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Ultrastructure of *Toxoplasma gondii* RH Tachyzoites Attenuated by Gamma Radiation and Histopathological Alterations in Mice Vaccinated with it

Mona Mohamed Amin and Eman Naser Hafez*
Health Radiation Research Department, National Centre for Radiation Research and Technology (NCRRT), P. O. Box: 29 Nasr City, Egypt

Abstract. *Toxoplasma gondii* (*T. g)* infection causes severe lethal disease on fetus and recipients. The study was designed to study the ultrastructure of 300Gy attenuate irradiated tachyzoite and histopathological alterations induced in vaccinated host. The alterations in *T.* gtachyzoite irradiated with attenuating dose of gamma radiation using Transmission Electron Microscopy (TEM). The histopathological changes produced in spleen, lung, kidney and testis in mice infected with $2x10^3$ RH virulent tachyzoites and others vaccinated with 300 Gy gamma-irradiated tachyzoites and challenged at 21st day with $2x10^3$ with highly virulent RH *T. gondii* tachyzoites were evaluated. Transmission electron microscopy revealed a uniform size, a smooth surface and intact cell or nuclear membranes with an oval-shaped nucleus also conoids and micronemes were observedin control group. By contrast, gamma-irradiated tachyzoites group showed vacuolization in their cytoplasm with the substantial reduction in the number of dense granules and the blur of some organelles. On the other hand, infected groups revealed massive histopathological changes which were markedly decreased in vaccinated group.

Key words*: Toxoplasma gondii*, TEM, histopathology, gamma, radiation.

INTRODUCTION

Toxoplasmosis is a world-wide infection caused by an obligate intracellular protozoan called *Toxoplasma gondii*. It infects man and animals cause economic losses in the form of abortion and neonatal deaths (Nisar *et al.,* 2015). There are three infectious stages of *T. gondii* including tachyzoite, brachyzoite and sporozoite in oocyst (Dubey, 2006).

The radiation attenuated vaccine has enabled the dissection of different immune responses as putative effecter mechanism. Priming of protective response by radiation is a highly coordinated series of events leading to development of various effecter responses, ranging from Th1-associated cell-mediated activity, to anti-parasitic antibodies, all of which contribute to elimination of challenge to varying extents (Hewitson *et al.,* 2005). Gamma radiation (^{60}Co) induces changes on *T. gondii*, leading to decreased or abolished reproduction but maintaining viability and probably physiology of these parasites while other attempts using inactivated or chemically treated antigens resulted in lower or no response. The study of Hiramoto *et al*. (2002) suggested that efficient immune response to *T. gondii* depends of its viability, maintaining its physiology until complete recognition by the host immune system. Thus, radiation seems a better tool to produce a toxoplasmosis vaccine with special focus to maintenance *T. gondii*.

Electron microscope has been widely used in the classification and identification of parasites, and in exploring the effects of drugs or radiation on parasite ultrastructure. Kook *et al.* (1995) studied the ultrastructural changes induced by gamma irradiated *Toxoplasma* tachyzoites. Dubremetz and Ferguson (2009) used ultrastructural techniques to classify previously unrelated parasites within a single phylum, the Apicomplexa. Stettler *et al.* (2003) studied the *in vitro* parasiticidal effect of nitazoxanide (NTZ) on *Echinococcus multilocularis* metacestodes using electron microscopy. Zhou *et al*. (2010) investigated the effect of diclazuril on the morphology of second-generation merozoites of *Eimeria tenella* using TEM.

After initial growth at the site of entry, the parasite will be disseminated via blood stream and finally localized within the host cells causing rapid cell death with rupture and liberation of the

* Corresponding author: eman_naser612@hotmail.com
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organisms and soluble antigens (Ferro *et al.,* 2002). *T. gondii* causes many changes ranging from mild congestion to severe degeneration involving mainly liver, spleen and pancreas (Sukthana *et al.,* 2003). It infects nearly all types of cells in the lung during the course of the disease and produces little change in these cells until a massive number of organisms were present and the cells ruptured (Parker *et al.,* 1981). Marked histopathological findings in damaged kidneys were detected and attributed to intestinal and/or kidney dysfunction during the infection. Few clinical studies have reported that *T. gondii* affect reproductive parameters of men. In this regard, Zhou *et al*. (2002) showed that infection with *T. gondii* in infertile couples is significantly higher than the fertile and the level of anti-sperm antibody is significantly higher in *Toxoplasma* infected than in non-infected couples. Another study was done in Chinese infertile men showed that among 100 cases of men's sterility, 36% were serologically *Toxoplasma* positive, while the seropositivity of infection in fertile men was 11% (Qi *et al.,* 2005). Moreover, there are several reports which show association of male genital tract impairment with special features of testicular toxoplasmosis (Crider *et al.,* 1988), *Toxoplasma* orchitis (Haskell *et al.,* 1989) and hypogonadism caused by congenital toxoplasmosis (Suresh *et al.,* 2007). These evidences suggest *Toxoplasma* infection in men may be associated with male sterility.

