Assessment of Radiation-Attenuated Vaccine or Thyme Oil Treatment on Controlling DNA Damage and Nitric Oxide Synthesis in Brain of Rat Infected with Toxocara canis

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Received: 15/3/2015 Accepted: 20/5/2015

ABSTRACT

Toxocara canis is a worldwide zoonotic roundworm that infects a number of hosts including humans. It exhibits marked affinity to the nervous tissues. This study deals with the changes in the brain of Toxocara canis infected rats regarding parasitological, nitric oxide (NO) level and DNA damage compared to the effect of vaccination with gamma radiation-attenuated embryonated egg or thyme oil treatment. Eighty rats were classified into four groups (twenty each): GI (normal control); GII infected with 2500 T. canis infective eggs/ml/rat (infected control); GIII vaccinated with 800Gy gamma-attenuated embryonated eggs (vaccinated group) and GIV infected with 2500 T. canis eggs and treated with thyme oil (thyme treated group). At the 14th day post-infection, ten rats from each group were sacrificed and the remaining were re-infected (challenged) with the same number of eggs. At the 14th days post challenge, brain tissues were taken for larval recovery, nitric oxide level evaluation and DNA damage using fragmentation and comet assay. The results exhibited a significant decrease in larval count and nitric oxide level with less damage in brain cells in thyme treated and gamma radiation-attenuated vaccinated groups compared to control infected group. It is also, concluded that vaccination using γ-rays is more effective in protection compared to using thyme oil.

Key Words: Gamma radiation; Thyme; DNA Comet; Toxocara canis; brain

INTRODUCTION

Toxocara species are worldwide occurring helminthes with high zoonotic potential (1). Larvae undergo a full development into the adult stage in the definitive host “carnivore” which sheds large amounts of eggs into the environment. Under suitable environmental conditions, the infective third stage larva develops in the egg (2, 3) which may be taken up by paratenic hosts, including humans. The two main clinical presentations of toxocariasis are visceral larva migrans (VLM) and ocular larva migrans (OLM). In VLM, the larvae invade multiple organs as the liver, heart, lungs, brain and cause responsive changes and tissue damage. In the murine host, larvae exhibit a predilection for the central nervous system and previous studies mainly revealed accumulation of T. canis larvae in brains of infected mice. It is also, assumed that most human cases of neurotoxocarosis result from infection with T. canis (4, 5).

Parasitologists have realized that chemoprophylaxis is unsustainable due to increasing drug resistance and the costs of constantly developing new drugs (6). Certain crop plants can uptake antibiotics from livestock manure applied to the soil and this has implications for human health (7). Elimination of internal and external parasites by the use of thyme, a natural remedy has been
recognized for centuries as an effective anti-microbial and anti-parasitic medication. Exploring
the alternative therapy that makes use of this herb containing significant amounts of thymol; a
compound that inhibits the growth of ringworm, mites and other parasites, and carvacol, which
combats E.coli and other bacteria. Treatment of parasitic conditions ranging from amoebas and
intestinal worms to fungi of the foot and toenail, using infusions, essential oils and poultices made
from the thyme plant, Thymus vulgaris which was used in the treatment of Entamoeba histolytica (8),
Leishmania major, Trypanosoma bruceia (9) and Trypanosoma cruzi (10). Subsequent studies have
confirmed thyme’s antioxidant properties, and how it helps the body to maintain higher levels of essential fatty
acids within the brain (11).

The use of ionizing radiation in the production of radiation-attenuated vaccines against various
parasitic infestations has been taken into consideration since the last 5 decades. The irradiated
attenuated vaccines represent a recent successful and promising approach in control of the parasitic
diseases. The most common types of ionizing radiation are gamma and UV rays that were used for
attenuating the infective stage of the parasite (12-14). The effects of ionizing radiation on parasites
depend on the radiation dose, developmental and physiological status of worms, and other various
physical parameters during or after the irradiation (15). The effect of toxocariasis on the tissues of rats
infected with irradiated Toxocara canis eggs was studied and it was noticed that the histopathological
changes caused by infection with Toxocara canis decreased with increasing the dose of irradiation of
the infective stage (16).

