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Vaccination with Irradiated Tachyzoites Show Better Therapeutic Effect Than Some Plants or Drugs on Toxoplasmosis

Mervat Abdel Fattah Moawad, Mona Mohamed Amin and Eman Naser Hafez
Health Radiation Research Department, National Centre for Radiation Research and Technology, P. O. Box: 29 Nasr City, Egypt.

ABSTRACT

Toxoplasma gondii infection occurs through ingestion of meat containing cysts, intake of food or water contaminated with cat feces or by handling infected animals. The immunization of animals by an appropriate vaccine could interrupt the circle between animals and humans. The present work is aimed to study the effect of vaccination with radiation-attenuated tachyzoites of *Toxoplasma gondii* RH strain and compare it with the therapeutic effect of some herbs as curcumin (*Curcuma longa*) or pomegranate (*Punica granatum*) as alternative medicine for toxoplasmosis. To assess the cellular immune response the level of TNF-α and IL-10 response were also studied. Tachyzoites were attenuated with both ultraviolet radiation at 2.47 mw-min/cm ^{2} and gamma radiation at 0.3 KGy. Male mice were infected with 2x10 ^{3} tachyzoites/ml and treated with *C. longa* and *P. granatum* at 100 and 200 mg/Kg body weight /day for three successive days. Other groups were injected with attenuated tachyzoites and challenged with 2x10 ^{3} active tachyzoites after three weeks. To compare the efficiency of vaccination and herbal treatment, a group of animals was treated with septrin at dose of 100 and 200 mg/Kg body weight/day. The group vaccinated with UV radiation-attenuated tachyzoites showed better growth inhibition (96.6%) of tachyzoites compared with gamma attenuated group (94.5%). Also, *P. granatum* showed better growth inhibition at dose 200 mg/Kg (88.9 %) compared to *C. longa* which gave 82.3% growth inhibition, while septrin showed 88.9% growth inhibition of tachyzoites.

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MAFM designed the study and collected data. MMA analyzed the data. ENH wrote the article.

Key words
Curcuma longa, Punica granatum,* septrin, radiation, *Toxoplasma gondii.

INTRODUCTION

Toxoplasma gondii is a cosmopolitan protozoan parasite that infects a wide range of mammal and bird species. Infection usually occurs by ingestion of tissue cyst in meat and food or water contaminated by cat's faeces (Chaudhary *et al*., 2006). It is transferred from animals to humans and leads to high economic losses in animals in the form of abortions in sheep and congenital or neuro toxoplasmosis in humans (Hiszczyńska-Sawicka *et al.,* 2014).

To control toxoplasmosis, a combination of antifolates, such as pyrimethamine and sulfadiazine had been used with success and they are the first choice drugs in most clinical settings (Dantas-Leitz *et al*., 2005; Zeng *et al*., 2013). However, this therapeutic regimen is not always suitable for prolonged treatment because of appearance of drug-resistant parasite variants and adverse side effects. Consequently, new therapies are critically needed (Djurković *et al*., 2002; Ferreira *et al*., 2006). There is an increasing awareness of the therapeutic potential of natural products and medicinal plants that are frequently considered to be less toxic and free from side effects than synthetic drugs (Abu El-Ezz, 2005). In this respect, *Punica granatum* is described for its medical properties and ancient Egyptians realized its benefits such as antiparasitic, anthelmintic and the anticoccidial activity (Dkhil, 2013). It exhibited a significant anti-inflammatory activities serving to protect against the tissue injuries induced by *Eimeria* and it is highly recommended for use as a food additive in poultry farms (Amer *et al.*, 2015).

Curcumin is a polyphenol derived from the root of the turmeric plant *Curcuma longa* and responsible for yellow-orange color and spicy taste of curries (Calabrese *et al*., 2003). It exerts anti-infectious, anti-tumor, anti-inflammatory and immuno-modulatory properties apart from their original indication for disease treatment (Kobashigawa *et al*., 1995). Preventive as well as therapeutic anti-inflammatory effects of curcumin treatment have been observed in various animal models (Sugimoto *et al.,* 2002). It was evaluated for its antitoxoplasmic activity (Al-Zanbagi, 2009) and it could reduce the severity of an infection of the upper and middle part of small intestine caused by *Eimeria acervulina* (Castañeda and González, 2015).