This study is aimed to evaluate the effect of attenuating dose of gamma radiation (300 Gy) on the ultrastructure of *Toxoplasma gondii*, Rh virulent tachyzoites and histopathological changes in different organs of mice vaccinated with the radiation-attenuated tachyzoites and then challenged with virulent tachyzoites.

MATERIALS AND METHODS

The RH virulent strains of *T. gondii* were maintained by routine intraperitoneal passage in mice every 72 h after that they were obtained from the Medical Research Centre, Ain Shams University. The tachyzoites concentration was determined by means of a haemocytometer. It was re-suspended at a density of 2×10^3/ml in saline (Grujie *et al.,* 2005). Two suspensions were prepared; one as non irradiated to act as control for TEM and the other irradiated with Cobalt-60 (Gamma cell-400) at dose levels 300 Gy at the dose rate 2.5 KGy/h at the time of experimentation. This was carried out at the National Center for Radiation Research and technology (NCRRT), Cairo, Egypt.

Transmission electron microscopy

Parasites both control and irradiated were incubated at 37°C in a 5% CO_2 atmosphere for 2 h. After incubation, the samples were washed twice in PBS and centrifuged at 1000 rpm for 10 min at 4°C. The sediment was pre-fixed with 2.5% glutaraldehyde for 8 h at 4°C, rinsed three times with 0.1 M PBS (pH 7.2), post-fixed with 1.0% osmium tetroxide (OsO_4) for 2 h, and washed three times with 0.1M PBS (pH 7.2). The tachyzoites were dehydrated by sequential incubations in increasing concentrations of ethanol (one time in 30%, 50%, 70%, 90% and 95% and three times in 100%) for 10 min. The dehydrated parasites were soaked in anhydrous acetone for 10 min and then dried after repeating three times. Parasites were subsequently embedded in epoxy resin at 60 °C for 12 h. Ultrathin sections were then prepared with a LKB-V-ultra microtome; they were stained with uranyl acetate and lead citrate and viewed with a JEM-2100 TEM (Aikawa, 1971).

Experimental mice

Thirty male Swiss albino mice six week-old and weighing 18-20 g at the beginning of the study (obtained from Experimental Animal Unit of Medical Research Center, Faculty of Medicine, Ain Shams University, Cairo, Egypt) were used. They were kept for at least one week to get them acclimatized to the conditions before being subjected to any experimentation. Three groups (ten mice each) were used. Group I was control, group II was infected with $2x10^3$ RH virulent tachyzoites and group III was vaccinated with $2x10^3$ gamma-irradiated RH virulent tachyzoites and then challenged with $2x10^3$ RH virulent tachyzoites on 21st day after vaccination. All animals were scarified, five days post infection and post challenge. Small pieces of spleen, lungs, kidneys and testis tissues were taken out, fixed in 10%

neutral buffered formalin and processed routinely for histology. Tissues were embedded in paraffin and then 6 µm thick sections cut with Reichert Rotary microtome which were stained with Harris hematoxylin and eosin (Culling, 1974).

RESULTS

Tachyzoites of uniform size had smooth surface, intact cell and nuclear membranes when observed under TEM. In addition, an oval-shaped nucleus, conoids, rhoptries and dense granules were also observed (Fig. 1A, B).

By contrast, many parasites from the gamma irradiated tachyzoites were deterimentally affected with several ultrastructural changes as there was an extrusion of the conoid, which significantly changed the appearance of the apical region. Rhoptries were markedly decreased in size, the outer structures showed wave-like folding and there was conspicuous projection lined only by plasma membrane. Vacuolization in the cytoplasm with the substantial reduction in the number of dense granules and the nuclear chromatin was disintegrated with destroyed granules scattered within the nucleus (Fig. 1C, D).

