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Pathogenic Potential of Fresh, Frozen, and Thermally Treated *Anisakis* spp. Type II (L3) (Nematoda: Anisakidae) after Oral Inoculation into Wistar Rats: A Histopathological Study

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Abstract: The third-stage (L3) larvae of *Anisakis* are the etiological agents of human anisakiasis caused by consumption of raw or undercooked seafood infected with anisakid nematodes. Infection with these worms is associated with abdominal pain, nausea, and diarrhea and can lead to massive infiltration of eosinophils and the formation of granulomas in the gastrointestinal tract if the larvae are not removed. Food allergy affects populations worldwide, and despite several reports on the presence of the potentially zoonotic nematodes among edible fishes in Egypt, there are few immunological and molecular studies investigating the epidemiology of these parasites. Anisakidosis, a human infection with nematodes of the family Anisakidae, is caused most commonly by *Anisakis* spp. In the present study, seventy specimens of the European seabass *Dicentrarchus labrax* commercialized in Alexandria city along the Mediterranean Sea were acquired during the period from July to December, 2015. Fish were necropsied and dissected to investigate the presence of nematode larvae. Thirty fish (42.9%) of the total were parasitized by nematode larvae which were morphologically identified as *Anisakis* spp. Type II (L3) according to light and scanning electron microscopy. The pathogenic potential of oral inoculation of fresh, frozen, and thermally treated larvae into Wistar rats was elucidated by histological examination of their thymus and spleen. Results obtained indicated that neither cooling nor freezing of the parasite could destroy their allergenic capacity. So, it is important to create a wider awareness of this potential risk to human health. It is becoming increasingly likely that the impact of *Anisakis* spp. on human health has been underestimated, and it is perhaps time to consider more sweeping measures than those currently enforced to protect the public health.
Key words: Anisakis spp. Type II (L3), histopathology, host–parasite relationship, spleen, thymus, Wistar rats.

Parasitic nematodes comprise one of the largest and most diverse groups of parasitic helminthes mostly of fresh, brackish, and marine water fishes. Heavily infected fish show emaciation, imbalanced swimming, and reduction of their vitality (Klimpel et al., 2011; Mehlhorn et al., 2011; Morsy et al., 2013, 2015; Abdel-Ghaffar et al., 2014, 2015). Anisakiasis is a fish-borne parasitic disease caused by consumption of raw or undercooked fish or cephalopods parasitized by *Anisakis* spp. third stage larvae (Takahashi et al., 1998; Chai et al., 2005). Symptoms of acute gastric infection include sudden epigastric pain, nausea, and vomiting that can occur 1–12 hr after the infected meal. Anisakiasis is often associated with a strong allergic response, with clinical symptoms ranging from isolated swellings to urticaria, angioedema, and life threating anaphylactic shock (Audicana et al., 2002; Baeza et al., 2005). The third stage (L3) larvae-infecting fish are arrested in their development until ingested by some sea mammals (seals and dolphins), whereupon they develop through two more stages until the adulthood is achieved. The first recorded human infection by a member of the family Anisakidae (*Anisakis* spp.) was reported in the Netherlands by Van Thiel (1962) who described the presence of a marine nematode in the center of an eosinophilic intestinal phlegmon of a patient suffered from acute abdominal pain as a "very unusual finding." Later, this nematode was identified as *Anisakis* spp., and the human parasitosis was named anisakiasis (Van thiel, 1962). Gutting the fish as soon as possible after capture is thought to prevent the migration of the parasite into the muscle tissues, thereby reducing the contamination of the edible parts of the fish (Smith, 1984). Although cooking at 70°C or freezing to −20°C for 72 hr is believed to destroy the infectivity of such parasites, the allergenic capacity of its denaturized protein is not eliminated (Caballero and Moneo, 2004). The secretory antigens produced by the worms can induce specific antibodies even following ingestion of fully cooked infected fish containing *Anisakis* antigens (Audicana et al., 1995). It has been postulated that *Anisakis* allergy may be more prevalent than any specific food allergy in the adult human population and comprises as much as 10% of idiopathic anaphylaxis (Baeza et al., 2005). Human anisakid infections frequently cause gastrointestinal symptoms which may be associated with mild to severe immunological responses, usually allergic-type reactions (Yazdanbakhsh et al., 2002). In the present study, the fish *Dicentrarchus labrax* was examined for the prevalence and morphological analysis of *Anisakis* spp. larval infection. Also, the pathogenic potential of intragastric administration of fresh, frozen, and thermally treated larvae inoculated into Wistar rats was a main objective of the study through histological examination of their thymus and spleen.

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Materials and Methods

Fish examination and parasitological study: Seventy specimens of the European seabass *Dicentrarchus labrax* (family: Moronidae) were collected during the period

from July to December, 2015 from boat landing sites and fishermen at the coasts of Alexandria city along the Mediterranean Sea in Egypt. Live fish were transported to the laboratory of Parasitology, Zoology Department, Faculty of Science, Cairo University; they were identified according to Randall (1992). Nematodes (Fig. 1A,B) were collected as encapsulated larvae from the surface of visceral organs such as the stomach, intestine, and muscles; subsequently, they were rinsed in phosphate-buffered saline for further experiments. For morphological characterization by light microscopy, some of the collected worms were fixed in hot 70 % ethanol (60°C).

Scanning electron microscopy: Parasite specimens were prepared according to Madden and Tromba (1976); worms were fixed in 3% phosphate-buffered glutaraldehyde (pH 7.3) for 18–24 hr, washed in the same buffer, and dehydrated in ascending alcohol series. After passing through an ascending series of Genosolv-D, they were processed in a critical point drier "Bomer-900" with freon 13 and sputter coated with gold–palladium in a Technics Hummer V and examined with an Etec Autoscan at 20 kV Jeol scanning EM.