An appropriate vaccine for animals could interrupt the link between animals and humans. Therefore, the immunization of the animals against *T. gondii* could be

* Corresponding author: dr.mona.amin@gmail.com
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effective in reducing the risk of human contamination (Abdollahi *et al.,* 2012). Vaccination with γ-irradiated tachyzoites not only protects the mice against challenge infection, but may circumvent the danger of the vaccine being life-threatening because of remaining tachyzoites inside macrophages posing as a latent infection (Assmar *et al.,* 1999). Hiramoto *et al.* (2002) reported that *T. gondii* RH virulent strain tachyzoites irradiated with 255Gy do not cause residual infection and induce the same immunity as a natural infection.

Vaccination with UV-attenuated RH *T. gondii* tachyzoites has high validity to protect murine models against acute toxoplasmosis (Osman *et al.,* 2009). Kannan *et al.* (2014) developed a protocol to inactivate *T. gondii* tachyzoites employing 1 min of ultraviolet (UV) exposure and showed that this treatment completely inhibited parasite replication and cyst formation *in vitro* and *in vivo***.**

During infection of animals, immune response to parasite antigens is implicated by induction of pro-inflammatory cytokines such as IL-12 and TNF-α (Bliss *et al.,* 1999). IL-10 plays a vital role in controlling the inflammatory response during acute *T. gondii* infection as it inhibits the production of IL-12, IFN-gamma, TNF-alpha, and IL-6 from both hematopoietic and non-hematopoietic cells in the brain (Wilson *et al.,* 2005).

MATERIALS AND METHODS

Experimental mice

Six-week-old male Swiss albino mice, weighing 18-20 g at the beginning of the study were obtained from Experimental Animal Unit of Medical Research Center, Faculty of Medicine, Ain Shams University, Cairo, Egypt. The mice were housed five per cage, given drinking water and regular mouse food *ad libitum.* They were maintained according to the Ethics Committee of the National Research Center and in accordance with the "Guide for the care and use of laboratory animals" published by the US National Institutes of Laboratory Animal Resources. The local IAUCC or ethical committee has reviewed and approved the actions and protocols detailed in the report.

Parasites under study

The RH virulent strains of *T. gondii* were obtained from the Medical Research Centre/Ain Shams University. The tachyzoites count was determined by means of a haemocytometer. It was re-suspended at a density of 2×10^3/ml in saline to be inoculated intra peritoneal into the male mice (Nikolić *et al.,* 1999).

Preparation of herbs

P. granatum and *C. l*onga were purchased from local market, made free of dust and other foreign materials and powdered finely with an electric grinder. Different dilutions were prepared using specific sterile water (Egypt Otsuka Pharm. Co.) and administered at doses of 100 and 200 mg/kg body weight (Grujić *et al.,* 2005).

Reference drug

Septrin were used as reference drug and administered at doses of 100 and 200 mg/kg body weight. It was crushed and dissolved in water specific for injection at the same dilutions 100 and 200 mg/Kg body weight according to Harris *et al.* (1988).

Radiation- attenuation of RHT

Tachyzoites of RH virulent strain were attenuated by UV- radiation by exposing them to 2.47 mw-min/cm^2 using UV light lamp, 254nm output, at distance of 10 cm. The intensity of UV- radiation was measured by Cole-Palmer Radiometer (7425 North Oak Park Avenue). Gamma radiation –attenuation was done by transferring tachyzoites into sterile rubber stopper vial and submitting them to ^{60}Co (Gamma cell-400) to be irradiated at the dose rate 2.5 KGy/h at the time of experimentation at the National Center for Radiation Research and Technology (NCRRT) Cairo, Egypt.

Experimental design

Mice were randomly divided into seven groups (n=18 for each group) A, B, C, D, E, F and G and further subdivided as shown in Tables I-III. They were sacrificed on the third day post infection and post challenge.

