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Effect of Vaccination with Irradiated Tachyzoites on Histopathological Changes and DNA Damage in Hepatocytes of Experimental Toxoplasmosis

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ABESTRACT

Current strategies for the control of toxoplasmosis are based on chemotherapy, however successful vaccine has also been demonstrated. The present study aims to assess the effect of the vaccination with radiation-attenuated tachyzoites in challenged mice regarding histopathological alteration and DNA damage of hepatocytes. Sixty mice were equally divided as follow: Group I left as a normal control group II was infected with $2x10^3$ RH virulent tachyzoites (infected control). Groups III and IV were subdivided into two subgroups a and b where subgroups III_a and IV_a were vaccinated with 2.47 mw-min/cm^2 UV and 0.3 KGy gamma radiation – attenuated tachyzoites respectively without challenge (as vaccine control). Subgroups III_b and IV_b were vaccinated with UV and gamma radiation-attenuated tachyzoites and challenged after three weeks with $2x10^3$ RH virulent tachyzoites. Livers were examined for histopathological changes and DNA comet assay. It was observed that acute infection with *Toxoplasma* tachyzoites produced toxic effects which lead to severe damage in liver tissues and DNA of hepatocytes. Meanwhile, the protective effect of UV or gamma radiation-attenuated tachyzoites vaccine resulted in the maintenance of normal histopathological characteristics and DNA of hepatocytes and UV irradiation is better in its protective capacity.

Key wards: Toxoplasma gondii, DNA comet assay, Histopathology, Liver, radiation.

INTRODUCTION

Toxoplasma gondii (T. gondii) is the main cause of the toxoplasmosis rarely causes any clinical presentations in infected immuno-competent individuals; however, immuno-suppressed adults or congenitally infected infants may suffer from the severe pathological effects of the disease [1, 2]. It can be transmitted by eating or drinking infected meat or milk, respectively, during contact with food, water or dust contaminated with cat feces, or by handling infected animals [3, 4]. Therefore, the immunization of the animals against *T. gondii* could be effective in reducing the risk of human contamination.

Almost 13% of the world population is affected by this parasite. It is an obligate intracellular parasite that moves with gliding activity and penetrate the host cells of many tissues including muscles, brain, intestine, placenta and liver [5, 6]. 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 organisms and soluble antigens [7]. The liver is the organ in which nutrients absorbed from the digestive tract are processed and stored so it was expected that it will be affected greatly through this pathway. *Toxoplasma* parasite causes many changes when it invades the cells due to the DNA damage that is provoked by the infection [8]. To evaluate the

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magnitude of DNA damage, single cell gel (comet) assay was applied in this setting which is rapid, simple, and reliable biochemical technique for evaluating DNA damage in mammalian cells. The basic principle of the single cell gel (comet) assay is the migration of DNA fragments in an agarose matrix under electrophoresis. When viewed under a microscope, cells have the appearance of a comet, with a head (the nuclear region) and a tail containing DNA fragments or migrating towards the anode [9]. The measurement of DNA damage as ratio of fluorescence intensities without any assumption of the morphological shape of investigated comet was reported by Boker et al. (1997)[10] while quantification of DNA damage in individual cells based on the migration of DNA in electric field was first described by Ostling and Johanson (1984)[11] and classification of comet cells from type 1-5 based on morphological basis was reported by Marin-Huachaca et al. (2005) [12].

Vaccination attempts with live, attenuated or killed parasites, as well as, different antigenic fractions of the parasite, have been conducted with varying success [13]. The application of radiation to control infectivity of parasites such as *T. gondii* has received much attention. It has been demonstrated that radiations from various sources has strong effects in killing or attenuating *Toxoplasma* cyst or tachyzoites [14].

Gamma radiation of tachyzoites severely affects their capability of proliferation in the host cells and the effect is dose-dependent [15]. UV-attenuated vaccine of *T. gondii* strain could induce certain protective immunity against challenge infection [16]. There are two hypothesizes regarding effects of UV rays on the protein and peptide compounds of living organisms. The first is that the protein particles are absorbed and photons of UV-rays in the specific location of this protein were qualified with the extinction coefficient and the numbers of specific locations of proteins. The second is that chemical reactions occurred in the absorbance location and general structure of protein changed or transformed into other structure[17].

