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A Study on Biochemical, Hematological and Immunological Changes After Exposure to Morphologically Identified Dead *Anisakis simplex* Larvae and Antigen in Experimental Rats

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Abstract: The toxic properties of remaining dead *Anisakis* larvae may play important role in human sickness. The aim of this work is to determine the possible impact of exposure to morphologically identified larvae of *Anisakis simplex* and its antigen on the health condition by measuring of some biochemical, hematological and immunological analytes besides histopathological studies. Sixty rats were divided into equal four groups. Group 1 as a control, group 2 rats were injected intraperitoneal by *A. simplex* larvae crude antigen (250 µg/rat), group 3 rats were inoculated orally by freshly collected *A. simplex* larvae (ten larvae /rat) and group 4 rats were inoculated orally by frozen *A. simplex* larvae at -20°C for 6 months (ten larvae /rat). The animals were injected and inoculated at the zero and seventh day of the experiment. Blood and tissue samples were collected at the 7th and 14th days for performing different tests. Most changes in biochemical and hematological parameters were observed at 14th day of the experiment especially in groups inoculated orally with larvae. Animals injected by larvae crude antigen showed these changes early at 7th day of the experiment. Serum immunoglobulin E levels showed highly significant increase in all experimental groups at 7th day of the experiment. Histopathological changes confirmed the results. These results suggest that the consumption of parasitized fish with *A. simplex* larvae even in dead condition and after freezing can cause human health disturbance. More sweeping measures than the ones present currently upon consumption of fish must enforce to protect the public.

Key words: *Anisakis* · Antigen · Biochemical · Hematological · Immunoglobulin E

INTRODUCTION

Global changes in eating habits, especially the eating of exotic and raw food, variable food production systems and climate change besides population growth and movements and increasing global trade of foodstuff are some of the important factors which affecting the emergence or re-emergence a lot of food borne parasitic diseases in the last decades [1].

Fish play an important role in human nutrition that provide a range of health benefits. It's the main source of proteins which contain large amounts of polyunsaturated fatty acids, which consider healthy fats [2].

Parasitic nematodes involve one of the biggest and most varied groups of parasitic helminths which infect mainly marine, brackish and freshwater fish [3].

Nematodes of the family *Anisakidae* (genera *Anisakis,* species *Anisakis simplex*) are zoonotic parasites which firstly described as "worm-herring disease" in the 1960s in the Netherlands after noting of different cases which suffering from acute abdominal pain with the consumption of lightly salted herring. The final hosts of *Anisakis* are marine mammals such as (whales, dolphins, porpoises, seals and sea lions), while the intermediate and/or paratenic hosts are crustaceans, cephalopods and fish. In human, infection is most frequently diagnosed in countries where is common to eat raw and undercooked fish [4, 5]. The exposure of fish to low or high temperature or adequately concentrated brine kill the *Anisakis* larvae and thus prevent the danger of human infection with live larvae. The toxic properties of remaining dead *Anisakis* larvae, such as a body fluids and tissues in the muscles and viscera of fish may play important role in the occurrence of human sickness cases after consumption of heavily infested fish [6].

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Only a few data dealt with the possible changes in mammalian body after exposure to dead larvae of *Anisakis simplex*. The aim of this work is to determine the possible impact of exposure to the dead morphologically identified larvae of *A. simplex* and its antigen on the health condition by measuring of some biochemical and hematological, immunological analytes besides histopathological studies.

MATERIALS AND METHODS

Experimental Animals: Sixty apparently healthy male albino rats (8 weeks old and weighing 125–150g) were purchased from the laboratory animal house of Faculty of Veterinary Medicine, Zagazig University, Egypt. They were kept under hygienic conditions in suitable metal cages and fed on a standard pellet diet with water *ad libitum*. They were acclimatized for one week before starting of experiment. The stool examination was done by direct wet saline smear and flotation method to confirm the absence of any parasitic infection in experimental rats.

Ethical Statement: All experimental procedures were performed strictly in accordance to national and institutional guidelines for the animal welfare and approved by the Ethics Committee of the Faculty of Veterinary Medicine at Zagazig University, Egypt.

Parasite Collection and Identification: *Anisakis simplex* larvae were collected by using sterilized needle and forceps from viscera and body cavities of herring fish which purchased from different local markets in Zagazig city. They were washed with saline several times to remove attached debris, then were cleared in lactophenol and were examined under light microscope [7]. Their identification based on morphology of the digestive tract, shape and presence of the boring tooth, position of the excretory pore and the shape of post anal tail and its terminal mucron [8, 9].