Biochemical parameters

The peritoneal and thoracic cavities of anaesthetized mice were exposed and the left ventricle was punctured with a fine needle. Blood was collected in sterile tubes and centrifuged for separation of the sera of the mice in sterile eppendrof. Serum Mouse TNF-α. And IL-10 were assayed by Enzyme linked-Immunosorbent Assay (ELISA) using the Biosource International California kits. Measurements were done in the NCRRT, Egypt according to methods of Sutterwala *et al.* (1998).

Statistical analysis

Results were subjected to Student's *t*-test using SPSS program version 8 to determine the significance of the data. Data are expressed as mean ± standard error. Values with $P<0.05$, $P<0.01$ and $P<0.001$ are significant, highly significant and very highly significant respectively.

RESULTS

C. longa at a concentration of 100 and 200 mg /Kg body weight caused reduction in the number of *T. godii* tachyzoites from 73.4% to 82.3% with the mean of tachyzoites as 12,000.4±313.4 to 7,000.6±210.0, respectively. While with the two doses of *P. granatum* the reduction ranged from 77.8% to 88.9% with the mean of tachyzoites as 10,000.4±309.9 to 4,000.6±121.2 respectively (Table I). Also subgroup F_1 that vaccinated with UV-attenuated tachyzoites showed growth inhibition of 96.4% with the mean of tachyzoites as 1,000.2±104.7 relative to subgroup G1 that vaccinated with gamma-attenuated tachyzoites showed 94.7% of inhibition and the mean was 2000.2±120.5. Regarding drug treated subgroups E1 and E2, the inhibition were 84.5% and 88.9% with the mean number of tachyzoites as 7,000.0±240.2 and 40,00.6±141.6, respectively (Table II).

Table I.- Effect of herbal treatment on the number of tachyzoites in peritoneal fluid, % of growth inhibition.

Groups	n	No. of tachyzoites ($X10^3$)	% Growth inhibition
+ve control B	6	43±1.16	-
Curcuma longa			
C1 (100 mg/kg)	6	12±0.31*	73.4
C2 (200 mg/kg)	6	7±0.21*	82.3
Punica granatum			
D1 (100 mg/kg)	6	10±0.31*	77.8
D2 (200 mg/kg)	6	4±0.12*	88.9
Septrin			
E1 (100 mg/kg)	6	7±0.24*	84.5
E2 (200 mg/kg)	6	4±0.14*	88.9

A, Non infected–non treated; B, Infected with $2x10^3$ RH virulent tachyzoites; C1, Infected with $2x10^3$ RH virulent tachyzoites–treated with100mg/Kg body weight with curcumin 15 min post infection for three successive days; C2, Infected with $2x10^3$ RH virulent tachyzoites – treated with 200mg/Kg body weight with curcumin 15 min post infection for three successive days; D1, Infected with $2x10^3$ RH virulent tachyzoites – treated with100mg/Kg body weight with *Punica* 15 min post infection for three successive days; D2, Infected with $2x10^3$ RH virulent tachyzoites – treated with 200mg/Kg body weight with *Punica* 15 min post infection for three successive days; E1, Infected with $2x10^3$ RH virulent tachyzoites – treated with100mg/Kg body weight with septrin 15 min post infection for three successive days; E2, Infected with $2x10^3$ RH virulent tachyzoites – treated with 200mg/Kg body weight with septrin body weight.

Table II.- Effect of vaccination with UV or gamma radiation-attenuated tachyzoites on the number of tachyzoites in peritoneal fluid, % of growth inhibition.

Group	n	No. of tachyzoites ($X10^3$)	% Growth inhibition
+ve control	9	43±1.16	-
UV	9	1±0.10*	96.4
Gamma	9	2±0.12*	94.7
Septrin			
E1 (100 mg/kg)	6	7±0.24*	84.5
E2 (200 mg/kg)	6	4±0.14*	88.9

F, Vaccinated with $2x10^3$ UV- irradiated tachyzoites and challenged after 3 weeks with $2x10^3$ RH virulent tachyzoites; G, Vaccinated with $2x10^3$ gamma - irradiated tachyzoites and challenged after 3 weeks with $2x10^3$ RH virulent tachyzoites. For other abbreviations, see Table I.