The present study aimed to evaluate the effect of vaccination with UV or gamma radiation-attenuated *Toxplasma gondii* tachyzoites on ameliorating hepatic histpathological changes and DNA damages in experimental toxoplasmosis.

MATERIALS AND METHODS

Experimental Mice

Six-week-old male Swiss albino mice, weighing 18-20 grams 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. Animals were acclimatized to laboratory conditions before starting the experiment. All animals were maintained according to the ethics committee of the National Research Center and in accordance to the "Guide for the care and use of laboratory animals" published by the US National Institutes of laboratory animal Resources (National Research Council). The local IAUCC or ethical committee has reviewed and approved the actions and protocols detailed in the report [18].

Parasites and Vaccine Preparation

Tachyzoites of high virulent RH strains of *T. gondii* were obtained from the Medical Research Center, Ain Shams University. Their concentration determined by means of a haemocytometer. 2 $X10^3$ tachyzoites /mouse were attenuated by exposing them to 2.48 mw - min/cm^3 UV energy and to 0.3 KGy at a dose rate of 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. Sixty mice were divided into four groups: Group I (ten mice) as included control normal, group II (ten mice) was infected with $2x10^3$ RH virulent tachyzoites and served as infected control group. Groups III and IV (twenty mice

each) were subdivided into two subgroups (a) and (b) (ten each), the subgroups IIIa and IVa were vaccinated with irradiated tachyzoites without challenge to be as control for the vaccine groups. Subgroups III_b and IV_b were vaccinated with 2.47 mw-min/cm^2 UV and 0.3 KGy gamma radiation–attenuated tachyzoites respectively and challenged after three weeks with $2x10^3$ RH virulent tachyzoites. At the 5th day post infection and challenge, livers were removed and a portion of each was processed for histopathological examination. They were kept for few days in 10% neutral buffered formalin solution. Then each liver biopsy was cut into 1 cm. thick slices, dehydrated by alcohol, cleared with xylol and finally embedded in paraffin. Paraffin blocks were made by using Reichert Rotary microtome serial paraffin sections of 5µm. Thickness were made and the sections were then stained with Harries Hematoxylin and Eosin [19].

Single Cell Gel (Comet) Assay

The remaining of liver tissues was subjected to study of DNA damage. Slides were prepared in duplicate per treatment. Thus, a volume of 10 µl of treated or control cells (~1 X 10^4 cells) was added to 120 µl of 0.5% low-melting point agarose at 37°C, layered onto a pre-coated slide with 1.5% regular agarose, and covered with a coverslip. After brief agarose solidification in refrigerator, the cover slip was removed and slides immersed to lysis solution (2.5M NaCl, 100 mM EDTA – Merck, St Louis, USA; 10 mM Tris-HCl buffer pH = 10 – Sigma Aldrich, USA; 1% sodium sarcosinate – Sigma Aldrich, USA; with 1% Triton X-100 – Sigma Aldrich, USA; and 10% DMSO –Merck St. Louis, USA) for about 1 hour. Prior to electrophoresis, the slides were left in alkaline buffer (0.3 mM NaOH, Merck USA; and 1mM EDTA, Merck, USA; pH > 13) for 20 minutes and electrophoresed for another 20 minutes, at 25 V (0.86 V/cm) and 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris-HCl (pH = 7.5) for 15 minutes, fixed in absolute ethanol and stored at room temperature until analysis. All of the steps described above were conducted in the dark to prevent additional DNA damage [9].

