Effect of vaccination with irradiated *Toxocara canis* larvae or thyme oil treatment on testicular histochemical and immunohistochemical changes of rats

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Abstract. *Toxocara canis* is widely distributed parasite that not only presents in definitive hosts but also occurs in paratenic hosts including human. Larvae migrate throughout the somatic tissue causing severe inflammatory and pathological reactions. This study aims to detect the effect of infection with *Toxocara canis* on testis of rats regarding histopathological, histochemical and immunohistochemical changes and amelioration of these changes with either vaccination with gamma radiation-attenuated embryonated eggs or with herbal treatment with thyme. The study was conducted on eighty rats classified into four groups (20 each): Group A (normal control); Group B (infected control); Group C infected and treated with thyme oil (thyme-treated); and Group D vaccinated with 800 Gy gamma radiation-attenuated embryonated eggs, and challenged with the same number of eggs (vaccinated-challenged). Testicular tissues were stained with Haematoxylin and Eosin (H &E) for histopathological study. Periodic acid Schiff’s (PAS), bromophenol blue (BPB) and Feulgen’s reaction for carbohydrates, proteins and DNA, respectively were done to examine histochemical changes. Immunohistochemical study was done through expression of TGF-β1 and caspase-3. Infected control group B showed severe histopathological changes with marked decrease in PAS +ve materials, total proteins and DNA and enhanced expression of Transforming growth factor- β1 (TGF-β1) and caspase-3. Moderate changes were observed in testicular tissues of group C treated with thyme. Slight changes were detected in vaccinated-challenged group D. It was concluded that *Toxocara canis* infection causes marked hispathological, histochemical and immunohistochemical changes in testicular tissues of rats that can be ameliorated by vaccination with radiation-attenuated infective stage or treated with thyme; however vaccination is more effective in protection.

INTRODUCTION

Toxocaraisis is an important socio-economically zoonotic disease caused by ascarid roundworms, *Toxocara canis* and *T. cati* (Maleki et al., 2017). It leads to debilitating disease causing ocular larva migrans or visceral larva migrans resulting in tissue damage of multiple organs. It may remain asymptomatic until encysted larvae are reactivated in immunocompromised individuals leading to further migration with new symptoms (Kwon et al., 2017; Moreira et al., 2014).

Many cases of toxocarosis in human caused by ingesting embryonated eggs from soil or infective larvae from undercooked meat have been observed. Transmission of somatic larvae to the definitive hosts’ offspring plays an important role in the lifecycle of *Toxocara* species. Infection of the fetus may be induced placental or lactogenic transmission (Coati et al., 2004; Schnieder et al., 2011).
Vaccination using live-attenuated pathogen supports the hypothesis that activating multiple innate receptors is better than activating only one receptor (Vasou et al., 2017). Gamma radiation affects the protein and peptide compound of the DNA of living microorganisms. Subsequently, they lose their ability of multiplication and division (Amin & Hafez, 2015). An advantage of live attenuated vaccine is its potent immunogenicity because organisms keep to behave and can be recognized by immune system as a natural infection (Viljoen & Lukins, 2012).

It was realized that chemoprophylaxis is unsustainable due to increasing drug resistance. Elimination of internal and external parasites using Thyme, a natural remedy, has been recognized for centuries as an effective anti-microbial and anti-parasitic medication. It was used in the treatment of Entamoeba histolytica, Leishmania major, Trypanosoma brucei, and Trypanosoma cruzi (Behnia et al., 2008; Santoro et al., 2007). 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 (Chevallier, 2001).

Transforming growth factor-beta (TGF-β1) is a type of cytokine secreted by many cell types including macrophages. It plays a central role in immunity and controls proliferation, differentiation, and other functions in many cells (Schoenhoff et al., 2009). It was reported that it is an important factor for the local survival and function of T regulatory cells observed during T. canis invasion in the mouse small intestine, liver, muscle, and brain (Fan et al., 2013).

Caspases (cysteinyl aspartate-specific proteases) are a family of important signaling molecules with various tasks depending on the subtype and organ involved. Their activation is a marker for cellular damage in diseases such as stroke and myocardial infarction. Though the role of apoptosis in initiation and progression is unknown for all caspases, their involvement as an indicator alone and as a potential leverage point for drug research makes them widely researched molecules (Lavrlik et al., 2005).

This study aims to identify the histological, histochemical, as well as immunohistochemical changes that might occur in the testis of male rats due to infection with Toxocara canis and the effect of vaccination with irradiated infective stage or treatment with thyme on controlling these changes.