Experimental study: Thirty-two male Wistar rats, 6–8 wk old and 130–150-g weight provided from (National Research Centre animal house, Cairo, Egypt) were kept in suitable cages with perforated covers at a temperature of 22 ± 2 °C, 55% ± 5% humidity, and 12-hr dark/light cycle. Rats were fed on standard food and water *ad libitum*. Animals were determined to be free of parasites as shown by repeated stool examination and applying direct wet saline smear and flotation techniques. All experimental procedures were performed following the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, USA.

Rats were divided into four groups (eight rats each):

Group I (control): rats were orally inoculated with saline.
Group II: rats were orally inoculated with freshly collected L3 larvae.
Group III: rats were orally inoculated with 1 wk previously frozen L3 larvae at −20°C.
Group IV: rats were inoculated orally with thermally treated L3 larvae in 100 °C water bath for 10 min.

Gastric intubation method: After washing the isolated worms from the host fish with 0.85 NaCl, they were transported into a petri dish containing 5 ml of 50 mM HCl solution. Larvae were orally inoculated into rat stomach by the gastric intubation method according to Figueiredo et al. (2012). This step was applied under anesthesia of rats with sodium pentobarbital (50 mg/kg).

The experimental rat groups II, III, and IV were inoculated with L3 larvae according to the time line as shown in Figure 1.

After the end of the experiment, rats were euthanized under an overdose of sodium pentobarbital (100 mg/kg) after 14 d of the second administration.

Histological examination: The thymus and spleen from the experimental rat groups were isolated, washed, and fixed in 10% buffered formalin until sectioned. Tissues were dehydrated in series of alcohols, cleared in xylol, embedded in paraffin wax, and sectioned by a microtome at 5 μm thick. The tissue sections were processed and stained with eosin and hematoxylin according to Drury and Wallington (1967). The stained sections were examined and photographed using a Zeiss research photomicroscope (Carleton, 1967) supplied with a Canon digital camera.

Image morphometry: The morphometric analysis was performed at the Pathology Department, National Research Center, using routine hematoxylin and eosin stained slides and a Leica Qwin 500 Image Analyzer (LEICA Imaging Systems Ltd., Cambridge, England) which consists of a Leica DM-LB microscope with a JVC color video camera attached to a computer system Leica Q 500IW.

Statistical analysis: Kolmongrov–Smirnov analysis confirmed that the data obtained for the thymus and spleen from the experimental rat groups were non-normally distributed and consequently analyzed by Kruskal–Wallis

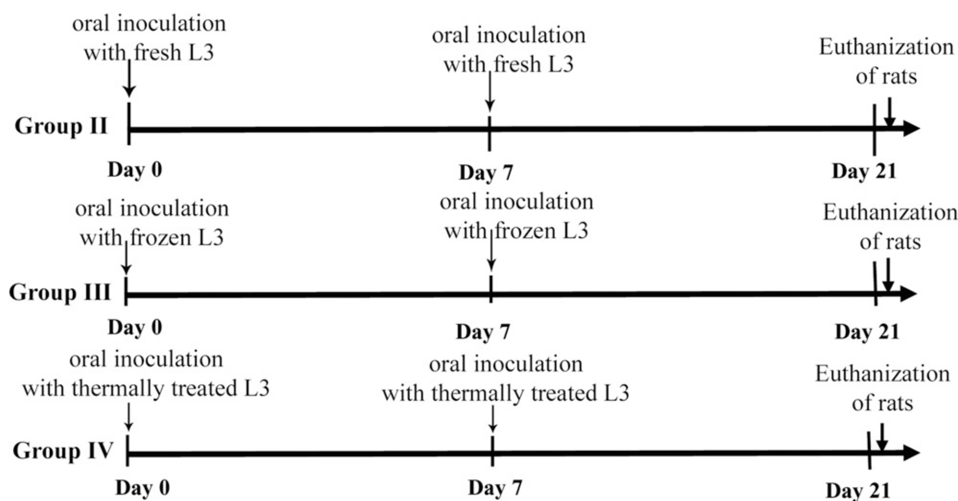

Fig. 1. Timeline of the experiment showing inoculation and euthanization days.

analysis and Mann–Whitney U test. Data were expressed as mean ± SEM.

Results

Morphological characterization of Anisakis Type II (L3): Thirty fish specimens of seventy (42.9%) were found to be naturally infected by nematode worms as encapsulated larvae recovered from the peritoneal cavity of the infected fish with an intensity ranged from 13 to 20 worms per fish (Fig. 2A,B). Morphological and morphometric characterization generally showed that all the recovered and examined parasite specimens closely resembled *Anisakis* spp. Identification was performed according to the key published by Rocka (2004). The recorded larvae (Fig. 1C–H) had a slender body 0.6–3.5 cm long and 0.3–1 mm wide. Prominent boring teeth were observed at the anterior extremity of the mouth area with four small papillae (two dorsolateral and two ventrolateral) surrounding the triangular mouth. The