Statistical Analyses

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

Histopathological Examinations (Fig.1- 6)

Histological examination of group I reveals normal lobular pattern of liver cells with radial arrangement of hepatocytes around the central veins. Between the hepatocytes, sinusoidal capillaries were clearly seen (fig. 1). Group II revealed marked histological changes including severe inflammatory cellular infiltration mainly lymphocytes which was very obvious especially around the portal area. This is also associated with dilatation and congestion of blood vessels and hepatic sinusoids. Haemorrhagic infiltration is also observed (fig. 2). Subgroup III_a (vaccinated with UV irradiated tachyzoites- unchallenged) showed vacuolizations with few inflammatory cells in hepatic sinusoids and mild dilatation of central vein (fig.3). Liver of mice from subgroup IV_a (vaccinated with gamma irradiated tachyzoites- unchallenged) showed dilated blood sinusoids and moderate focal hepatic necrosis associated with few inflammatory cells infiltration (mainly neutrophils) (Fig. 5). Vaccinated-challenged subgroups III_b and IV_b (UV and gamma irradiated tachyzoites) showed that hepatocytes almost normal with mild congestion (Figs. 4, 6).

Histopathological examinations:

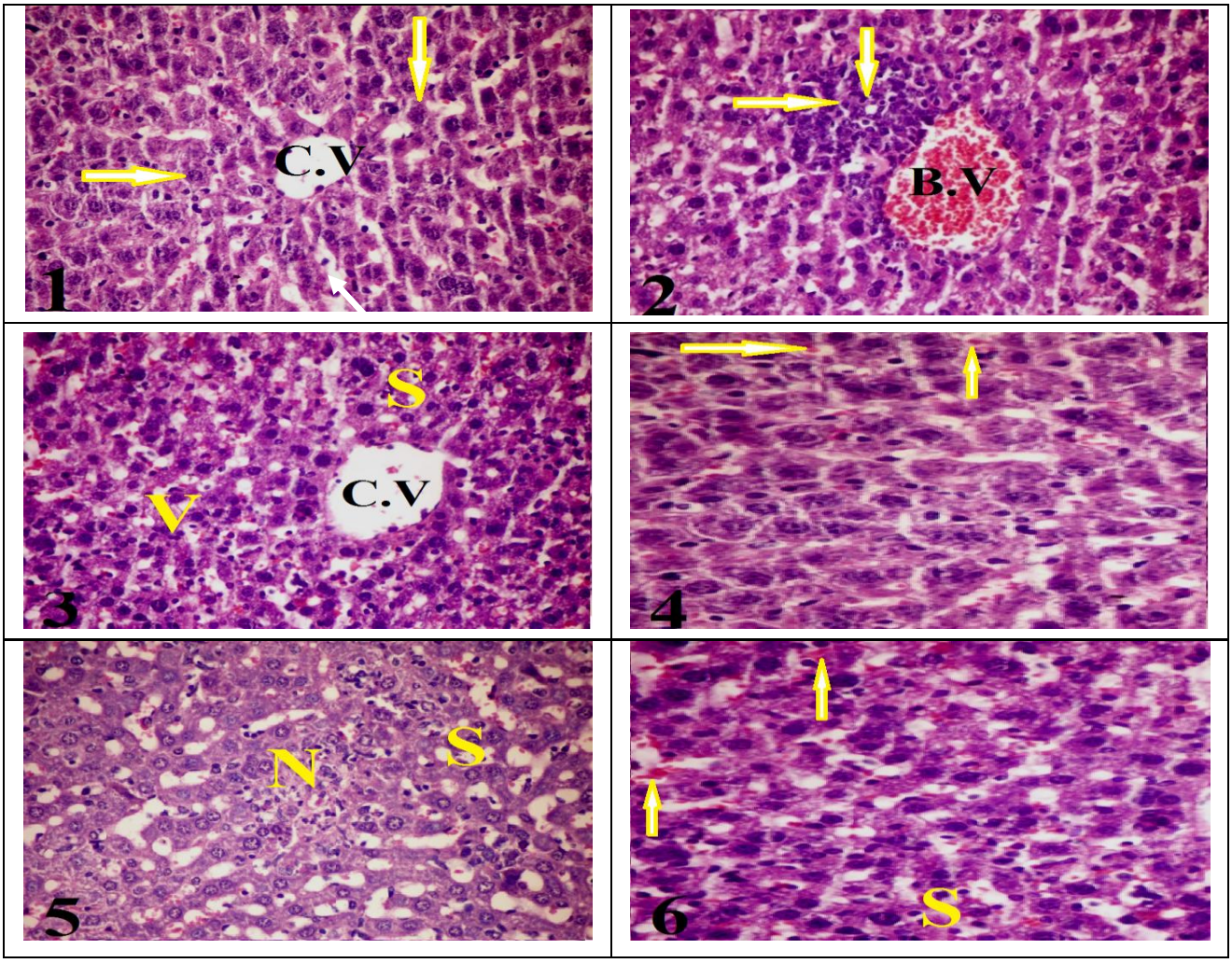


Fig. (1): Liver of mice of control group I (normal) showing radially arranged hepatocytes (→) around the central vein (C.V.) (H&E X400)

Fig. (2): Liver of group II (infected control) showing dilatation and congestion of hepatic blood vessel (B.V.) with severe infiltration with inflammatory cells (→) (H&E X400)

Fig. (3): Liver of subgroup III_a (UV irradiated- unchallenged) showing mild dilatation of central vein (C.V.) with few inflammatory cells in hepatic sinusoids (S) and the presence of vacules (V) (H&E X400)

Fig. (4): Liver of subgroup III_b (UV irradiated- challenged) showing nearly normal histological structure of hepatic lobules with mild interstitial congesion (→) (H&E X400)

Fig. (5): Liver of subgroup IV_a (gamma irradiated-unchallenged) showing dilated blood sinusoides (S) and focal hepatic necrosis (N) associated with inflammatory cells infiltration (H&E X400).

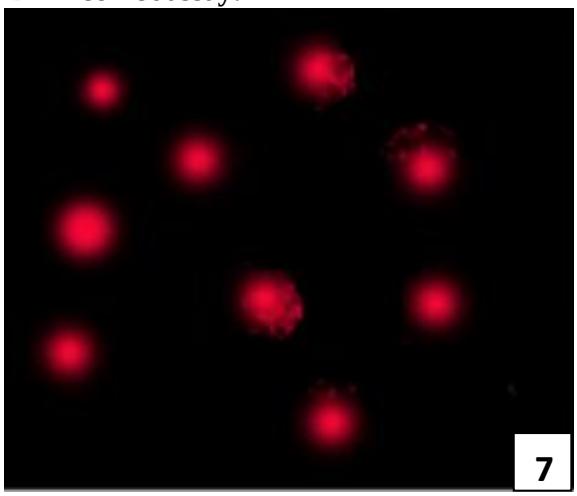
Ftg. (6): Liver of subgroup IV_b (gamma irradiated- challenged) showing an almost normal hepatic cells with signs of haemorrhages in between (→) . Mild sinusoidal (S) dilatation and congestion are also observed (H&E X400)