NO is produced by a number of different cell types in response to cytokine stimulation and thus
has been found to play a role in immunologically mediated protection against a growing list of
parasites (17). It plays an important role in the immune mechanisms of human and inhibits enzymes
necessary for energy metabolism and cell growth. In addition, NO is produced by cells other than
those involved in immune response, such as hepatocytes and endothelial cells which are significant in
the life cycle of many parasites. The overproduction of NO is harmful for the cells and the surrounding
tissues (18-20). Moreover, it produced at high level by activated macrophages can be metabolized by
auto-oxidation to form peroxynitrite which is potentially toxic to DNA, and may dissociate to from
hydroxyl radical leading to DNA oxidation or l and strand breaks (21).

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 (22). The classification of comet cells from types 1 to 5 based on morphological basis was
reported by Marin-Huachaca et al. (23). Quantification of DNA damage in individual cells based on
the migration of DNA in electric field was first described by Ostling and Johanson (24), while measurement
of DNA damage as a ratio of fluorescence intensities without any assumption of the morphological
shape of investigated comet was reported by Bocker et al. (25).

The aim of this study is to confirm whether infection with T. canis provokes oxidative stress
in the host through measuring changes in levels of nitric oxide synthesis and DNA damage in brain
and controlling of rat infected with T. canis with radiation–attenuated vaccine or Thyme oil treatment.

MATERIAL & METHODS

Animals

Eighty male albino rats (Rattus norvegicus), weighing 100–160 g each were kept in cages under
controlled environmental conditions. They were acclimatized to laboratory conditions before starting
the experiment. All animals were maintained in conformance 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 (26).
Preparation of Parasite Eggs

*T. canis* eggs were obtained from the uteri of female nematodes collected from the naturally infected dogs. Eggs were incubated in 0.5% formalin solution at 28°C for 4 weeks. Embryonated eggs were kept at +4°C until used \(^{(27)}\).

Thyme Oil Preparation

The essential oil of the *Thymus vulgaris* (Thyme) is obtained from the Egyptian Company for Oils and Soap, Cairo, Egypt. The required concentration of the plant oils was prepared by diluting the stock of the oil with few drops of Tween 80 as emulsifier and water was added (42.5 mg/kg body weight) \(^{(28)}\).

Radiation Source

The irradiation process throughout the time of study was performed using cobalt 60-gamma cell 220 irradiator type G6500 (manufactured by the atomic energy of Canada Ltd., Ottawa, Canada) at the National Center for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority (AEA), Cairo, Egypt. The dose rate in the irradiation chamber was 2.5 Kgy/h at the time of experimentation. *Toxocara* eggs were exposed to 800 Gy radiation.

Experimental Design

The experiment was carried out using four groups; each group consisted of 20 rats, the first group (GI) was used as control group. In the second group (GII): each animal received orally infective embryonated *T. canis* eggs (2500 infective eggs per ml/rat). The third group (GIII): received 800 Gy irradiated infective *T. canis* eggs and the fourth group (GIV) received the infective *T. canis* eggs and treated with thyme oil. However, at 14th day post infection, rats were re-infected (challenged) with the same number of infective *T. canis* eggs (2500 eggs/ml/rat). FiveTen rats were scarified at the 14th day post infection and post challenge. The brain was removed for biochemical analysis. The homogenates of brain tissues (10%) were prepared in normal saline for biochemical and DNA fragmentation assays.

Worm Recovery and Larvae Count

On days 14 and 14R after inoculation, ten rats at each group were killed by cervical dislocation and larvae were recovered from brain, after acid digestion, as described by Xi and Jin \(^{(29)}\). Larval counting in fixed samples was undertaken microscopic technique.

Determination of NO Production

The brain nitrite level was determined using colorimetric method based on the Griess reaction in which nitrite is treated with sulfanilamide and N-(1-naphthyl) ethylenediamine to produce an azo dye. NO is a labile compound, has a brief half-life and is rapidly converted to the stable end-products, nitrite and nitrate, in oxygenated aqueous solutions. Nitrite levels were measured after the enzymatic reduction of nitrate to nitrite with nitrate reductase. Sodium nitrite solution was used for standard measurements.

