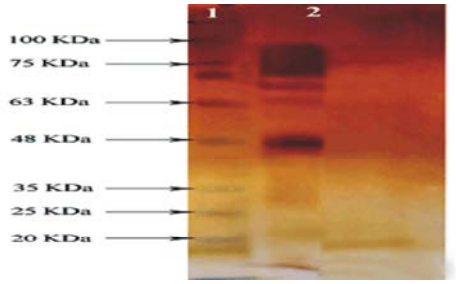

Fig. 19: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for parasite lysate extract:

Lane 1, shows protein marker from 20 to 100KDa.
Lane 2, shows protein bands separated from *Toxoplasma* trophozoite lysate showing 45 and 75 KDa proteins.

Gene expression of INF gamma was significantly higher in GI and lowest in GIV in relation to housekeeping gene GADPH in relation to different groups.

DISCUSSION

The current work was intended to study the effects of total parasite lysate vaccine in experimental murine toxoplasmosis and the role of Influenza virus vaccine

as adjuvant in minimizing the fatal pathological effects of toxoplasmosis. *Toxoplasma* brain cysts were reduced in vaccinated groups than control groups. This clarifies the effect of *Toxoplasma* lysate in defense against *Toxoplasma* infection, however this role was augmented by influenza virus vaccine adjuvant as shown before in this study. Reduction of brain cyst number was reported after vaccination with the major surface antigen of *Toxoplasma gondii*, SAG1 [28], *Toxoplasma* lysate [29], Cocktail DNA vaccine encoding GRA7 and ROP2 GRA7 and ROP2 antigens [30,31], attenuated type I vaccine strain [32], live attenuated parasites [33], antigen-adjuvant combinations that enhance the immunogenicity of antigen candidates for *Toxoplasma* vaccines as ROP2 and GRA4 recombinant proteins co-administered with CpG-oligodeoxynucleotides [34] and autoclaved *Toxoplasma* vaccine (ATV) combined with Bacillus Calmette-Guérin (BCG) as an adjuvant [35].

The anti *Toxoplasma* IgM and IgG were significantly higher in all groups as compared to control group GIV identifying that humeral response induced by vaccinations may share in protection. This effect was prominent with and without adjuvant (GI and GII). The high IgM was translated into lower brain cyst numbers in the same groups. This was also marked with IgG and INF gamma expression. The antibodies were produced rapidly and in increasing way corresponded with duration of immunizations and this was asreported by Li *et al.* [36]. High antibody titers as response to vaccination was reported by Vercammen *et al.* [28], Mishima *etal.* [29], Jongert *et al.* [30], Zhang *et al.* [31], Sánchez *et al*. [34], Couper *et al.* [37] andTan *et al.* [38]. Other vaccines as DNA vaccines induced similar effects [39,40]. However the role of IgG antibodies is unclear in anti-*Toxoplasma* immunity and serum IgG antibodies are thought to play a partial role in the prevention of *T. gondii* infection [41,42]. The successful use of recombinant Influenza and adenoviruses encoding *Toxoplasma* SAG in vaccination was shown to protect against oral challenge with *T. gondii* as reported by Machado *et al.* [20].

Gene expression of INF gamma was shown to increase in all vaccinated groups in relation to GADPH as compared to control group. More Th1 response was recorded with mice vaccinated with *T.gondii* lysate with Influenza virus vaccine. These results are in harmony with Jongert *et al.* [30], Zhang *et al.* [31], Sánchez *et al*. [34] Couper *et al*. [37], Tan *et al.* [38] and Ismael *et al.* [43]. IFN-γ, plays an important role in cell mediated response and Th1 cell-mediated immunity protects against the intracellular parasite *T. gondi* [44,45]. After stimulation with parasite Ag,lymphocytes from mice from all groups expressed higher levels of IFN-γ. *In vitro* experiments indicated that both CD4(+) and CD8(+) T-cell subsets produce IFN-gamma upon re-stimulation with a *T. gondii* lysate [46]. Thus cell mediated immunity associated IFN-γ expression is elicited by vaccination [8]and this can explain the augmenting role of Influenza virus vaccine. *T.gondii* infection inhibits STAT1 and downregulate surface Major Histocompatibility Complex (MHC) class II on infected cells [47]. IFN-gamma regulates many down stream effector proteins through the transcription factor STAT1 including inducible nitric oxide synthase (iNOS) and surface MHC class I and class II [48,49]. It seemed that the addition of a small quantity of Influenza virus vaccine was sufficient to obtain an enhancement of immune response against toxoplasmosis through induction of INFgamma expression.