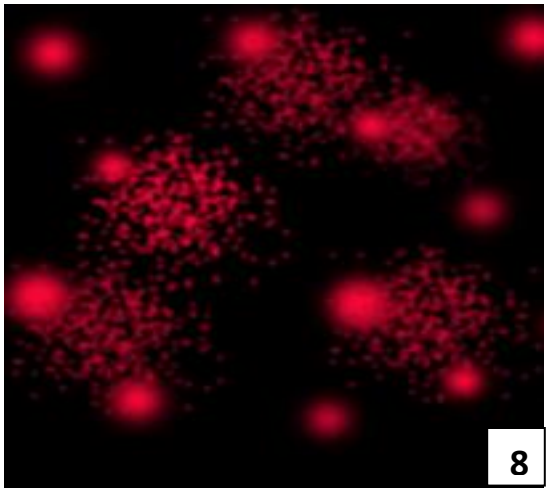
DNA Comet Assay

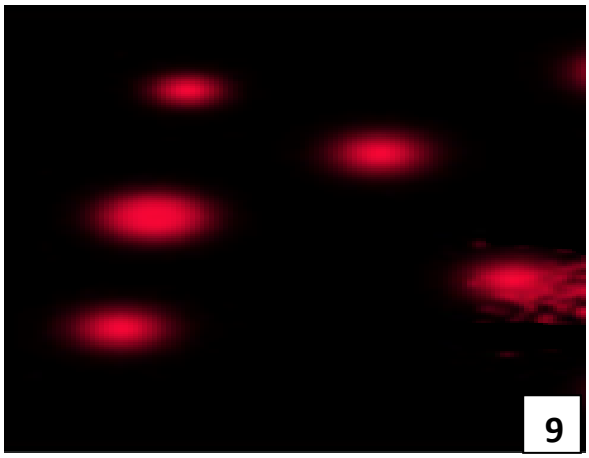
DNA comet assay in hepatocytes was expressed in table (1) figs. (7-14) showing high significant increase in tail moment in group II ($P<0.001$) compared to the normal group I. The results showed high significant decrease in tail moment in subgroup III_a ($P<0.01$) and % untailed was % 93 while, subgroup IV_a showed high significant decrease in tail moment ($P<0.001$) with % 92 untailed compared to the infected group. Subgroups III_b and IV_b which were vaccinated with irradiated

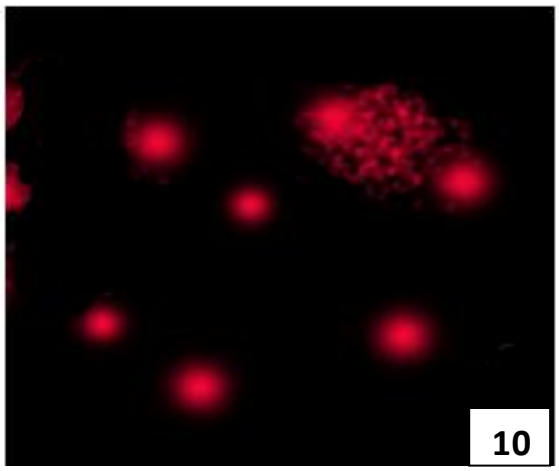
tachyzoites and challenged showed significant decrease in tail moment ($P < 0.01$) and increase in % untailed to % 92 and % 91 respectively compared to the control infected group II.

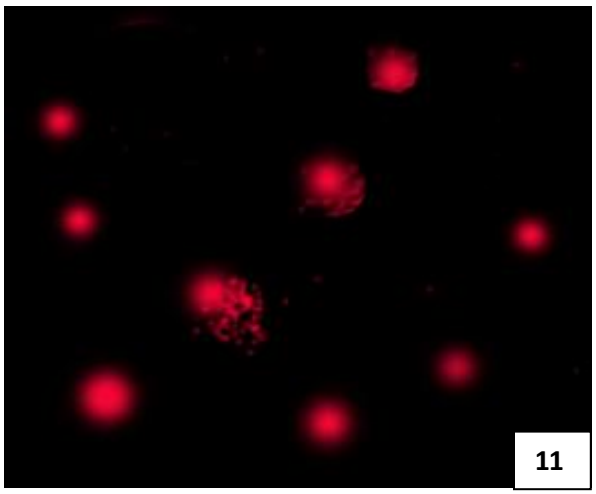
DNA comet assay:











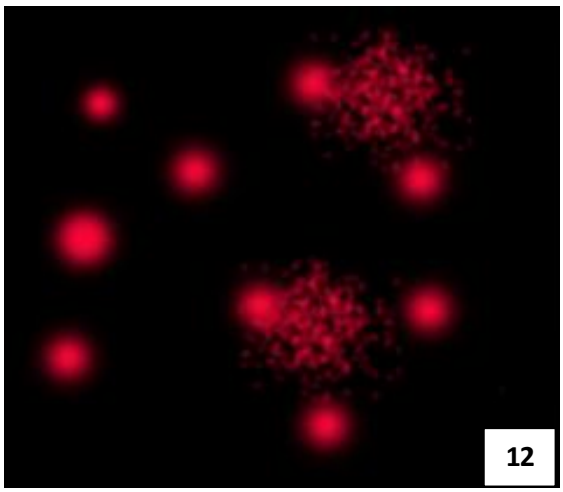


Fig. (7): Group I control normal
Fig. (8): Group II control infected
Fig. (9): Group III_a UV irradiated-unchallenged
Fig. (10): Group III_b UV irradiated- challenged