Immunological effects of herbal treatment are presented in Table III. Second subgroups in treated groups were chosen with dose of 200mg/Kg body weight as they gave better results in reduction of number of tachyzoites and increase in growth inhibition. Regarding IL- 10 assays, in normal control group was 145.2±18.6 and in control infected group B was 627.3±58.3. While, in infected treated subgroups C2, D2 and E2 showed 356.9±25.1, 331.6±22.5 and 230.8±17.1, respectively. There was high significant difference between all treated subgroups and the control non-infected one with $P<0.001$. On comparing non infected treated subgroups C3, D3 and E3 there was significant increase from control normal group ($P<0.01$) as were 210±9.8, 250±18.7 and 130±7.2, respectively. Regarding of TNF-α level, it was 10.8±0.6 in control normal group while in control infected was 44.2±2.9 with high significant increase ($P<0.001$). In infected-treated subgroups C_2, D_2 and F_2 the levels were 36.8±1.3, 25.1±1.8 and 42.4±2.2, respectively.

Immunological effects of vaccination were presented in Table IV, regarding IL-10 in vaccinated challenged subgroups F1 and G2 they were 410.4±85.2 and 470.3±68.3 respectively with significant increase compared to control normal group as $P< 0.01$. Also, Mean±SE of TNF-α level in vaccinated challenged subgroups F1 and G1 were 19.1±0.9 and 24.8±1.5 respectively with high significant difference from control normal ($P< 0.001$). All vaccinated-unchallenged subgroups showed a slight increase than normal with significant difference $p<0.01$.

Table III.- Effects of herbal treatment on immunological parameters in sera experimental mice.

Groups		n	IL-10 pg/ml		TNF-α pg/ml	
			No. of tachyzoites	Change (%)	No. of tachyzoites	Change (%)
ve control	A	18	145.2±18.6		10.8±0.6	_
+ve control	B	16	627.3±58.3**	+332.0	44.2±2.9**	+309
Curcuma longa						
Infected- treated 200mg/Kg[4]	C2	6	356.9±25.1**	+145.7	36.8±1.3**	+240
Uninfected-treated	C3	6	210±9.8*	+44.3	15.7±0.9*	+45.3
Punica granatum						
Infected- treated 200mg/Kg	D2	6	331.6±22.5*	+128.3	25.1±1.8*	+132
Uninfected-treated	D3	6	250±18.7*	+72.4	19.8±0.7*	+83.3
Septrin						
Infected- treated 200mg/Kg	E2	6	230.8±17.1*	+58.4	42.4±2.2*	+292
Uninfected-treated	E3	7	130±7.2*	-11.4	8.9±0.8*	-17.6

C3, Treated with 200mg/Kg body weight with Curcumin without infection; D3, Treated with 200mg/Kg body weight with Punica without infection; E3, Treated with 200mg/Kg body weight with septrin without infection.
For other abbreviations, see Tables I and II.

Table IV.- Effects of vaccination with UV or gamma radiation-attenuated tachyzoites on immunological parameters in sera experimental mice.

Groups		n	IL-10 pg/ml		TNF-α pg/ml	
			No. of tachyzoites	Change (%)	No. of tachyzoites	Change (%)
-ve control	A	18	145.2±18.6	_	10.8±0.6	_
+ve control	B	18	627.3±58.3**	+332.0%	44.2±2.9**	+309%
UV- irradiated						
Vaccinated- challenged	F1	9	410.4 ±85.2**	+182.7%	19.1±0.9*	+35%
Vaccinated-un challenged	F2	9	380.3±68.3*	+162.1%	14.6±0.4*	+76%
Gamma-irradiated						
Vaccinated-challenged	G1	9	470.3±52.5**	+224.1%	24.8±1.5*	+129%
Vaccinated-un challenged	G2	9	415.7±75.2*	+186.2%	18.8±0.6*	+74%
Septrin						
E_2 Infected- treated 200mg/Kg	E2	9	230.8±17.1*	+58.4 %	42.4±2.2*	+292 %
E_3 uninfected-treated	E3	9	130±7.2*	-11.4%	8.9±0.8*	-17.6%

F1, Vaccinated with $2x10^3$ UV irradiated tachyzoites and challenged after 3 weeks with $2x10^3$ RH virulent tachyzoites; F2, Vaccinated with $2x10^3$ UV irradiated tachyzoites without challenge; G1, Vaccinated with $2x10^3$ gamma - irradiated tachyzoites and challenged after 3 weeks with $2x10^3$ RH virulent tachyzoites; G2, Vaccinated with $2X10^3$ gamma - irradiated tachyzoites without challenge.
Values represented mean ±SE of 5 mice in each group.
** High significant at ($P<0.001$) compared to –ve control.
* Significant at ($P<0.01$) compared to –ve control.
(%) Change to –ve control.
For other abbreviations, see Tables I, II and III.