The histopathology of kidneys in normal control group I revealed the normal histological structure of renal parenchyma (Fig. 2A). In infected control group II (Fig. 2B) revealed enlargement with inflammatory condition affecting the glomeruli with cellular proliferation, congestion of renal blood vessel, degeneration and interstitial fibrosis. Group III vaccinated with gamma-irradiated tachyzoites (Fig. 2C) showed peritubular inflammatory cells infiltration. Regarding the spleen of group I, it shows normal architecture (Fig. 3A). The infected spleen group II (Fig. 3B) showing haemorhge, extramedullary mega karyocytosis, necrosis, fibrosis and degeneration of spleen tissues. In group III, vaccinated with gamma-irradiated tachyzoites the tissues showed no histopathological changes (Fig.3C). The normal lungs group I showed normal architecture (Fig. 4A) while infected lungs group II (Fig. 4B) revealed solidification of alveolar lining, dense congested parenchymal cells with darker areas of necrosis. The observed congestion,

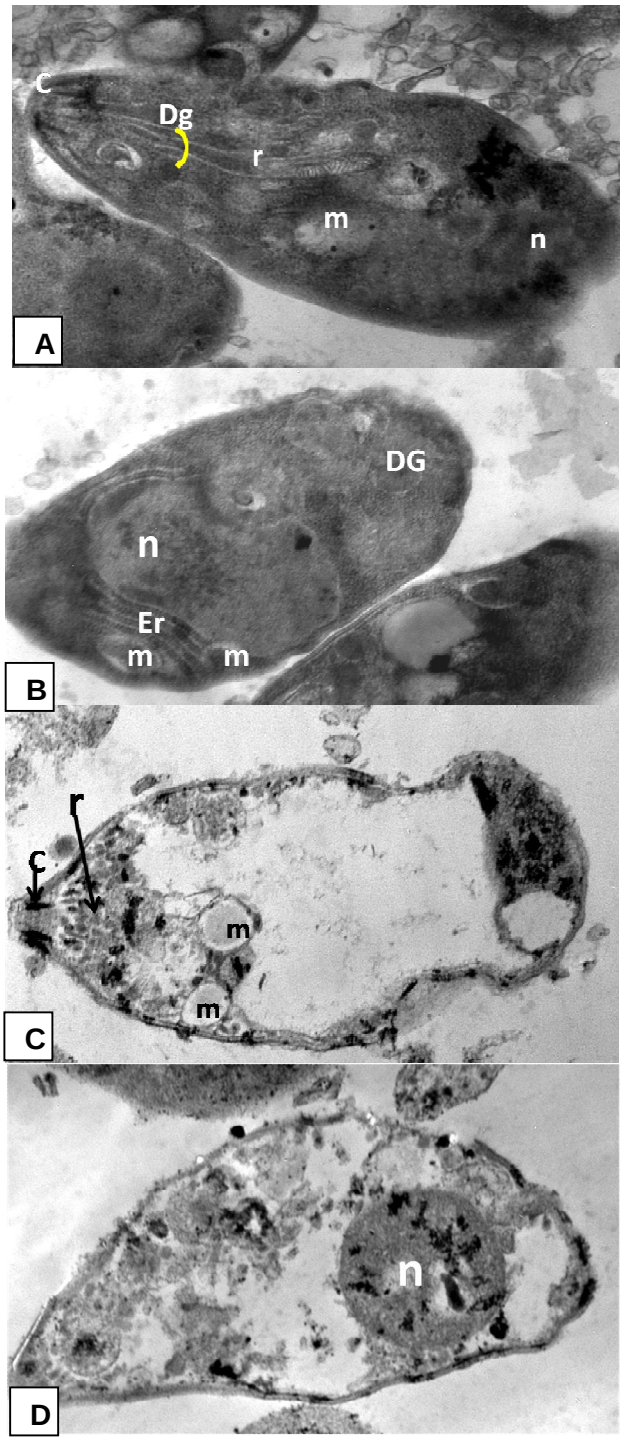
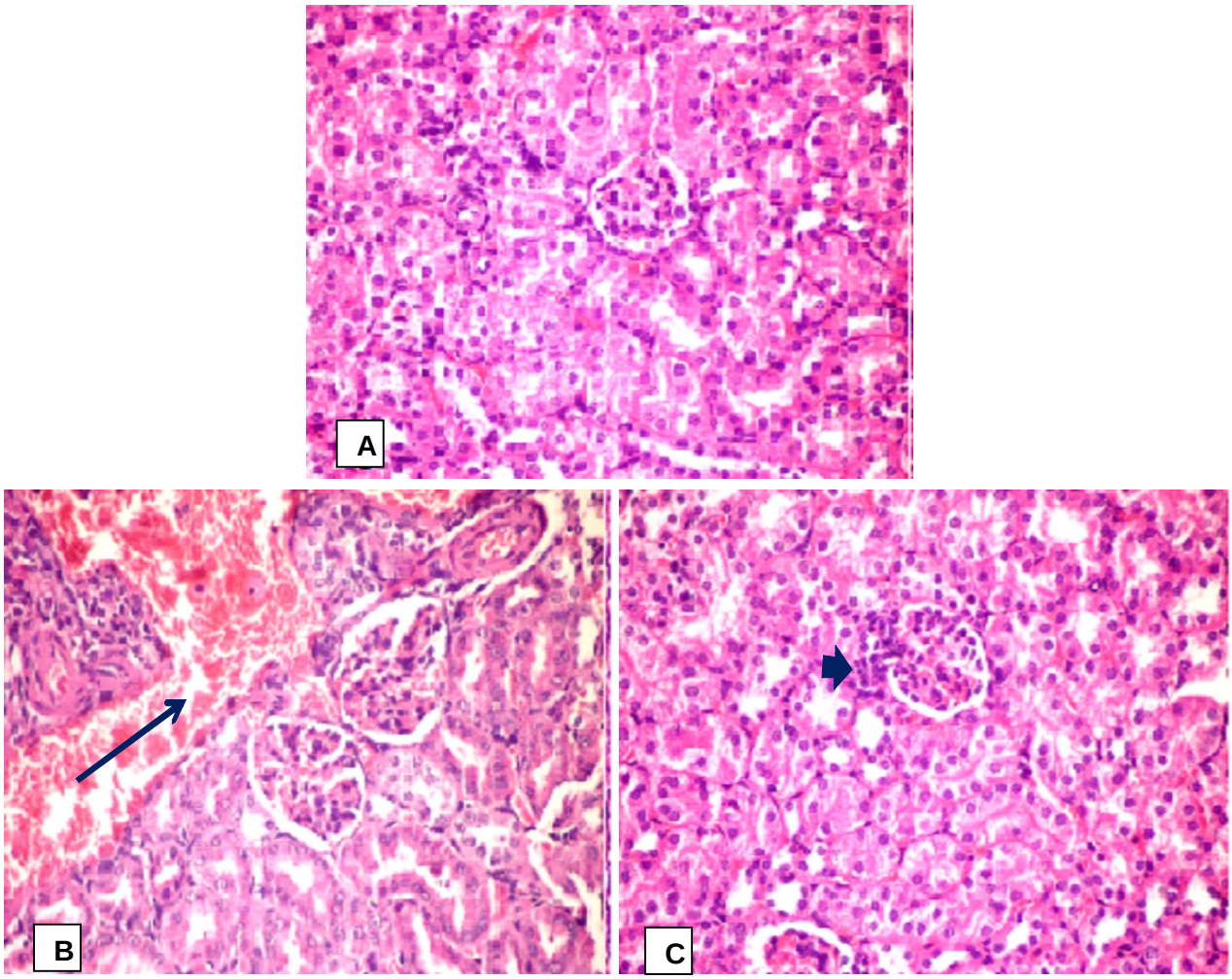


Fig. 1. Transmission electron micrograph of normal (A, B) and gamma irradiated (C, D) tachyzoites n, nucleus; m, micronem; r, rhoptries; Er, endoplasmic reticulum; Dg, dense granules.
Magnification: 25,000x.