Fig. (11): Group IV_a gamma irradiated-unchallenged
Fig. (12): Group IV_b gamma irradiated-challenged

Table (1): Mean number of tail moment, length and % of untailed DNA in comet assay.

	Tailed%	Un tailed%	Tail length um	T DNA%	TAIL Moment
I	6	94	2.25±0.1*	2.58	6.53±0.1*
II	11	89	4.20±0.2*	3.66	15.46±0.1*
III_a	7	93	2.90±0.1*	3.18	9.33±0.1*
III_b	8	92	3.38±0.3*	3.08	11.27±0.08*
IV_a	8	92	3.21±0.3*	2.51	7.71±0.07*
IV_b	9	91	4.07±0.1*	2.41	9.78±0.02*

* Statistically significant at P<0.05

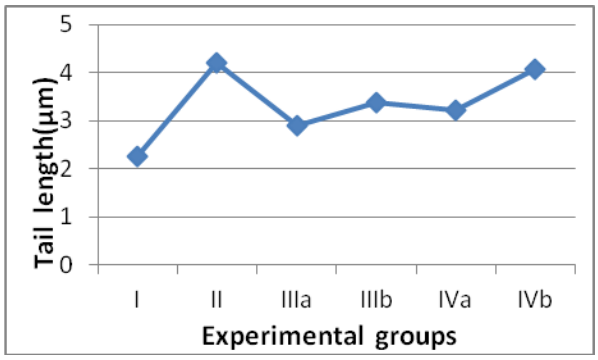


Fig. (13): Changes in tail length in all groups

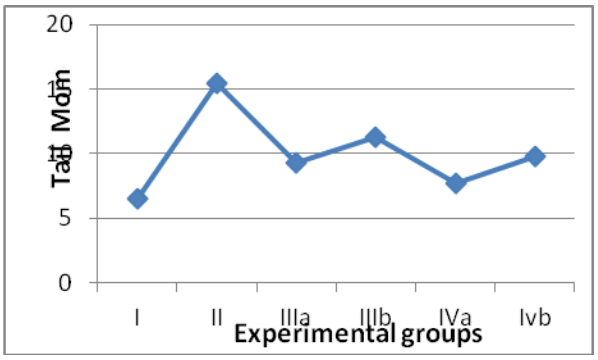


Fig. (14): Changes in tail moments in all groups

DISCUSSION

Toxoplasmosis remains a serious disease and many studies were carried out on *Toxoplasma gondii* parasite and its effect on tissue histology. It is generally accepted that the liver is the essential body organ responsible for detoxifying and withdrawal of many injurious substances in the body and it is one of the most important organs affected by infection and a preferable site for growth and multiplication of the parasite [20]. In experimental models of infection, acute mortality can result either from a failure to control the parasite number or due to the excessive immunological response against the infection [8]. In the present study, marked histopathological alterations were observed in control infected group such as dilatation of bile duct, edema, inflammatory cells infiltration and congestion of portal vein. These changes were in agreement with previous study by Mordue et al. (2001) [21] which reported that the major site of tissue pathology during lethal toxoplasmosis was the liver and damage including enlargement, cytoplasmic vacuolization, and release of liver enzymes was not directly attributable to intracellular parasite replication or apoptosis. However it is mediated through a soluble parasite-derived factor(s) or an induced host factor(s) that reaches toxic levels during lethal infections. Other studies on the virulence of toxoplasmosis described severe alterations as cell destruction and liver necrosis [22, 23].