Previous studies proved the lymphocyte proliferation effect and the induction of high IFN-γ correlates with protective immunity against *T. gondii* [18,50,51,52,].

The pathological manifestations were reported to be severe with spleen of mice that showed hemosiderosis is in red pulps and progressed to necrosis in lymphoid cells in white pulps in non vaccinated control group. The brain showed congestion of blood vessels, edema in meningesand diffuse gliosis in cerebrum with appearance of brain cysts. These manifestations were reduced in vaccinated groups as shown before. Similar pathological manifestations were reported in toxoplasmosis[11, 53] and reduction in pathological manifestations was reported by Eissa *et al.* [35] due to autoclaved toxoplasma vaccine.

Analysis of *T.gondii* lysate showed marked protein bands at 45 and 75 kDa. Peptides from tachyzoite were shown to elicit immune response and their presence in the cytoplasm is the signal for the MHC Class I pathway. HLA-A*0201 transgenic mice immunized with these peptide pools plus adjuvant could induce peptide-specific high IFN-γ production and protect mice against challenge from type II parasites [38,54,55].

The peptide epitopes reported here to elicit IFN-γ are primarily derived from proteins of *T. gondii*[56,57].Some of these proteins have been shown to be involved in invasion and attachment [58,59].

As reported before; vaccination with SAG1 in mice elicits protection against *T. gondii* infection [9,44]. Vaccination by vaccines encoding GRA1 and SAG1 primes a strong humoral and cellular immune response and enhances protection against *T. gondii* challenge through induction of high levels of

IgG antibody, IFN-gamma, change of the CD4(+)/CD8(+) lymphocyte ratio and stimulates NK cell-killing activity [60]. Mice receiving *T.gondii* HSP70 gene vaccine showed prolonged survival with marked production of IFNgamma from splenocytes of infected mice [61]. Immunization of BALB/cmice with MIC3 and SAG1 vaccines stimulated both the cellular and humoral immune responses with the production of anti *T.gondii* antibodies, up regulation of levels of IFN-Î³, IL-4 and IL-10and increased survival time [62].

Future investigations should study incorporation of adjuvant in *Toxoplasma* vaccine to enhance the protective effect against *T. gondii* [9]. Adjuvant can increase protection against *T. gondii* [44]. For example; the biodegradable and biocompatible PLG polymers [51] and encapsulation in microparticles [8,51]. Autoclaved *Toxoplasma* vaccine (ATV) when combined with BCG as an adjuvant was effective in triggering cell mediated immunity as shown by a significant increase in the percentage of splenic CD8+ T-lymphocytes [35].

Finally, The influence of the parasite genotypes (types 1, 2 and 3) [63] and host susceptibility on disease progression and severity and the effect of *Toxoplasma* vaccines should be assessed using different *T. gondii* strains and different host models [8,64].

This study had shown that a *T.gondii* lysate vaccine with Influenza virus vaccine can elicit a broad range of immune responses that are capable of decreasing pathological manifestations in mice acutely infected with *T. gondii* and this vaccine induced mixed Th1and Th2 responses.

ACKNOWLEDGMENT

The author is thankful for Prof. Dr. Adel Bekeer for his kind assistance in interpretation of histopathological results and Dr. Mohamed Sharaf for RT-realtime PCR.

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