MATERIAL AND METHODS

Animals
Male albino rats weighing 100–160 gm. each of the same colonies were used. 80 rats were kept in the laboratory at room temperature and housed in cages (10 rats in each cage). They received a diet of standard rodent pellets produced by the Cairo Company for Oil and Soap. Ad libitum water and food were available.

Preparation of irradiated eggs of parasite
T. canis embryonated eggs are obtained from the uteri of female nematodes collected from the naturally infected dogs. They were incubated in 0.5% formalin solution at 28°C for 4 weeks then kept at +4°C until used (Galvin, 1964). Toxocara eggs were exposed to 800Gy gamma-radiations rays at a dose rate of 2.5 KGY/h at the time of experiment. This was performed at the National Center for Radiation Research and Technology (NCRRRT), Cairo - Egypt.

Thyme Oil Preparation
The essential oil of 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) (Elhabazi et al., 2006).

Infection of rats
The subjects are 80 rats divided into four groups (each 20 rats). The experiment was performed on the four groups; group A (normal control); group B infected orally with 2500 T. canis infective eggs/ml/rat
(infected control); group C infected with 2500 T. canis eggs and treated with thyme oil (thyme treated); and group D vaccinated orally with 800 Gy gamma radiation-attenuated embryonated eggs and re-infected (challenged) with the same number of eggs (vaccinated-challenged).

**Histopathology**

Animals of different groups were euthanized under anesthesia 30 days post infection and vaccination. Testis were carefully separated and washed in normal saline. Specimens were fixed in 10% phosphate buffered formalin (pH 7.4). Fixed materials were embedded in paraffin wax and sections of 5-micrometer thickness were cut. Slides were stained with Haematoxylin and eosin for histological examination. For histochemical demonstration of total carbohydrates periodic acid Schiff’s technique (PAS), total proteins bromophenol blue (BPB) and DNA stained by Feulgen’s were applied (Kiernan, 1981).

**Immunohistochemical Localization of TGF-β1**

Each paraffin embedded tissue section was deparaffinized with subsequent immersion in xylene and rehydration in solutions of decreasing ethanol (100, 95 & 80%). Endogenous peroxidase was blocked by incubating the tissue sections in 3% hydrogen peroxide (H2O2) (Sigma) at room temperature for 10-15 min. slides were washed twice with phosphate buffered saline (PBS) solution 5 min each. Subsequently, the tissues were submitted to heat induced antigen retrieval protocols. The slides were submerged in a 10 mm sodium citrate buffer at pH 6.0 and incubated in 830-W microwave oven for not less than 15 minutes.Slides were left to cool were incubated in PBS solution twice, 5 min each. A set of immunohistochemical detection kit was employed to detect the TGF-β1 by incubating each tissue section with 100 µl of the ready to use goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Thermo n Scientific, Cat.N: 36000) in a humidified chamber for 10 minutes. Then, slides were washed twice with PBS solution, 5 min each. Thereafter, the sections with oxidase was identified by the chromogen 3, 3-diaminobenzidine (DAB) and incubated in a humidified chamber for 10 minutes which turned to be brownish. Then the slides were washed with tap water. Sections were counter stained with Harris hematoxylin (Vector Laboratories, Burlingame, CA, USA), dehydrated and mounted with mounting medium (Neomarkers). Furthermore, in order to ascertain the specificity of the tissue staining, negative and positive control sections were treated as above to evaluate results (Wu, 2008).

**Caspase-3**

A portion of each testicular tissue was fixed in Bouin’s solution and processed in paraffin for immunohistochemical examination by caspase-3 stain. Sections were mounted on ploy-L-lysine coated slides. The streptavidin-biotin-peroxidase method was performed. Primary monoclonal antibody against caspase-3 (1:100 dilutions, Neomarkers, Fremont, USA) was used on deparaffinized sections. Endogenous peroxidase activity was blocked by 0.3% solution of hydrogen peroxidase in phosphate buffered saline (PBS) at room temperature for 10 min. After microwave treatment, primary antibody was applied for 30 min at room temperature and washed in PBS. Linking antibody and streptavidin-peroxides complex were added consecutively for 10 minutes at room temperature and then washed by PBS. The peroxidase activity was visualized by diaminobenzidine (Sigma, St. Louis, USA) which is applied for 5 minutes. The appropriate positive and negative controls were also labeled with the primary antibody. Positive cells for caspase-3 immune stain show intracellular brown punctuations. (Haque et al., 2009).