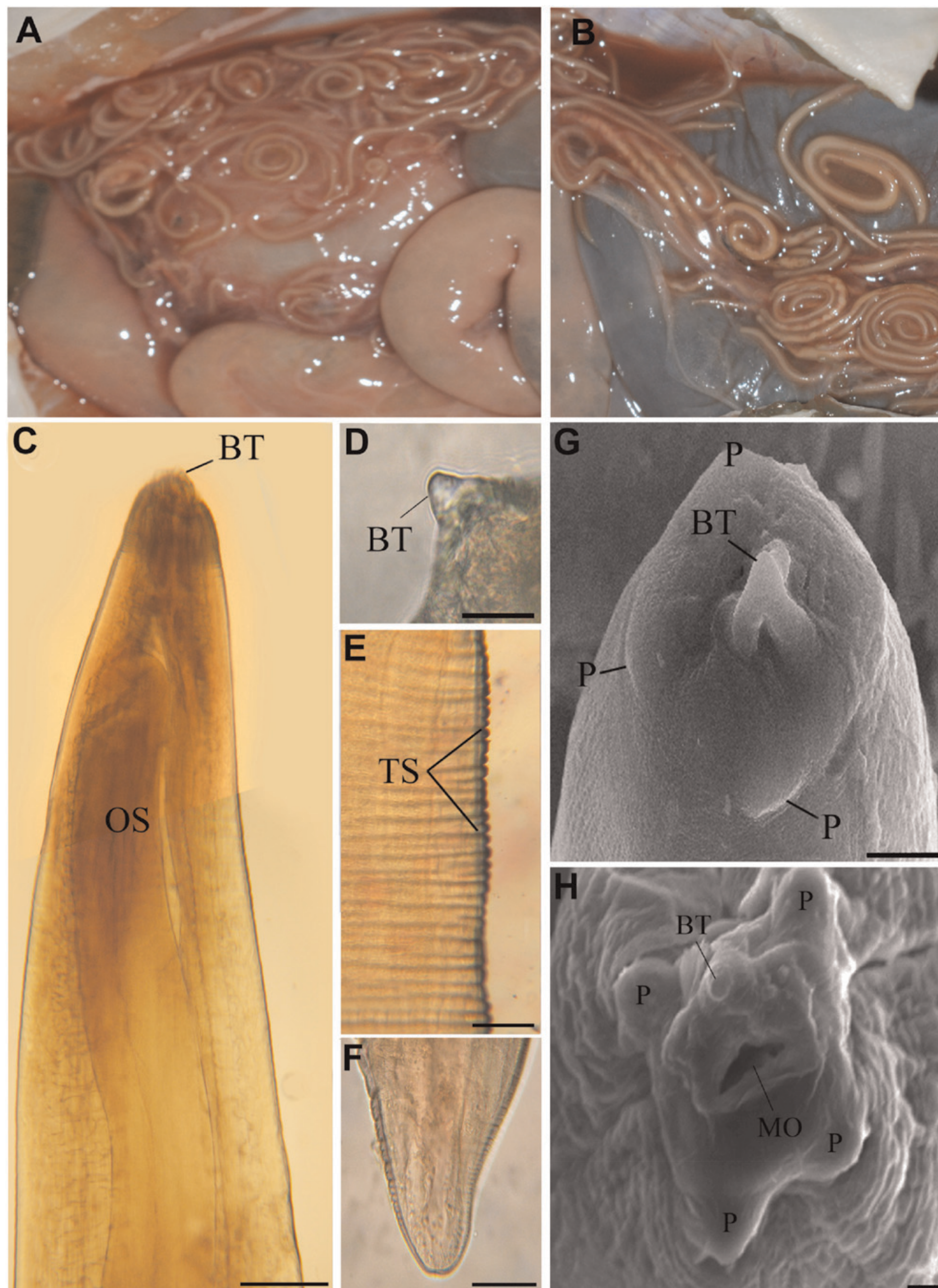

Fig. 2. A, B. Photographs of *Dicentrarchus labrax* showing the heavy infestation with *Anisakis* spp. Type II (L3) in the peritoneum and visceral organs of infected fish. C–F. Photomicrographs showing high magnifications of the worm L3: C. The anterior part with a boring tooth (BT) and esophagus (OS). D. The boring tooth (BT). E. Transverse striations (TS) of the cuticle. F. The posterior part of the worm. G, H. Scanning electron micrographs of *Anisakis* spp. Type II (L3) showing the anterior part of the worm body, with a boring tooth (BT) and four papillae (P) surrounding the triangular mouth (MO), 10 μm. (Scale bars: C = 100 μm; D–H = 10 μm).

cuticle had transverse striations that started from the cephalic region and extended to the anus. The postanal tail was rounded without a terminal mucron.

Thymus: Light microscopic examination of the histological sections from the control rat group treated with saline revealed the normal size of thymus (Fig. 3A,B) with a normal distribution of thymus lobules. The ratio between cortex and medulla was normal (2: 1). Rare apoptotic bodies were seen in some control sections.

Histological examination of thymus sections of the experimental rat group orally inoculated with freshly isolated larval worms (Fig. 3D,F) revealed their atrophy (Fig. 3D) and a striking tinctorial change which was obvious especially in the cortical region because of marked apoptosis of the lymphocytes in this region. However, there was a robust recovery of the medullary thymocytes evidenced by the darker medullary region. The cortical–medullary ratio in these sections was difficult to be determined. Focal hemorrhages and more apoptotic lymphocytes were seen in the examined sections (Fig. 3E,F).

Thymus sections of the experimental rats orally inoculated with frozen larvae (Fig. 4A–D) showed a dramatic decrease in the overall size of the organ (Fig. 4A)