The effect of gamma radiation on microorganisms is a decrease in the number of cells capable of reproducing [24]. UV radiation affects the protein and peptide compounds of DNA of living microorganisms, so the ability of division and multiplication was lost or changed [25]. In this study,

attenuating dose of either UV or gamma radiation was chosen instead of killing dose according to previous studies reported that live attenuated tachyzoites as vaccine confer stronger protective immunity than dead one [26, 27]. Also, it was reported that live parasites produce certain proteins which enhance and increase the host resistance against *T. gondii* challenged mice [28]. Regarding vaccinated-challenged subgroups III_b and IV_b recorded in the study, few histopathological alterations were detected in comparison with those described in group II. These findings suggest that the protective capacity of the vaccinated mice to control this pathogen is dependent on the regulation of immune response of vaccinated mice. This supports the studies reported that vaccination of mice increased their ability to construct a defense mechanism against toxoplasmosis [29, 30]. Also, this was in agreement with Zhao et al. (2013) [31] who reported that mice vaccinated with UV-*T.gondii* and then challenged had even significantly increased survival rate and extended survival time, decreased parasite burden, as well as improved liver histopathology. On other hand, vaccinated non challenged subgroups III_a and IV_a showed minimal histopathological alterations and hepatic lobules showed normal architecture. These can be explained by the effect of radiation that weaken the parasite, although retaining their metabolic functions lose their proliferation ability through damaging DNA molecules by UV radiation [32] or direct effect as in gamma radiation that induces changes in tachyzoites, leading to decreased or abolished reproduction, but maintaining viability, a respiratory response and preserved protein and nucleic acid synthesis. So, they present antigens to the host's immune system and elicit cellular immunity and cytokine responses in a highly similar way to natural infection [33].

Although immune-regulatory cytokines help in eradication of *T. gondii*, they expose the organs to certain endogenous genotoxic agents which induced DNA damage. As the hepatocytes were active metabolic cells and when such parasite invaded the cell it can lead to disturbances in its metabolic activity which intern leaded to shape distortion, which could be due to edema and accumulation of fluid in cells. Extent of DNA damage in hepatocytes was assessed by single cell gel electrophoresis. The comet cells were classified on morphological basis into type I short tail cells with relatively little DNA degeneration; type 2, long tail; type 3, long tail wider at the end; type 4, long tail separated from the head of the comet and type 5, almost no DNA is left in the head of the comet and the tail appears as a cloud far from the head [10]. In the present study, control normal group showed intact cells with no or very short tails because there was no DNA damage or stretching of band, while control infected group showed more comet cells with long tails, representing DNA fragments migration, the tail was more wider and thicker than the head of comet. Similar changes were also found by other workers and some related the disturbances in the hepatocyte function and shape to the DNA damage in the liver cells provoked by infection with *Toxoplasma gondii* [3, 34]. This was in agreement with the previous study reported significant DNA damage in liver and lymphoid tissues during lethal strain infections [23]. Also, Harba and Afifi, (2012) [35] reported marked DNA damage by comet assay in leukocytes and spleenocytes in mice infected with virulent strain *T. gondii* tacyzoites. Regarding vaccinated-challenged subgroups either UV or Gamma radiation, the majority of cells appeared normal rounded and few cells with very short tails. Vaccinated non challenged subgroups showed very minimal degeneration with no tails indicating that irradiation prevents propagation and division of the parasite so cannot affect liver cells.

CONCLUSION

The present work proves the validity of vaccination with UV or gamma-attenuated tachyzoites to protect murine models against acute toxoplasmosis and this was represented by amelioration of the histological changes and DNA damages in hepatocytes and increasing the cellular immune response. Also, the study showed that UV radiation gives better protective immune response than gamma radiation. It is advisable in the future using radiation-attenuated vaccine in controlling toxoplasmosis, however manipulation of non-ionizing UV- light to irradiate tachyzoites (present in the food) more preferred instead of the higher energy ionizing sources as they are cheaper and convenient to perform.

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