**RESULTS**

**Histopathological investigations**

Figure 1 reveals that Group “A” (normal control) showed normal seminiferous
Figure 1. (a) Control group showing normal testicular tissue of rat. (b) Testis section of infected control rat showing distorted ST with irregular outlines (→). Tubular atrophy and increased intertubular spaces (*) are observed. Cytoplasmic maculation (V) of germ cells and sloughed (S) necrotic cells are seen. Interstitial dilated capillaries (C) and degenerated Leydig cells (L) are observed. (c) Another testis section of the same group showing detachment of germ cells (*) from basement membrane and exfoliated cells (Ex) in tubular lumen note vacuolated (V) germ cells and gaps formation (→) in-between. The interstitium shows excess fibrous tissue (F) in and around blood capillaries. (d) Testis section of thyme-treated rat showing gradual appearance of spermatogenic cells within ST. However, some tubules (T) show disarrangement and reduction of germ cells. Enlarged intertubular spaces (*) and atrophied Leydig cells (L) are still observed. (e) Testis section of vaccinated rat showing restoration of normal histological structure in most seminiferous tubules (ST). (f) Another testis section of the same group showing regenerative changes in many ST while in others partial separation of germ cells (*) is present. Also, the interstitial spaces show some exudates (E) and fibrous thickening (F) of blood capillaries (H&E X200).
tubules (ST) embedded in a loose connective tissue stroma containing interstitial Leydig cells (a). Tubular atrophy, distorted ST with increased intertubular spaces, sloughed necrotic cells, vacuolation of cytoplasm and degenerated Leydig cells was detected in Group “B” (Control infected). Also, interstitium showed excess fibrous tissue in and around blood capillaries (b, c). Group C (thyme treated) demonstrated disarrangement and reduction of germ cells with atrophied Leydig cells; however gradual appearance of spermatogenic cells within ST were detected (d). Group “D” (vaccinated-challenged) showed restoration of normal histological structure in most seminiferous tubules with regenerative changes in many ST, while in others partial separation of germ cells is present (e, f).

**Histochemical results**

**Polysaccharides (PAS + ve materials) (Fig. 2)**

The total polysaccharides exist in the form of intense coarse deeply stained pink granules in the cytoplasm of germ cells of normal group A (a). In infected control group B revealed marked decrease of PAS granules in germ cells (b). Testis section of thyme-treated rat (group C) showed moderate content of PAS +ve materials (c). Thymus section of thyme-treated rat (group C) showed moderate content of PAS +ve materials (c). Thymus section of thyme-treated rat (group C) showed moderate content of PAS +ve materials (c). Thymus section of thyme-treated rat (group C) showed moderate content of PAS +ve materials (c).

Figure 2. (a) Testis section of normal control rat showing reddish deeply stained granules of PAS +ve materials. (b) Section of infected control rat showing decreased stain affinity of PAS. (c) Thyme-treated rat showing moderate content of PAS +ve materials. (d) Vaccinated-challenged rat showing nearly normal content of PAS +ve materials (X200).
Vaccinated-challenged group D revealed nearly normal content of PAS +ve materials (d).

**Total proteins (Fig. 3)**

In normal control rats (group A), total protein appeared as deeply stained blue granules in the cytoplasm of germ cells of the seminiferous tubule (a). Marked reduction in total protein was recorded in the in control infected group B (b) while thyme treated group C showed moderated reduction of stained protein granules (c). Testis section of vaccinated rat (Group D) showed restoration of normal amount and distribution of protein materials (d).

**DNA content (Fig. 4)**

In group A (normal control), DNA-chromatin particles appeared as dense granules positively stained with Feulgen's reaction in all layers of spermatogenic cells (a). Rats of group B (infected control), the nuclei of infected cells had weakly stained chromatin bodies denoting few DNA content (b). Thyme treated group C revealed that DNA content was moderately reduced (c). On the other hand, testis sections of vaccinated rat showing strong reaction of DNA content of most germ cells (d).

Effect of thyme oil treatment or vaccination with gamma radiation-attenuated infective stage on testicular PAS,

![Figure 3. Testis sections of normal control rat showing dense stain ability of protein materials in and around seminiferous tubules (a). Sections of infected control rats showing highly decreased stain ability of protein materials (b). Thyme-treated group rats showing moderate content of total proteins (c). Vaccinated-challenged group showing restoration and distribution of normal amounts of protein materials (d) (Bromophenol blue, X200).](image-url)
Figure 4. Testis section of normal control group rat showing red color of DNA particles in all layers of spermatogenic cells (a). Testis sections of infected control rat showing reduction of DNA content (b). Thyme-treated rat showing moderate reaction of DNA content (c). Vaccinated-challenged group of rats showing strong reaction of DNA content of most germ cells (d) (Feulgen, X200).