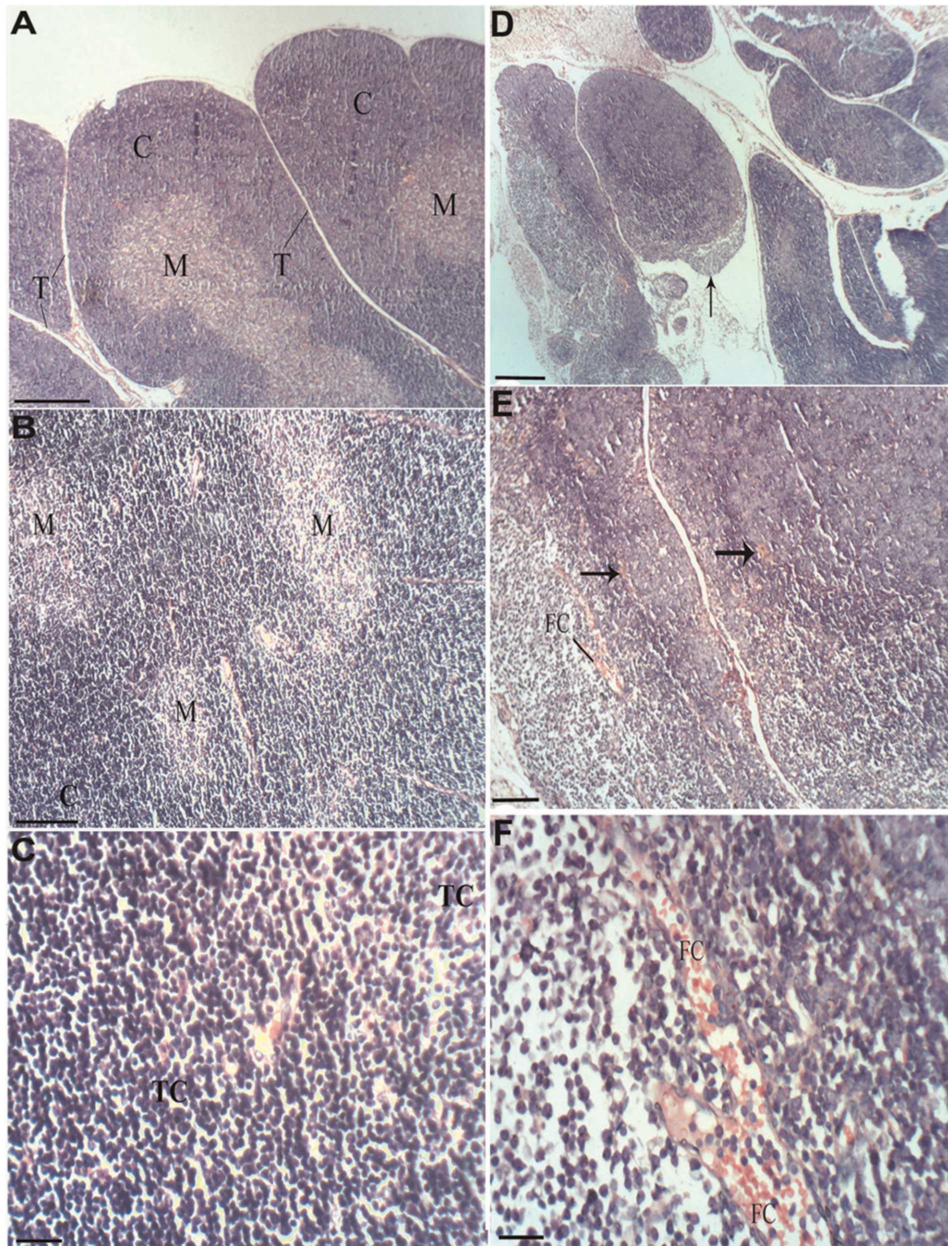

Fig. 3. A–C. Photomicrographs of sections in thymus (H&E) from control rats (saline treated) showing A. the normal architecture of thymus lobules, separated by trabeculae (T), with outer cortex (C) and inner medulla (M). B, C. High magnifications with rare tinctorial change. D–F. Photomicrographs of sections in thymus (H&E) from rats orally inoculated with freshly isolated L3 of *Anisakis* simplex showing D. atrophy of the thymus (arrow) and striking tinctorial change in the cortical region because of marked apoptosis of the lymphocytes. E,F. lymphocytic necrosis, depletion (arrows), and focal hemorrhage (FC). (Scale bars: A, D = 500 μm; B, E = 200 μm; C, F = 50 μm).

and were severely affected by necrosis and inflammation of the cortical tissue in addition to focal hemorrhage (Fig. 4B,C). Also, it was observed that several apoptotic lymphocytes with intracytoplasmic apoptotic bodies were spread in the medulla region (Fig. 4D).

In contrast to the control, histological sections of thymus isolated from the experimental rat group orally inoculated with thermally treated larvae (Fig. 4E–G) showed no marked change in the size of the organ (Fig. 4E). However, a decreased cellularity of the medulla region led to difficulty in determining the ratio between cortex and medulla (Fig. 4F). Oncotic necrosis and lymphocyte depletion were observed in the cortical region in addition to the presence of a large number of apoptotic bodies, blood vessel congestions, and hemorrhages (Fig. 4G).

Spleen: Histological sections from the spleen of control rats (Fig. 5A–C) inoculated orally with saline showed its normal architecture with a normal ratio between white and red pulps (Fig. 5A,B). Also, the periarteriolar lymphatic sheath surrounding artery was observed with a normal distribution. The white pulp was dark because of the presence of predominant small lymphocytes and lymphoid follicles. Rare apoptotic cells were observed (Fig. 5C) and no congestion or hemorrhages were seen in the examined sections.