All samples were deproteinized before assay. Briefly, for every 200 μl sample, 400 μl of 0.5 N sodium hydroxide and 400 μl of 10% zinc sulfate were added. The samples were then vortexed and centrifuged at 25 000 xg for 5 min at 4°C. Nitric oxide metabolites (nitrate and nitrite) were assayed by first reducing nitrate to nitrite. Nitrate reductase (0.05 unit/ml) along with reduced nicotinamide adenine dinucleotide (90 μmol/L) and flavin adenine dinucleotide (3.12 μmol/L) were added to each sample to convert nitrates to nitrites. Nitrite production was then determined with the spectrophotometric Greiss reaction. For this study, 100 μl of sample or water blank was mixed with 50 μl of 0.32 mol/L potassium phosphate, pH 7.5 and 10 μl of nitrate reductase with cofactors. To the
sample mixture, 40 μl of Greiss reagent (10% sulfanilamide and 1% naphtylethylenediamine dihydrochloride in 85% phosphoric acid) was added. The mixture was incubated for 10 min at room temperature and the absorbance was read at 550nm. Sample nitrite levels were expressed in _mol/L_.

### DNA Fragmentation

The brain tissue was harvested by centrifugation and lysed by 600ml lysing buffer (Sigma). The cell suspension was shaken gently and kept overnight at 37 °C. DNA was extracted by addition of 200μl saturated NaCl to samples, then shaken gently and centrifuged at 12000rpm for 10 min. The supernatant was transferred to a new eppendorf tube and DNA was precipitated by addition of 700μl cold isopropanol, followed by centrifugation. The sediment was washed in 500 μ, 70% ethyl alcohol for 8 min at 12000rpm. The supernatant was decanted and the resulting pellet was gently re-suspended in 50μl or appropriate volume of TE buffer then supplemented with 5% glycols for 30 min. To get rid of RNA, an appropriate volume of RNAase was added and incubated at 37 C for 1 h. The DNA samples were mixed with 6x loading buffer and analyzed on a 2% agarose gel stained with 1 mg.ml ethidium bromide (Sigma). Results were quantified by densitometric analysis of bands corresponding to fragmented DNA on a BioDoc II digital imaging system.

### Single Cell Gel (Comet) Assay

Brain tissues were collected from the rat samples, homogenized in 0.075 M NaCl and 0.024M ethylenediaminetetraacetic acid (EDTA) buffer, pH 7.5, at a ratio of 1 g of tissue to 1 ml of buffer, and then cooled to 4°C. Volumes of 6 μl of brain homogenates were suspended in 0.5% low-melting agarose (LMA) (Sigma-Aldrich, St Louis, MA, USA) and placed onto microscope slides that were cleaned and coated with 300 μl of 0.6% normal melting point agarose (NMP) agarose beforehand. After solidification on ice for 10 minutes, the slides were covered with 0.5% low melting point (LMP) agarose. Once the agarose gel was solidified, the slides were immersed for one hour in an ice-cold lysis solution, consisting of 100 mM Na2EDTA, 2.5 M NaCl, 10 mM Tris–HCl, and 1% sodium sarcosinate, which was adjusted to pH 10, using 1% Triton X-100 and 10% dimethyl sulfoxide (DMSO) that were added immediately prior to use. Before electrophoresis, the slides were removed from the lysis solution and placed for 20 minutes in a horizontal electrophoresis unit (near the anode) that was filled with an alkaline buffer to allow the unwinding of DNA and to express alkali labile damage. The electrophoresis alkaline solution consisted of 1 mM Na2EDTA and 300mM NaOH, pH 13. After the unwinding of DNA, electrophoresis was carried out in the freshly prepared alkaline solution for 20 minutes at 25 V (300 mA). Electrophoresis at high pH resulted in structures resembling comets, as observed by fluorescence microscopy; the intensity of the comet tail relative to the head reflected the number of DNA breaks. Afterwards, the slides were neutralized by adding Tris buffer (pH 7.5), stained with 30 ml of ethidium bromide (Sigma-Aldrich, St Louis, MA, USA) (20 mg/L), and then covered and stored in sealed boxes at 4°C for further analysis.