DISCUSSION

Toxoplasmosis is one of the most common parasitic infections of humans and other mammals. Human vaccines are not available and current anti-toxoplasma treatment is disappointing. The medical value of plants lies in some chemical substances that produce a definite physiological action on human body (Abdel Hady *et al.,* 2008). This study was aimed to compare treatment with *Punica,* curcumin and vaccination with gamma or UV radiation-attenuated tachyzoites on controlling toxoplasmosis.

Pomegranate and curcumin were evaluated for their antitoxoplasmic activity using an intraperitoneal infection by the RH virulent strain of *T. gondii*. Curcumin showed growth inhibition of 73.4 and 82.3 % in doses of 100 or 200 mg/kg body weight/day, respectively. This was in accordance with Al-Zanbagi (2009) who showed 97.4 and 96.8% of growth inhibition with doses of 100 or 200 mg/kg body weight/day using water dilution of curcumin. Bereswill *et al.* (2010) demonstrated that hyper-acute inflammation in the small intestine following *T. gondii* infection is ameliorated by Curcumin treatment and untreated mice displayed severe pan-ileitis.

The maximum survival rate for *T. gondii* tachyzoites using 100 and 200 mg/kg body weight/ day of water solvent of pomegranate was 22.2 and 11.1% which is better than water extract of Curcumin with survival rate of 26.6 and 17.7% respectively. This was in agreement with previous experiments were carried out to show antiparasitic activity of *P. granatum* (pomegranate, fruit-rind). It was successfully used to treat the dysentery and diarrohea (Warrier *et al.*, 2002). Its efficiency was 100% in treatment of buffaloes infected with schistosomiasis after second dose of 225mg/kg body weight. On comparing its effect with praziquantel, it showed significant increase in milk production, body weight and feed intake in infected buffaloes (Niaz *et al*., 2012). Also El-Sherbini and Soukry (2012) reported that ethanol extract of pomegranate peel has a remarkable effect on treatment of human *Trichomonas tenax*. Pomegranate juice was used as larvicide against the myiasis producing 3rd stage larvae of *Lucilia sericata* (Mazyad *et al.,* 1999). The sun-dried rind of immature fruit is presently used as herbal formulation in Orissa and India for the therapy and prophylaxis of malaria which shows their antiparasitic activities (Dell'magli *et al.,* 2010).

The results presented here showed a growth inhibition of *T. gondii* tachyzoites as 84.5% and 88.9% on treatment with septrin in 100 or 200 mg/kg body weight/day, respectively. This result is quite comparable to that recorded by Al-Zanbagi (2008) who found that spiramycin inhibited the *Toxoplasma* tachyzoites growth at 71% and 94% at the same doses used. But when Spiramycin was administered at higher doses, the inhibition of growth was better, as recorded by Al-Zanbagi (2011) whose percentage of growth inhibition was 98% in 400 mg/kg/day for seven days post infection.

Attenuated *T. gondii* with different source has been used in number of studies (Duarte *et al.,* 2002). Irradiated *T. gondii* with ^{60}Co irradiation has been used to vaccinate cats and mice against infection (Omata *et al.,* 1996). Killing of oocysts (Dubey *et al.,* 1996) and tissue cysts (Dubey and Thayer, 1994) by ^{137}Cs irradiation and protective immunity induced by vaccination with irradiated *T. gondii* (Lin *et al.,* 1999). El-Bararwy (2012) showed that the immunization with X-Ray irradiated *T. gondii* tissue cysts could partially protect the mice from infection. Using gamma-attenuated *Toxoplasma* tachyzoites in this study with dose o.3 KGy was in accordance to Kook *et al.* (1995) who studied different doses of radiation from 200 to 300 Gy and reported that the inhibition of tachyzoites proliferation in tissue ranged from 80% to 90%. These results reported 94.7% of growth inhibition of tachyzoites in vaccinated groups. Camossi *et al*. (2014) reported that immunization of female rats with 255- Gy-irradiated tachyzoites of *T. gondii* induced reduction of parasitic load in most organs analyzed.