Table 1. Effect of vaccination with 800 gray gamma radiation-attenuated eggs or thyme oil treatment on testicular PAS, total proteins and DNA contents in rats of experimental groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group A Normal control</th>
<th>Group B Infected control</th>
<th>Group C Thyme treated</th>
<th>Group D Vaccinated-challenged</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS mg/g tissue</td>
<td>1447.4±104.3</td>
<td>290.6±30.5a</td>
<td>592.6±38.9a</td>
<td>814.4±62.2b,a</td>
</tr>
<tr>
<td>Total proteins mg/g tissue</td>
<td>1240±15.3</td>
<td>250.6±17.9a</td>
<td>666±24.1a,a</td>
<td>923.4±32.9b,a</td>
</tr>
<tr>
<td>DNA</td>
<td>854.6±60.7</td>
<td>82.9±5.3a</td>
<td>218.2±14.7a</td>
<td>613.4±43.8e,a</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE. P values a < 0.001; b < 0.01; c < 0.5 and n.s non-significant compared to control normal group. P values a1 < 0.001; b1 < 0.01; c1 < 0.5 and n.s. non-significant compared to control infected.

total proteins and DNA contents was shown in Table 1. The PAS, total protein and DNA contents were high significantly (P < 0.001) suppressed in infected group (B) while this content was significantly suppressed (P < 0.01) in thyme-treated group C. On the other hand, vaccinated-challenged group D showed non-significant decrease as compared to normal control group A.
**Immunohistochemical results**

Immunohistochemical results displayed in (Table 2, Fig. 5) revealed expression of Caspase-3 in normal control group “A” (66.9±4.2). On the other hand, dense deposition of Caspase-3 was recorded in infected control group “B” (383.5±86.3) with high significant difference (P<0.001). In group “C” (Thyme treated), the positive traces were reduced compared to group “B” (178.2±27.3). Vaccinated-challenged group C showed weak deposition of collagen (87.1±18.4).

Regarding expression of TGF-β1 in normal control group A was recorded by 50.6±9.2. In infected control group B, Table 2. Immunohistochemical changes in testis of rats of experimental groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group A (Normal control)</th>
<th>Group B (Infected control)</th>
<th>Group C (Thyme treated)</th>
<th>Group D (Vaccinated-challenged)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3</td>
<td>66.9±4.2</td>
<td>383.5±86.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>178.2±27.3&lt;sup&gt;b,a₁&lt;/sup&gt;</td>
<td>87.1±18.4&lt;sup&gt;c,a₁&lt;/sup&gt;</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>50.6±9.2</td>
<td>755.9±40.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>310.2±22.9&lt;sup&gt;a,a₁&lt;/sup&gt;</td>
<td>90.1±4.5&lt;sup&gt;b,a₁&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE. Number of mice in each group is ten.
P values a < 0.001; b < 0.01; c < 0.5 and n.s non-significant compared to control normal group.
P values a₁ < 0.001; b₁ < 0.01; c₁ < 0.5 and n.s.1 non-significant compared to control infected group.

Figure 5. (a) Immunohistochemical staining of Caspase-3 in group A showing no expression. (b) staining of Caspae-3 in group B showing strong positive expression. Group C showing moderate positive expression (c) while, group D showing weak positive expression (d) (indicated by brown colour x400).
strong densely TGF-β1-stained cells which could be distinguished by their brownish color (755.9±40.3). Group C (thyme treated) revealed that intensity of positive traces was reduced compared to group B (310.2±22.9) while vaccinated – challenged group D few dispersed brown traces were detected (90.1±4.5) (Table 2, Fig. 23-26).

**DISCUSSION**

Host sex has been reported to influence the development of parasitic infections resulting in different parasite growth or pathophysiological effects (Poulin, 1996). Santos *et al.* (2017) revealed clear differences in migration pattern of *T. canis* larvae according to the sex of *R. norvegicus* with greater larval elimination in male rats than female due to hormonal influences. Also, Harder *et al.* (1992) reported that rodent males would be more susceptible to parasitic infections because of the deleterious effect of testosterone which impairs the hosts’ immunological response.