All preparation steps were performed under dimmed light to prevent additional DNA damage. Images of 100 randomly selected cells (50 counts on each duplicate slide) were analyzed for each sample. For each group, a total of 500 cells were analyzed under a Leitz Orthoplan epifluorescence microscope (magnification 250x) equipped with an excitation filter of 515 to 560 nm and a barrier filter of 590 nm. The microscope was connected through a camera to a computer-based image analysis system (Comet Assay IV software, Perspective Instruments).

Comets were randomly captured at a constant depth of the gel, avoiding the edges of the gel, occasional dead cells, and superimposed comets. DNA damage was measured as tail length (TL = distance of DNA migration from the center of the body of the nuclear core), and tail intensity DNA (TI = % of genomic DNA that migrated during the electrophoresis from the nuclear core to the tail). By presenting all three parameters together, more information could be obtained on the extent of DNA damage.
Statistical Analysis

All data were expressed as mean ±SE (standard error). Data were assessed using a one-way ANOVA using SPSS 15.0 program and p<0.05 was considered statistically significant.

RESULTS

Larval Count

The mean larvae burden in infected mice group GII were 960 ± 29 at 14th day post infection and 740±13 in 14th day post challenge with 38.4% and 29.6% of worm recovery respectively. Regarding vaccinated group GIII, the mean larva burdens were 210 ±20 with 8.4% of worm recovery and 90± 15 with 3.6% at days 14th and 14th post challenge respectively. In the thyme treated group GIV, the mean larvae burden were 350 ±40 with 14 % and 160 ± 30 with 6.4% worm recovery at both 14th days post infection and post challenge respectively (Table 1).

Table (1) Toxocara canis larvae counts in brain tissue

<table>
<thead>
<tr>
<th>Days post infection</th>
<th>Groups</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M±S.E</td>
<td>960±29</td>
<td>210±20</td>
<td>350±40b</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>% of recovered larvae</td>
<td>38.4</td>
<td>8.4</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>% of immune response</td>
<td>------</td>
<td>78.13</td>
<td>63.54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M±S.E</td>
<td>740±13</td>
<td>90±15b</td>
<td>160±30b</td>
<td></td>
</tr>
<tr>
<td>14R</td>
<td>% of recovered larvae</td>
<td>29.6</td>
<td>3.6</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>% of immune response</td>
<td>------</td>
<td>87.83</td>
<td>78.37</td>
<td></td>
</tr>
</tbody>
</table>

Values are recorded as Mean ±S.E.
% of Immune response (percent of change between non-irradiated and irradiated eggs treated rats)
% of larvae recovered (per larval dose) from the brain rats treated with Toxocara canis eggs
b: significant change from infected group at P<0.05 .R: reinflection (challenge).

Nitric Oxide Level

The data presented in Table (2) show that NO levels increased significantly in infected group GII compared to those of the normal control group GI after the same number of days post-infection and post challenge. The vaccinated group GIII showed a significant difference compared to normal group and control infected group. Also, in the thyme treated GIV the level of NO level decreased compared to control infected group II.

Table (2) Nitric oxide levels (µmol/L) in rats brain cells between different groups on different days

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 14 Mean ±SE</th>
<th>Day 14R Mean ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>G I</td>
<td>18.1±0.13</td>
<td>18.1±0.13</td>
</tr>
<tr>
<td>G II</td>
<td>37.9 ± 0.8a</td>
<td>45.9±0.7a</td>
</tr>
<tr>
<td>G III</td>
<td>23.3±0.2ab</td>
<td>31.1±0.3ab</td>
</tr>
<tr>
<td>G IV</td>
<td>26.5±0.2ab</td>
<td>35.9 ±0.3ab</td>
</tr>
</tbody>
</table>

a: significant change from normal group I  b: significant change from infected group II
DNA Fragmentation

The optical density of DNA extracted from brain tissue cells showed fragmented DNA laddering or apoptotic bands with higher intensity in control infected group GII at 14th and 14th R days while the apoptotic bands are lower in vaccinated group GIII and in thyme treated GIV. The results also revealed that DNA fragmentations were more observable in thyme treated in comparison with those observed in the vaccinated group (Table 3) (Fig. 1).