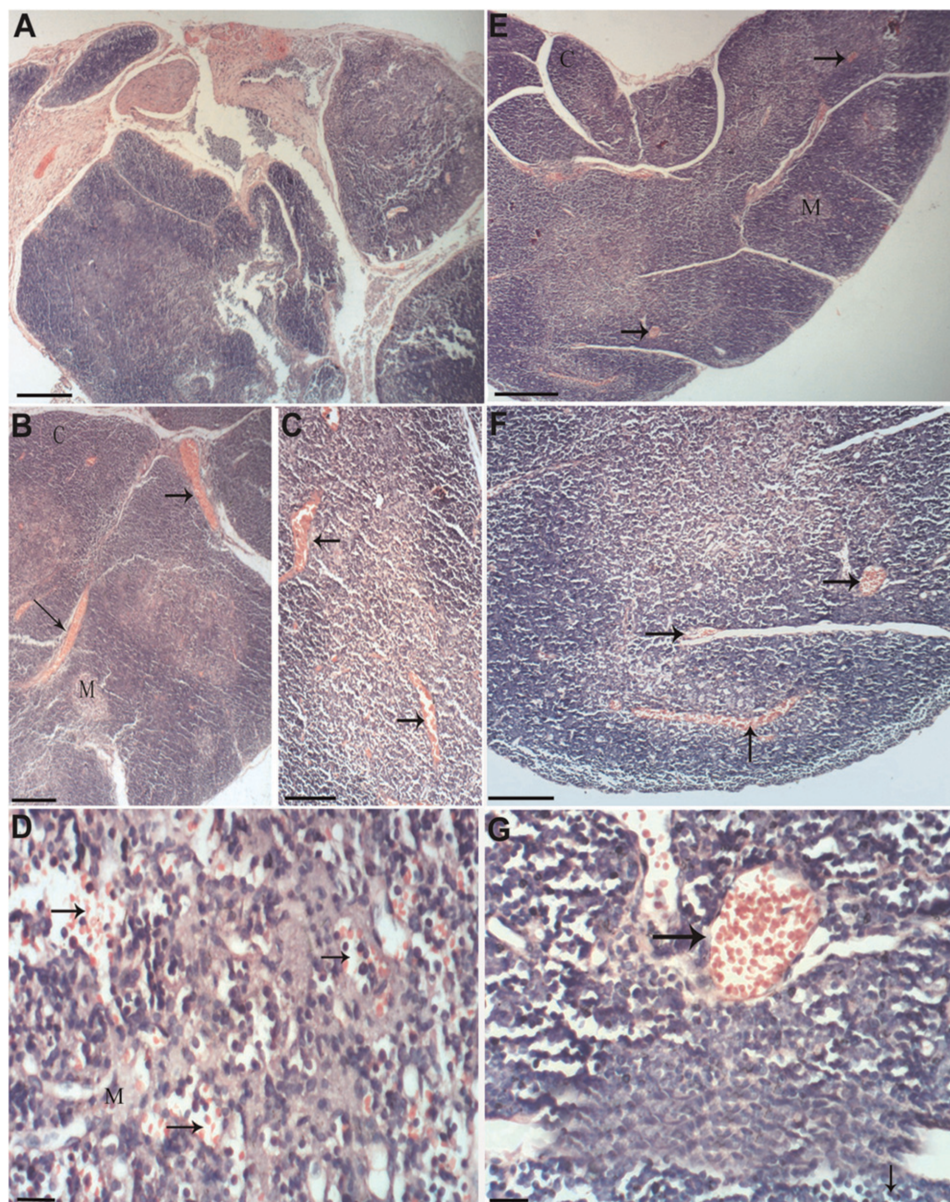

Fig. 4. A–D. Photomicrographs of sections in thymus (H&E) from rats orally inoculated with frozen *Anisakis* spp. Type II (L3) showing: A–C. Necrosis and inflammation of the cortical tissue and congested blood vessels (arrow). D. Lymphocytic depletion (arrows). E–G. Photomicrographs of section in thymus (H&E) from rat orally inoculated with thermally treated L3 showing: E. Decreased cellularity in the medulla region leading to the difficulty of determining the ratio between cortex and medulla and congested blood vessels (arrow). F, G. congested blood vessels (arrow). (Scale bars: A, E = 500 μm; B, C, F = 200 μm; D, G = 50 μm).