Figs. 2. Histological structure of mouse kidney of control; A, showing normal renal structure; B, mouse kidney infected with $2x10^3$ RH virulent tachyzoites showing congestion and dilatation of renal blood vessel (→); C, mouse kidney infected with $2x10^3$ γ-irradiated RH_3 virulant tachyzoites and challenged at 21st day after vaccination with $2x10^3$ RH virulent tachyzoites showing mild epithelial infiltration (→)
Magnification: 400x, stain: Haematoxylin and Eosin.

desquamation of the epithelia and infiltration with cells led to thickening of the alveolar walls that became collapsed. Group III vaccinated with gamma-irradiated tachyzoites revealed mild histopathological changes (Fig. 4C). Testis of group I showed normal structure of testis (Fig. 5A). Infected control group II (Fig. 5B) revealed interstitial infiltration with leucocytes, focal hemorrhage, destruction of germinal layers further damage, plugging of seminiferous tubules by epithelial debris, fibrosis and atrophy in the majority of the seminiferous tubules were detected in the infected testis. Gamma-vaccinated group III (Fig.5C) revealed spermatid giant cells in the lumen of seminiferous tubules or no histological changes.

DISCUSSION

Studies regarding the effect of ionizing radiation on protozoa have successfully used a variety of metabolic and morphological changes that are associated with loss of viability (James *et al.*, 1991). It could be an excellent tool for abolishing the reproductive ability of *T. gondii* tachyzoites,

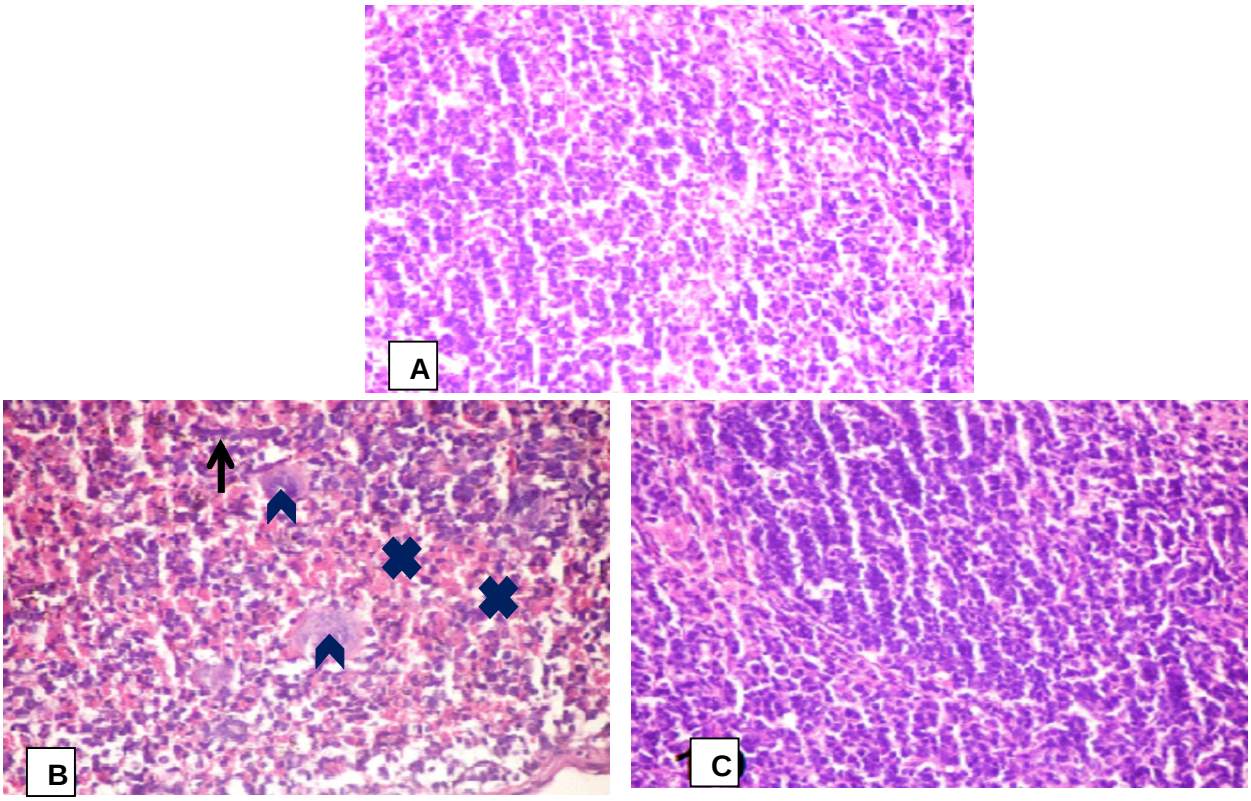
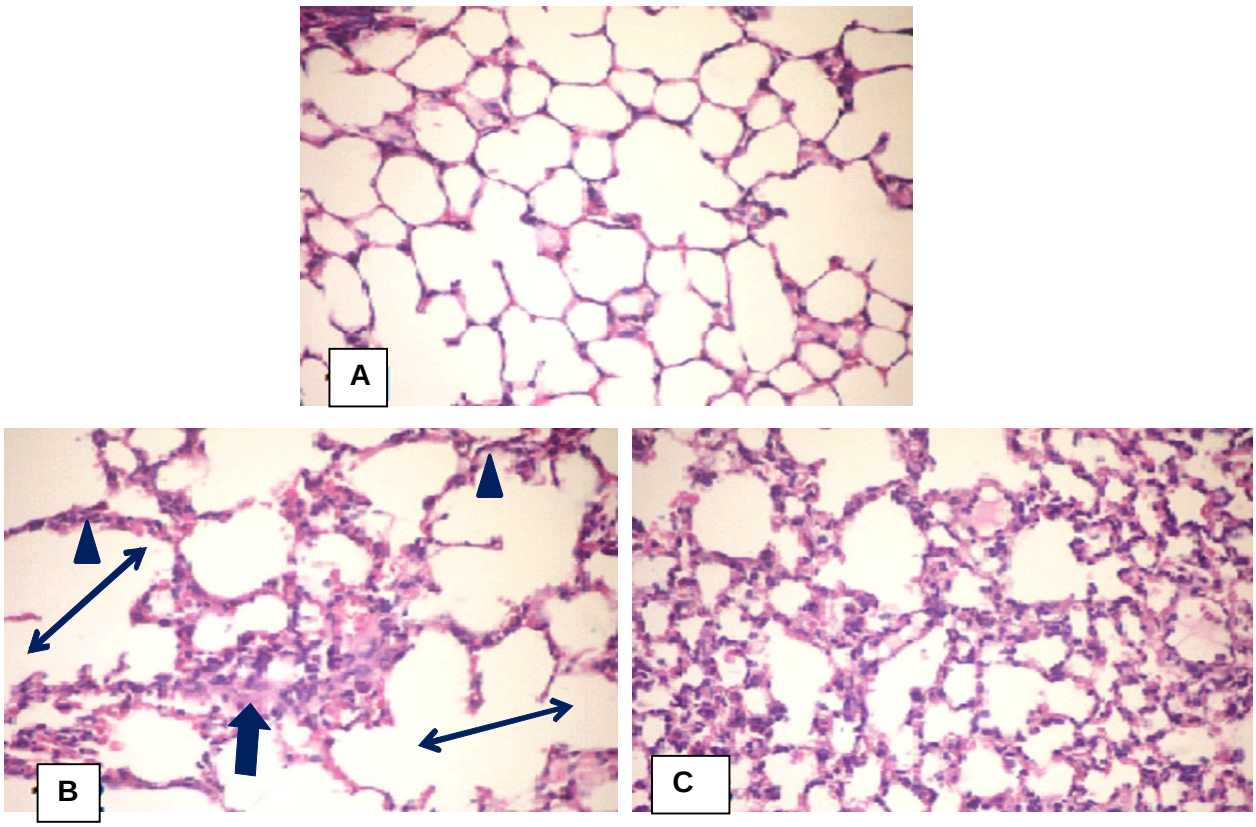