Previous studies reported presence of disseminated larvae of some nematodes in testicular tissues of experimentally infected animals. Chabaud (1972); Ruiz & Morera (1983) reported ectopic localizations of *Angiostrongylus costaricensis* nematode causes testicular lesions. Granulomatous inflammatory reaction, heavy eosinophilic infiltration with extensive parenchymal hemorrhagic necrosis and worms obstructing the arteries of the spermatic...
cord were also detected. Leccia et al. (2012) reported two scrotal cases of *Dirofilaria repens* and revealed that although its infection presents clinically as subcutaneous and conjunctival nodules, lesions involving the male sexual organs (scrotum, epididymis, spermatic cord) were also detected. As lesions may mimic malignant tumors, diagnosis is based on histological examination. Some protozoa infection as *Toxoplasma gondii* was reported not only affects female reproduction but also causes male reproductive impairment. High prevalence of toxoplasmosis in sterile men has been reported (Dalimi & Abdoli, 2013).

Histopathological study in control infected group revealed marked inflammatory reaction with distorted, atrophy and increased intratubular spaces between seminiferous tubules. This complies with the work done by Moawad et al. (2015) who reported degenerative changes and congestion in renal parenchyma of *Toxocara canis* infected rats. Amin & El-Kabany (2013) reported histopathological changes in liver tissues caused by infection with *T. vitulorum*. Also, Janecek et al. (2014) studied histopathological changes in brain of model host orally infected with *T. canis* and *T. cati* and revealed structural damage, demyelination and perivascular lymphocytosis.

In the current study, severe depletion of total polysaccharides and protein was observed in germ cells of the seminiferous tubule in infected control group. DNA particles were markedly reduced in the nuclei of infected germ cells. Eid et al. (2015) observed the deposition of PAS-positive material in the walls of blood vessels in brain of mice infected with *Toxocara canis*. Moawad et al. (2016) reported that histochemical alterations were not only restricted to the site of infections but also to various parts of organ tissues. This is due to parasite replication, invasion and secretion of some toxins which may reduce the immune response. Brossier et al. (2005) reported that some toxins may cause disruption in glucokinase activities and subsequently defectiveness in the process of glycogenesis. Acceleration of both hexokinase and phosphorylase activities promote glycolysis and glycogenolysis. Kuzna-Grygiel & Kolodziejczyk (2000a) found that increasing activity of oxidative enzymes in parasitic infection can be considered as a source of highly reactive free radicals “hydroxyl radicals”. This leads to damages of cellular membranes, genetic material, and death of the host cells. Excess of superoxide radicals interact with hydrogen peroxides and organic peroxides with generation of highly reactive entities that can attack DNA, membrane lipids and other essential cell components.

Immunohistochemical staining in testis of infected control group revealed strong positive expression of TGF. This was in according to Oshiba & Aiad (2016) who reported positive staining of TGF-β1 immunohistochemistry in small intestine of *Toxocara canis* infected mice. Wu et al. (2008) detected TGF-β1 mainly in infiltrating leukocytes in hepatic lesions with strong expressions due to infection with *Toxocara canis* eggs. It was recorded that TGF-β1 is an important factor for the local survival and function of T regulatory (Treg) cells observed during *T. canis* invasion in the mouse small intestine, liver, muscle, and brain (Fan et al., 2003).

Regarding caspase-3, it showed strong positive expression in control infected group that was in according to Wesley et al. (2013) who detected significant expression of cleaved caspase-3 in *T. canis* larval excretory/secretory antigen.

Thyme treated group revealed moderate alterations in all histopathological, histochemical and immunohistochemical changes in testis. This was in accordance to results exhibited by Amin et al. (2016) showing significant decrease in larval count and nitric oxide level with less damage in brain cells of thyme treated compared to control infected group. Caldera et al. (2013) reported that Thyme administration before and after *Toxocara vitulorum* infection resulted in a significance reduction of larval burden compared to infected group.
Current work revealed that most testicular cells in vaccinated challenged group showed mild changes compared to control infected group and also to thyme treated group. This agreed with those found by Moawad et al. (2015) reported that vaccination with 800Gy attenuated larvae showed amelioration in all histopathological, biochemical and hematological changes in kidney of Toxocara canis infected rats. Also Amin et al. (2016) revealed that radiation-attenuated vaccine using 800 Gy gamma rays irradiated embryonated eggs of T. canis-infected rats caused a decrease in larval count, NO level and improvement in the histopathological lesions and DNA fragmentations as well as damage in brain tissues of infected rat.

CONCLUSION

It is concluded that Toxocara canis infection affect rat testis causing hispathological, histochemical and immunohistochemical changes that can be ameliorated by vaccination with infective stage irradiated with 800Gy gamma radiation or with thyme-treatment. However, vaccination is more effective in protection. It might be useful if future researches study the effect of Toxocara infection on host fertility.

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Declaration of interest

The authors declare that they have no conflict of interest.

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