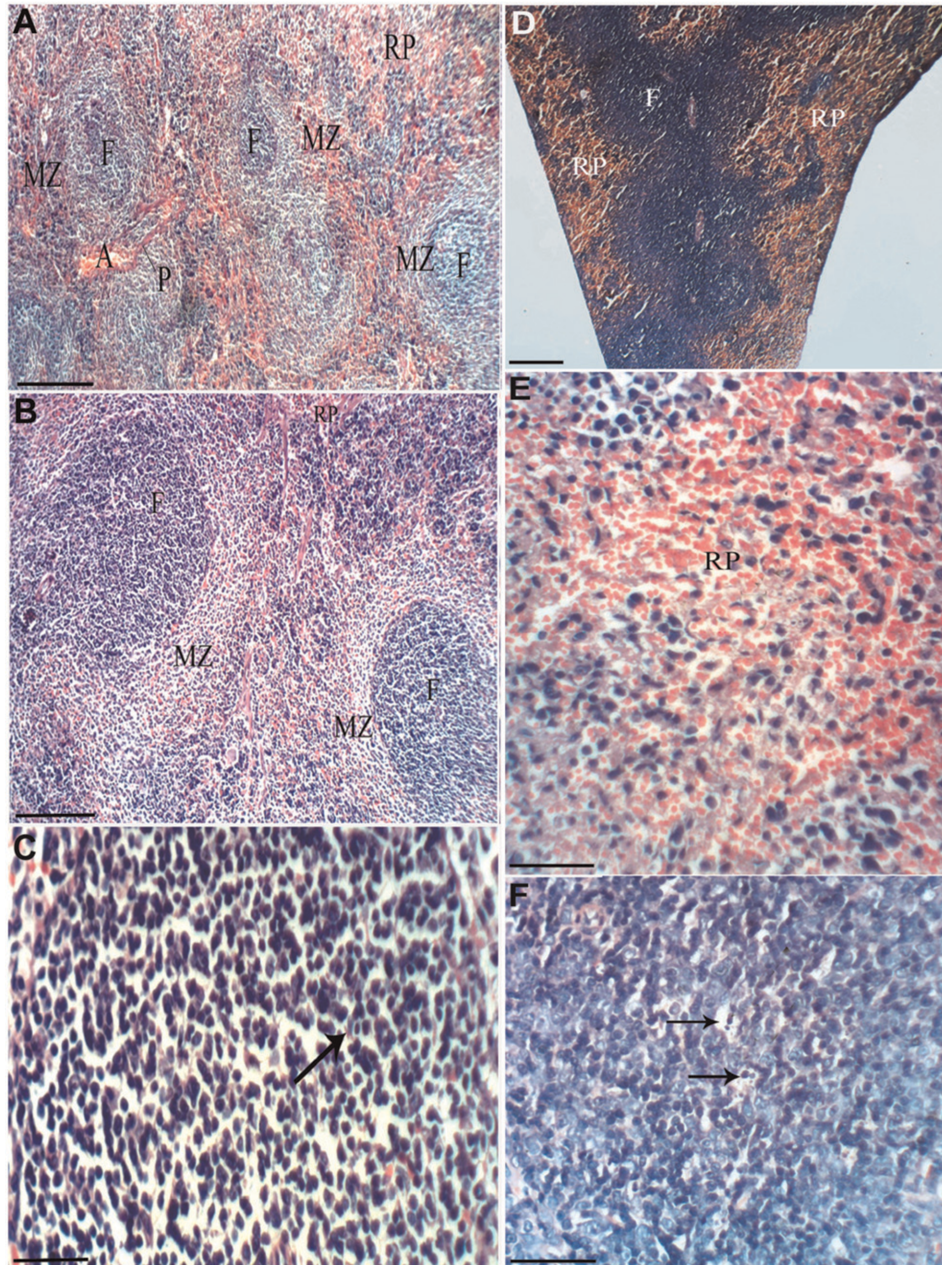

FIG. 5. A–C. Photomicrographs of the spleen sections (H&E) from control rats (saline treated) showing A, B. the normal architecture of the spleen tissue with red (RP) and white pulps; the white pulp is composed of PALS area (P) surrounding central artery, the lymphoid follicle (F), and the marginal zone (MZ) surrounding the follicle. C. A high magnification, apoptotic bodies were observed (arrow). D–F. Photomicrographs of the spleen section (H&E) from rats orally inoculated with freshly isolated L3 showing D, E. congestion of red pulp (RP). F. Lymphocytic necrosis and depletion (arrow). (Scale bars: A = 300 μm; B = 200 μm; D = 500 μm; C–F = 50 μm).

Examination of the spleen sections from the rat group orally inoculated with freshly isolated larval worms of *Anisakis* spp. Type II (Fig. 5D–F) revealed a severe red pulp congestion (Fig. 5D,E) with a severe recovery of lymphocytic cells in the periarteriolar lymphoid sheath (PALS) in addition to the distribution of large number of apoptotic cells with aggregates of pigmented materials of apoptotic bodies (Fig. 5F).

The spleen sections from the experimental rat group orally inoculated with frozen larvae (Fig. 6A–D) revealed different grades of lymphoid follicular atrophy; follicles were with indistinct germinal center (Fig. 6A). Also, severe congestion of the red pulp and lymphocyte infiltrations in the PALS were observed (Fig. 6A,B). Macrophages with aggregates of pigmented materials in addition to lymphocyte necrosis (Fig. 6C) were also observed in the examined sections.

The histological examination of the spleen sections from the experimental rat group orally inoculated with thermally treated larvae revealed an abnormal distribution of white and red pulps accompanied with severe red pulp congestion (Fig. 6E-G). The number of lymphocytes in