Table (3) Maximal optical density (MOD) of DNA fragments in brain tissues of different groups

<table>
<thead>
<tr>
<th>Basic pairs</th>
<th>G I</th>
<th>G II</th>
<th>G III</th>
<th>G IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact DNA 2000 bp</td>
<td>1750</td>
<td>1340</td>
<td>1340</td>
<td>1340</td>
</tr>
<tr>
<td>1700 bp</td>
<td>980</td>
<td>980</td>
<td>1000</td>
<td>980</td>
</tr>
<tr>
<td>1500 bp</td>
<td>820</td>
<td>820</td>
<td>980</td>
<td>940</td>
</tr>
<tr>
<td>1000 bp</td>
<td>600</td>
<td>725</td>
<td>940</td>
<td>770</td>
</tr>
<tr>
<td>600 bp</td>
<td>380</td>
<td>570</td>
<td>570</td>
<td>595</td>
</tr>
<tr>
<td>500 bp</td>
<td>390</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. (1) Fragmentation patterns of DNA from rat brain in each group: First lane: DNA ladder, Lane 1 Control normal GI, Lane 2 control infected GII at 14th day, Lane 3 control infected GII 14th R, Lane 4 and Lane 5 vaccinated: GIII at 14th and 14th R day respectively, Lane 6 and Lane 7 thyme treated GIV at 14th and 14th R day respectively.
DNA Comet

Evaluation results of comets by image analysis were evident in Fig. (2-8) as well as illustrated in table (4). The changes of comet tail DNA%, tail length and tail moment displayed a marked increase in control infected group II in both 14th and 14th R days compared to normal group I. While, the vaccinated group III and the thyme treated group IV showed a decrease in untailed % and exhibited significant difference in tail moment and tail length compared to that of control infected group II especially in the 14th R days.

Table (4) Mean number of tail moment and tail length and % of tail DNA in experimental groups

<table>
<thead>
<tr>
<th>DNA damage Groups</th>
<th>Tailed %</th>
<th>Untailed %</th>
<th>Tail length (µm)</th>
<th>Tail DNA %</th>
<th>Tail moment (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G I</td>
<td>3</td>
<td>97</td>
<td>1.52±0.13</td>
<td>1.91</td>
<td>3.17±0.02</td>
</tr>
<tr>
<td>G II 14th</td>
<td>18</td>
<td>82</td>
<td>4.14±0.12</td>
<td>3.97</td>
<td>16.06±0.02</td>
</tr>
<tr>
<td>G II 14th R</td>
<td>20</td>
<td>80</td>
<td>4.26±0.09</td>
<td>4.44</td>
<td>19.05±0.03</td>
</tr>
<tr>
<td>GIII 14th</td>
<td>15</td>
<td>85</td>
<td>3.89±0.03</td>
<td>3.58</td>
<td>15.24±0.19</td>
</tr>
<tr>
<td>GIII 14th R</td>
<td>10</td>
<td>90</td>
<td>3.54±0.03</td>
<td>3.72</td>
<td>12.14±0.01</td>
</tr>
<tr>
<td>GIV 14th</td>
<td>13</td>
<td>87</td>
<td>3.72±0.03</td>
<td>3.98</td>
<td>13.24±0.04</td>
</tr>
<tr>
<td>GIV 14th R</td>
<td>11</td>
<td>89</td>
<td>3.60±0.04</td>
<td>3.42</td>
<td>13.41±0.02</td>
</tr>
</tbody>
</table>

Tail DNA% = percent of DNA in the comet tail.
Tail length = length of the comet tail measured from right border of head area to end of tail (µm = pixels)
Tail moment = tail DNA% X tail length
a: significant change from normal group I
b: significant change from infected group II
n: no significant
Fig. (2) Normal group I (-ve), intact nuclear DNA (x250)

Fig. (3&4) Infected group GII (+ve), DNA damage expressed by tail moment in a rat brain cell. It showing strongly damaged spot (x250)

Fig. (5&6) Vaccinated group GIII, DNA damage expressed by tail moment in a rat brain cell. It showing less damaged spot (x250)

Fig. (7&8) Thyme treated group IV DNA damage expressed by tail moment in a rat brain cell. It showing damaged spot
DISCUSSION

The infection risk for humans with T. canis is high due to dog’s defecation habits that result in environmental contamination and increases the risk of infection and consuming undercooked or raw meat serves as a transmission route. 