Fig. 3. Histological structure of mouse spleen of control; A, showing normal architecture; B, mouse spleen infected with $2x10^3$ RH virulent tachyzoites showing haemorhge (✖)extramedullary megakaryocyte (➧), fibrosis (➧) and degeneration of spleen tissues; C, mouse kidney infected with $2x10^3$ γ-irradiated RH_3 virulant tachyzoites and challenged at 21st day after vaccination with $2x10^3$ RH virulent tachyzoites showing an improvement with normal architecture of spleen.
Magnification: 400x, stain: Haematoxylin & Eosin.

without affecting most of its functions, allowing the immune system to recognize the viable agent without risk of progressive infection or residual cysts, with remarkable importance in vaccine production (Hiramoto *et al*., 1999).

Structures of γ-irradiated *T. gondii* were changed significantly compared with control group. It was found that the initial contact of the apical region of infective forms of protozoa of the phylum Apicomplexa is fundamental to trigger host cell invasion (Nicholas *et al.*, 1983). It involves not only specialized secretory organelles, such as rhoptries and micronemes, but also a complex cytoskeletal component known as conoid which is involved in the process of infection of vertebrate cells (Dobrowolski *et al.*, 1997). In the present study, there were marked morphological changes in the *T. gondii* tachyzoites. These results are in agreement with those reported by Kook *et al*. (1995) who suggested that irradiation of *T. gondii* tachyzoites can cause marked decrease in rhoptries, disappearance of golgi complex and mitochondria, decrease in the number of electron dense granules and severe damage to various intracellular organelles including the nucleus. Miranda *et al*. (2013) studied the effect of γ-radiation on asexual intraerythrocytic *Plasmodium falciparum* and reported that irradiated parasite had defective mitosis, sparse cytoplasm, fewer ribosomes, disorganized and clumped organelles, and large vacuoles.

T. gondii is regarded as one of the diseases whose diagnosis depends on the histopathological change, pathogenicity of the strain of parasite, host

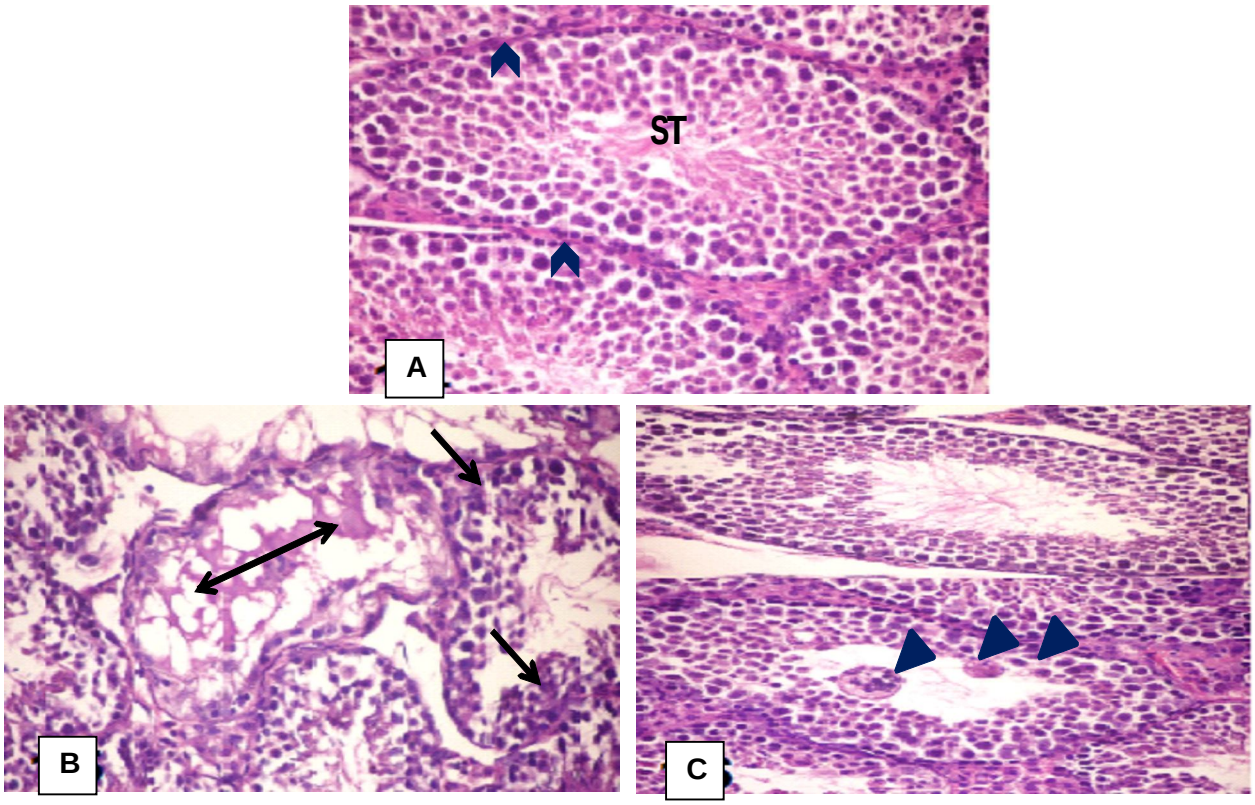


Figs. 4. Histological structure of mouse lungs of control; A, revealing normal structure of lung tissues; B, mouse lungs infected with $2x10^3$ RH virulent tachyzoites showing dilated alvoli(↔), thickened intra-alveolar septa(▲) and inflammatory cell infiltration (⬆); C, mouse kidney infected with $2x10^3$ γ-irradiated RH_3 virulant tachyzoites and challenged at 21st day after vaccination with $2x10^3$ RH virulent tachyzoites showing mild histopathological changes Magnification: 400x, stain: Haematoxylin and Eosin.

susceptibility and immune status (Dubey, 2006). The histopathological changes due to parasitism in different organs vary from one species to the other and are usually more pronounced in the young animals than adults. These changes occur even in subclinical infection, but clinical manifestations develop when the pathological changes are sufficiently numerous and large enough to cause dysfunction (Frenkel, 1990).