On comparing the feasibility of using ultraviolet light to attenuate the *Toxoplasma* tachyzoites, the study showed 96.4 % of growth inhibition of tachyzoites and decrease in the number of tachyzoites in peritoneal cavity. This was in agreement with Osman *et al.* (2009) who showed survival rate of 93% in challenged mice with attenuating dose of 2.47 mw-min/cm^2 UV-radiation while killing dose of 3.30 mw-min/cm^2 showed 80% survival rate. This is also in agreement with Yang *et al.* (2010) and Zhao *et al.* (2013) who reported that mice vaccinated with UV-attenuated *T. gondii* tachyzoites and then challenged had significantly increased survival rate and extended survival time, decreased parasite burden, improved liver histopathology, and increased mRNA expressions of Th1-type cytokines (IL-2, IFN-γ, and TNF-α).

Munoz *et al.* (2009) observed after *T. gondii* infection is characterized by an overproduction of pro-inflammatory cytokines such as IL-12, IFN-γ, TNF-α and IL-10 are key regulator of the immune response cascade. In this study, the level of Il-10 and TNF-α were studied in sera of the groups of mice treated with the higher dose of treatment as these groups showed better results of protection. Cytokine assays revealed that the serum levels of IL-10 and TNF-α were highly increased in control infected group compared to the normal ($P<0.001$). The treated groups either by herbs or drugs showed also

elevation in cytokine levels compared to normal group ($p<0.001$) but reduced level than control infected one. This was in accordance with Allam (2009) who studied immunomodulatory effects of curcumin treatment on murine schistosomiasis. He reported that infected mice treated with curcumin showed high serum level of interleukin IL-12, TNF-α and IL-10 level than normal group. Pomegranate and curcumin treated un-infected groups gave significant increase than normal ($p<0.01$) this was in accordance with Kobashigawa *et al.* (1995) and Niaz *et al.* (2012) who reported the immunoregulatory effect of curcumin and *Punica* while septrin un-infected treated group gave reduction of cytokines level compared to normal control.

Vaccinated groups showed elevation in the cytokine level compared to normal and reduction compared to control infected group. The elevation was more in challenged than non challenged groups which may be explained by triggering of more immunoregulatory mechanisms after vaccination. This was in accordance with Abdollahi *et al.* (2013) who reported an increased IL-10 level in infected treated group compared to normal after using excretory/secretory (E/S) *Toxoplasma* antigen as vaccine. Studies done by Hiramoto *et al.* (2002) reported that 200 Gy sterilized *T. gondii* tachyzoites elicited cellular immunity and cytokine response (IL-10, IL-12, IFN-γ and TNF-α) similar to natural infection in mice. Mice immunized with irradiated tachyzoites had extended survival time after subsequent tachyzoite challenge, and displayed minimal cerebral pathology after cyst challenge. Irradiated tachyzoites lose their reproductive ability whilst maintaining metabolic function and may provide a novel tool for the study of toxoplasmosis and vaccine development.

CONCLUSION

Results showed that using water solution of *C. longa* and *P. granatum* as growth inhibitors of the parasite, *T. gondii*. However, *P. granatum* is better than *C. longa* in survival rate and growth inhibition and this may be due to differences in the stability of its anti-microbial agent. Furthermore, vaccination of mice by gamma or UV radiation-attenuated vaccine and then challenge showed better effect than herbal treatment. Also, UV radiation had significantly prolonged survival time, decreased parasite burden and increased cellular immune response than gamma radiation attenuated-vaccine. Our data suggests that *P. granatum* can be used as an adjuvant with UV-attenuated *T. gondii* tachyzoites vaccine.

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Conflict of interest declaration

The author(s) did not declare any conflict of interest.

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