Also, several studies have demonstrated the persistence of T. canis and T. cati larvae in a number of paratenic hosts, which are sources of food for humans. Furthermore, viable larvae of T. canis have been found in meat even after prolonged periods of freezing. The migration route of T. canis larvae in the paratenic host has been examined in various studies resulting in a strong affinity to the brain. Commonly described clinical symptoms in humans have been behavioral disorders, focal or generalized seizures, ataxia, sensory disturbances and brain infarcts. Sporadically, cognitive dysfunctions and reduced ability of long and short term memory have been reported.

This study aims at evaluating the damages in the brain tissue of T. canis infected rats as regard larval count, nitric oxide level, and DNA damage and the effects of thyme treatment or vaccination with gamma radiation attenuated vaccine were also evaluated. In the current work, the recovery of T. canis larvae in control infected group was high in 14th day post infection and post challenge in brain tissue. Similar results were reported by previous studies who found that larval recovery showed a progressive increase over the course of infection, with special predilection for the central nervous system. In the thyme treated group, the number of recovered larvae was decreased compared to control infected group II and markedly reduced in the 14th day after reinfection. This is in accordance with a previous study which reported that thyme administration before and after Toxocara vitulorum infection resulted in a significance reduction of larval burden compared to infected group. For the group subjected to 800 Gy radiation attenuated-vaccinated group, it showed a marked reduction in number of larvae 14th of infection. This agree with those found by Kamiya et al who studied the effect of radiation on the viability and migratory ability of second-stage larvae of T. canis in mice and reported that most of the larvae irradiated with 80 or 160 Krad remained in the digestive tract, mainly in the stomach and the proximal half of the small intestine. It was found that radiation dose of 300 Krad prevented the migration of visceral larvae migrans while with using 80 -100 Krad a number of larvae was found in brain in 15th day post infection. The same vaccinated group showed a marked decrease in larval count at 14th day post challenge due to the stimulation of immune response.

With respect of NO level in the brain tissue, it demonstrated a high significant increase in control infected group in the 14th days post infection and post challenge. This outcome is compatible with that observed by Nassief et al who reported significant increase in serum NO in control infected group compared to normal one. In radiation-vaccinated group, there was a significant reduction in NOs level which was marked in 14th day post challenge due to stimulation of the immune response. Also, there was a significant reduction in NO level in both thyme treated group which agree with that observed by Youdim and Deans who showed that both thyme oil and thymol provided beneficial effects on the antioxidant status of the rat brain.

Concerning the to DNA damage of brain cells, the present results demonstrated higher intensity of DNA laddering on agarose gel electrophoresis with high significant increase in tail moment and tail length using comet assay in control infected group II on 14th days post infection and post challenge compared to control normal group I. Radiation-vaccinated group IV, less decrease in tail length and moment especially at 14th day post challenge compared to control infected group III as a result of stimulation of immune response by the attenuated vaccine similar investigations revealed that one of the advantages of live, attenuated vaccines is their potent immunogenicity since the organisms are still able to replicate and behave initially in a similar manner to a natural infection, thereby stimulating the immune system to secrete the immunoregulatory products and induce the cellular activation that would normally occur. For the case of the thyme treated group, laddering was lower in intensity with decrease in tail length and moment compared to control infected group II.
CONCLUSION

Radiation-attenuated vaccine using 800 Krad gamma rays irradiated embryonated eggs and thyme treatment for T. canis- infected rat caused a decrease in larval count, NO level and improvement in the histopathological lesions and DNA fragmentations as well as damage in brain tissues. Vaccination was more effective in protection against increase nitric oxide and DNA damage.

ACKNOWLEDGEMENTS

This work was supported by National Center for Radiation, Research and Technology. We are grateful to it for using gamma radiation unit and all facilities for animal housing and doing experiments.

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