Previous studies recorded many alterations in different organs in mice infected with virulent RH tachyzoites. In the present work, the histopathology of kidneys in infected control group revealed inflammatory and degenerative changes. These findings were similar to those described by Aspinall *et al*. (2003) and Fayed *et al*. (2004). While vaccinated group III with $2X10^3$ gamma-irradiated *Toxoplasma* tachyzoites and challenged after 3 weeks with another dose of virulent RH tachyzoites showed mild congestion of renal blood vessels with cellular infiltration compared to control infected group II.

The histopathological findings observed in spleen in infected control group II revealed necrosis, fibrosis and degeneration of spleen tissues with increased inflammatory cell population. These were in accordance with the results recorded by Buxton (1998) and Gres *et al*. (2003). Fayed *et al*. (2004) reported many histopathological abnormalities in spleen infected with low dose sporulated oocysts and nacrosis, fibrosis and degeneration in spleen of mice infected with high dose of sporulated oocysts. Meanwhile, $2X10^3$ gamma-irradiated *Txoplasma* tachyzoites vaccinated group III showed no changes in spleen tissue.



Figs. 5. Histological structure of mouse testes of control; A, showing normal seminiferous tubules (ST) cellnucleus and basement membrane (^); B, mouse lungs infected with $2x10^3$ RH virulent tachyzoites showing marked necrosis of spermatogoeal cells linning seminiferous tubules (↓), degeneration of seminiferous tubules(↔)and decline in spermatozoa; C, mouse kidney infected with $2x10^3$ γ-irradiated RH_3 virulant tachyzoites and challenged at 21^{st} day after vaccination with $2x10^3$ RH virulent tachyzoites showing no histological changes with spermatid giant cells (▲) in the lumen of seminiferous tubules
Magnification: 400x, stain: Haematoxylin and Eosin.

Regarding the changes in infected lungs group II, there was diffused alveolar damage interrupted by multifocal necrosis and interstitial pneumonitis. These findings were nearly similar to those observed by Elbanhawy *et al*. (1995), Issa *et al*. (1999) and Fayed *et al*. (2004). In vaccinated group III, mild histopathological changes were observed.

The infected testis in the present histological section showed degeneration of seminiferous tubules. This was in agreement with that reported by Antonios *et al*. (2000) and Lopes *et al*. (2011) who reported diffused testicular degeneration with calcification foci and a multifocal mononuclear interstitial inflammatory infiltrate, and a mononuclear interstitial infiltrate and focal necrotic areas of the muscle fibers surrounding the seminal vesicles. Abdolhossein and Abdoli (2013) suggested that *T. gondii* infection can cause temporary impairment of the reproductive parameters of human or animal male as well as impairment of different hormones which may cause insufficient male productivity. Vaccinated group III showed no pathological changes or spermatid giant cells in the lumen of seminiferous tubules.

The present results proved that histopathological alteration were reduced in vaccinated groups. This was in accordance with the study done by Sahran *et al*. (2009) who reported that mice acquired a strong and efficient resistance when vaccinated with irradiated tachyzoites. Also, these findings proved that exposing tachyzoites to gamma

radiation reduces their ability to propagate and consequently decreases their toxic effects. Hiramoto *et al.* (2002) observed that *T. gondii* RH strain failed to reproduce when exposed to γ-ray in vitro and *in vivo*. The study of Al-Barwary (2012) showed that infection with irradiated tissue cysts and then challenged gave high level of protection than those infected and then vaccinated with the irradiated cysts due to stimulation of immunological response from radiation-attenuated tissue cysts.

CONCLUSION

Vaccination with 300 Gy gamma radiation-attenuated tachyzoites stimulate immune response in challenged infection so gave protection and amelioration in histopathological changes in affected organs.

Conflict of interest declaration

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

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