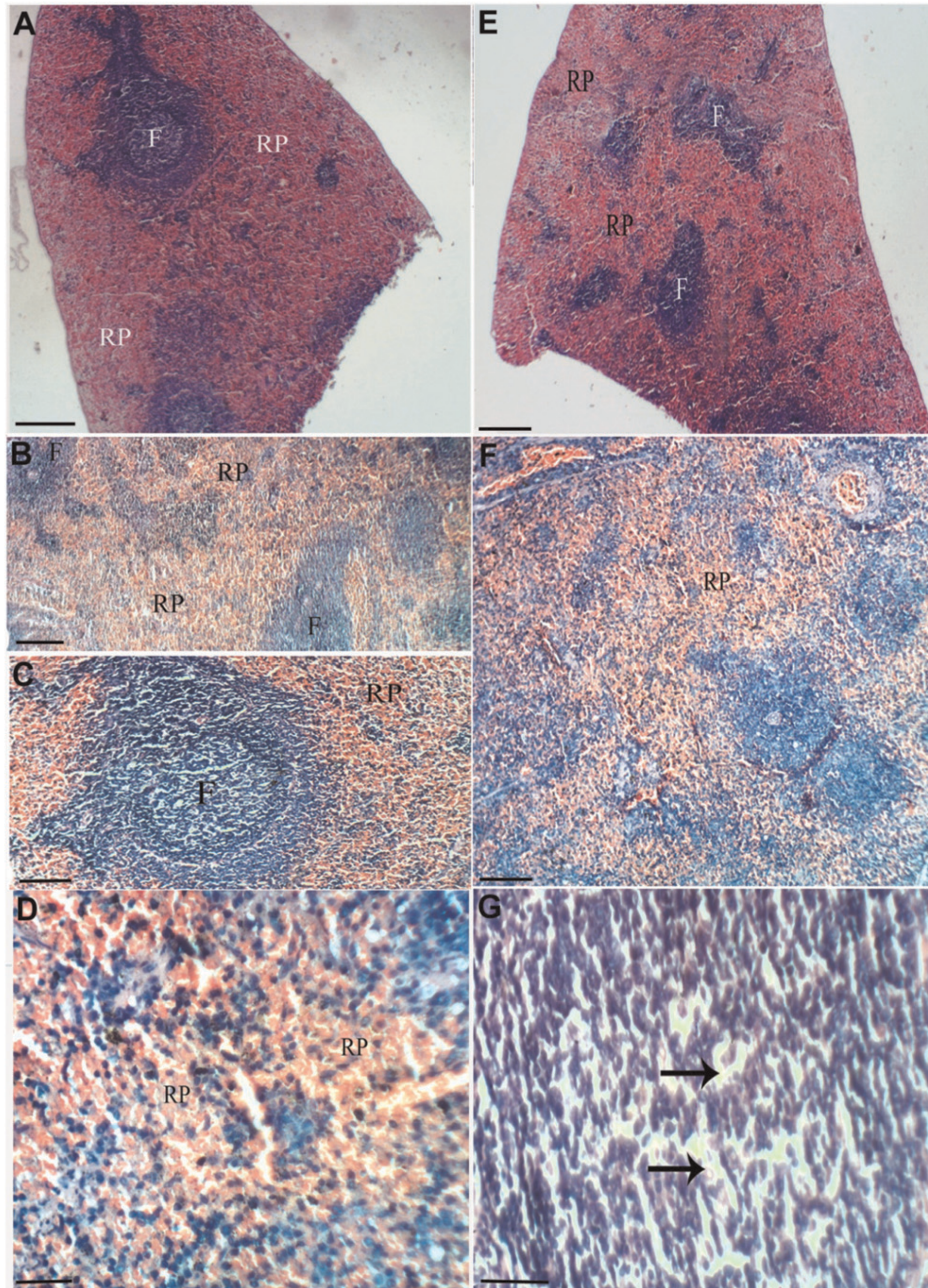

Fig. 6. A–D. Photomicrographs of the spleen sections (H&E) from rats orally inoculated with frozen L3 showing A–C. severe congestion of red pulp (RP) indicating a difficulty to determine the area percentage between the spleen pulps. E–F. Photomicrographs of the spleen sections (H&E) from rats orally inoculated with thermally treated L3 showing: E, F. Severe congestion of red pulp (RP). D, G. Lymphocytic necrosis and depletion in white pulp (arrows). (Scale bars: A, E = 300 μm; B, C, F = 200 μm; D, G = 50 μm).

the PALS was decreased. The distribution of scattered, occasional macrophages with aggregates of pigmented materials in addition to oncotic necrosis was also observed.

Image analysis: Histological sections from thymus (Table 1) revealed that there was a marked reduction in the cortical and medulla regions of the three parasite inoculated rat groups in addition to a significant change in the lymphocyte count compared with the control. The rat group orally inoculated with fresh larvae showed a significant decrease ($P < 0.01$) in the lymphocyte count compared with the control. At the same time, a significant increase ($P < 0.05$ and $P < 0.01$) was observed in the lymphocyte count of the rat groups orally inoculated with frozen and thermally treated larvae, respectively. Also, the follicular area of the spleen white pulp which is the key part of immune response was affected by a nonsignificant increase in its area fraction in case of fresh and frozen L3 inoculated rats in comparison with the control group. The lymphocyte count was significantly increased ($P < 0.01$) in the follicular area of all of the experimental rat groups (thermally treated L3

Table 1. Kruskal–Wallis analysis for different parameters of the thymus and spleen from control as well as experimental rat groups inoculated (oral) with *Anisakis* Type II (L3).

Experimental rat groups	Organ									
	Thymus							Spleen (white pulp)		
	Cortex				Medulla					
	Area	Area fraction	Area%	Lymphocyte count	Area	Area fraction	Area%	Follicle area	Follicular perimeter	Lymphocyte count
Control	4781.85 ± 1302	0.402 ± 0.11	40.25 ± 10.96	181.6 ± 4.37	5748.88 ± 1254.73	0.484 ± 0.10	48.39 ± 10.59	4335.09 ± 206.71	257.22 ± 9.98	148.40 ± 8.30
Per oral inoculation with fresh L3	2777.56 ± 2000.10	0.234 ± 0.01	23.38 ± 1.68	130 ± 4.60**	3811.41 ± 633.14	0.320 ± 0.05	32.08 ± 5.32	6056.71 ± 1833.78	300.24 ± 59.83	190.80 ± 8.30**
Per oral inoculation with frozen	4325.49 ± 836.97	0.364 ± 0.07	36.41 ± 7.04	200.60 ± 2.52*	5562.02 ± 739.48	0.468 ± 0.06	46.82 ± 6.22	5272.61 ± 905.76	272.25 ± 25.85	140.00 ± 12.96
Per oral inoculation with thermally treated L3	3657.39 ± 724.36	0.308 ± 0.06	30.78 ± 6.09	208.60 ± 6.55**	5040.2 ± 1050.13	0.424 ± 0.08	42.42 ± 8.83	4322.01 ± 583.52	248.06 ± 18.38	201.60 ± 6.04**

Data are expressed as mean ± SEM.
*, ** Significant difference ($P < 0.05$ and $P < 0.01$) in comparison with control group at $\alpha = 0.05$ and $\alpha = 0.0001$, respectively.

inoculated rats > L3 fresh inoculated rats > frozen L3 inoculated rats).

Discussion

In the present study, the recovered worms from the examined fish were identified belonging to the genus *Anisakis* Dujardin (1845). They possessed all the general morphological and morphometric characteristic features of that genus according to the key proposed by Pippy and Van Banning (1975) including inconspicuous three lips with a prominent boring tooth on the anterior end; a straight anterior gut consisting of esophagus, ventriculus, intestine; the posterior end terminated with or without mucron; and the cuticle is obviously transversely striated. As there are two morphotypes of that genus *Anisakis*: Type I and II, the isolated worms in the present study are morphologically identified as *Anisakis* Type II according to the key described by Berland (1961), where it is characterized by a triangular mouth in the head region surrounded by four papillae with no lips; the postanal tail is rounded without terminal mucron. By contrast, the first type is characterized by an anterior head with a rounded mouth opening with dorsal and ventrolateral lips equipped by papillae; the postanal region was terminated with a small mucron.

The thymus is a primary or central lymphoid organ in which T lymphocytes undergo differentiation and maturation autonomously within the cortex. It has been shown to be a sensitive target organ following exposure to pathogens (Verinaud et al., 2004; Pearse, 2006). Therefore, changes in thymus histology and architecture are considered to be of particular relevance for immunotoxicity screening. The spleen is the largest secondary lymphoid organ; it is dark red to blue–black color located adjacent to the greater curvature of the stomach and containing about one-fourth of the body's lymphocytes and initiates immune responses to blood-borne antigens (Nolte et al., 2002; Balogh et al., 2004; Haley et al., 2005). It comprises two functionally and morphologically distinct compartments, the red pulp and the white pulp. The red pulp is a blood filter that removes foreign material and damaged erythrocytes as well as particulate materials and circulating bacteria from the blood supply (Suttie, 2006; Mebius and Kraal, 2005). The white pulp is composed of three subcompartments: the PALS, the follicles, and the marginal zone (Cesta, 2006).

In the present study, most of the examined thymus sections obtained from experimental rat groups inoculated with fresh, frozen, and thermally treated L3 *Anisakis* Type II larvae showed congestion of blood vessels with an excess amount of blood in addition to the dilation of some capillaries and veins. These observations agreed with Stefanski et al. (1990) and AL-kindi et al. (2009). It is also well-known that decreased

cellularity of the thymus is the most frequently encountered histological finding associated with worm-induced effects. Elmore (2006) mentioned that the decreased cellularity is often associated with the presence of dead lymphocytes. Determination of the cell death type is important because it may provide insight into the pathogenesis of the lesion. The STP Committee on the Nomenclature of Cell Death recommends the use of the term "necrosis" to describe findings comprising dead cells in histological sections, regardless of the pathway by which the cells died (Levin et al., 1999). They also recommend the use of the modifiers "apoptotic" and "oncotic" to specify the predominant morphological cell death pathway. Oncotic necrosis is the cellular process that can be seen in areas of thymus infarction (death of tissue due to an absence of blood flow which is often due to obstruction of a blood vessel). However, as in the present study, apoptotic necrosis, on the other hand, is characterized by cell shrinkage, nuclear fragmentation, extrusion of membrane-bound cytoplasm, and nuclear debris in the form of small dense apoptotic bodies. The lymphocytic depletion and necrosis of the thymus may be related to its functional role or to the degree of lymphocytic maturation, which was greatly observed in both the cortex and medulla regions of thymus section from all of the experimentally inoculated rat groups. Therefore, cooking or freezing treatments did not protect the experimental rats against infection by anisakid larvae. These findings agreed with the previous study by Audicana and Kennedy (2008). Rat groups that were inoculated with fresh and frozen larvae revealed the atrophy of the thymus with striking tinctorial change and severe lymphocytic depletion and necrosis in both cortical and medulla areas with increased number of apoptotic lymphocytes that appeared at high magnification. These observations also agreed with Dutz et al. (1973) and Smythe et al. (1971) who stated that temporary thymus atrophy or necrosis may be due to nutritional or infectious stress in animals and may cause prolonged and profound changes in the immune status and host defense. This atrophy is characterized by intense structural and morphological alterations associated with an increased level of apoptosis (Suttie, 2006; Verinaud et al., 2004).

As the largest secondary lymphoid organ in the body, the spleen is involved in the capture and destruction of pathogens as well as the induction of immune responses (Cesta, 2006). Most of the spleen sections obtained from experimental rat groups inoculated with fresh, frozen, cooked, or thermally treated L3 showed a severe congestion of the red pulp with excessive distension of its sinuses by erythrocytes in accordance with (Stefanski et al., 1990; Ward et al., 1999). Also, pigmented materials in the red pulp were observed in some spleen sections, in accordance with Stefanski et al. (1990); Ward et al. (1999) and Suttie (2006) who stated that ceroid/lipofuscin or hemosiderin pigments is usually harbored in macrophages and may be present in the marginal zone of the red pulp following immune response eliciting. Most of the spleen sections from rat group inoculated thermally treated L3 revealed severe congestion of red pulp, and no demarcation between red pulp and white pulp was detected. Also, inflammatory cell infiltrations in splenic capsule and inflammatory lesions were observed. These observations coincide with those reported by AL-kindi et al. (2009) who concluded that the inflammatory lesions and degenerations of epithelial cells produced as a result of parasitic antigen administration is related to mononuclear cell infiltrates and migration of white blood cells mainly macrophages and lymphocytes to the immune organs for attacking and clearing parasite antigen debris.

Conclusion

The data obtained from the oral inoculation of *Anisakis* Type II (L3) into Wistar rats revealed that cooking and freezing of worms might not protect against stimulation of immune responses inducing allergic and hypersensitivity reactions as well as histological alterations with various degrees. So, it is important to note that although cooking or freezing kills the parasite, these procedures are not necessarily destroying the allergenic capacity of *Anisakis* Type II (L3). *Anisakis* Type II (L3) can not only cause symptoms directly associated with the gastrointestinal infection but also cause histopathological changes in the thymus and spleen which reflects to the body immune response against L3 larvae. Thus, it is important to create a wider awareness of this potential risk to human health.

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