Preparation of Crude *Anisakis* Antigen: Two hundred fifty of *A. simplex* larvae which obtained from the visceral organs of the infected fish were washed thoroughly with sterile saline solution and then were grounded in a mortar with 10 ml phosphate buffer saline (PBS). After that the homogenate was centrifuged for 20 minutes at 10000×g in a cooling centrifuge and the pellet was discarded. The supernatant was separated and its protein content was determined to be 250 µg/ml according to the Bradford method [10] and then was stored without any addition as a crude antigen (Ag) at -20°C until use.

Experimental Design: The animals were divided randomly into four groups of 15 rats / group as a following:

Group 1: Control group kept only on standard diet and water.

Group 2: Rats were injected intraperitoneal (I/P) by *A. simplex* crude antigen (250 µg/rat).

Group 3: Rats were orally inoculated with freshly collected *A. simplex* larvae (ten larvae /rat).

Group 4: Rats were orally inoculated with frozen *A. simplex* larvae at -20°C for 6 months (ten larvae /rat).

Experimental groups were injected and inoculated twice, first time at zero day of experiment followed by the second time at the 7th day of the experiment. The animals were observed daily for detection of any clinical signs.

Sampling: Samples of the blood were collected from the retro-orbital plexus of rats after overnight fasting from different experimental groups at the 7th and 14th days of the experiment, under anaesthesia with sodium pentobarbital. Blood samples were collected into the plain centrifuge tube without anticoagulant for serum separation for biochemical analysis and IgE estimation and clean Wasserman tubes containing dipotassium salts of ethylenediaminetetraacetic acid for hematological analysis. Rats under anaesthesia from each group were euthanized for collecting samples from the liver, kidneys and intestine for histopathological examination.

Determination of Some Clinical Chemistry Tests: Serum was used to determine alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities, total proteins, albumin, creatinine and urea concentrations. All of these analytes were measured according to manufacture's instructions by using of commercial diagnostic kits which were purchased from Diamond Diagnostic Company and Vitro by using of Photometer 5010 (Robert Riele GmbH and co-kg, Germany), except globulins concentration was estimated by subtracting albumin from total proteins.

Determination of Some Hematological Parameters: Total erythrocyte count, packed cell volume (PCV) value, hemoglobin (Hb) concentration, total and differential leucocytic counts were determined by using an automated blood cell analyzer (Sysmex XT-2000iV, Kobe, Japan).

Determination of Total Serum IgE Levels: Total serum levels of IgE were quantified by ELISA kit which was purchased from Cusabio according to the manufacturer's instructions by using of ELISA microplate readers (analytikjena- Carl Zeiss, Germany).

Histopathological Studies: Liver, kidneys and intestine of rats were dissected out and then were fixed at 10% neutral buffered formalin, were dehydrated in a graded ethanol series, were cleared in xylene and finally were embedded in paraffin wax. Paraffin sections of 5 µ thickness were stained by hematoxylin and eosin (H&E) and examined microscopically [11].

Statistical Analysis: Data were evaluated by using one-way analysis of variance (ANOVA), Tukey's HSD multiple comparison tests was used to test the significance differences between the mean values. Means in the same row followed by different letters were significantly different and the highest value was represented by the letter (a). Variability in the data was expressed as the pooled SEM and the alpha level for determination of significance was 0.05. All data were performed using SPSS software (v.21).

RESULTS

Morphological Features of *Anisakis simplex* Larvae: Larvae were observed creamy white in color, measuring 1-4 cm in length, coiled and encapsulated in the peritoneal cavity of the infected fish (Figures 1 & 2). They have a characteristic cuticle provided with a prominent transverse striation (Figures 4 a & b). Their gut consists of esophagus, ventriculus and intestine; while the ventricular appendix is absent. Esophagus has the twice length of ventriculus (Figure 3a). The ventriculus is longer than wide (Figure 3e). In addition, their anterior end carries a prominent boring tooth, labia papillae and excretory pore (Figures 3 b, c & d). The excretory pore locates below the tooth and the excretory duct locates below pore (Figures 3 b & d). Rectum surrounded by anal glands and opened with anus (Figure 4 a). The post anal tail is short, rounded with a terminal mucron (Figures 4 a & b).

Clinical Signs: Ruffled hair with a focal alopecia clearly was observed along the dorsum with a degree of depression after a few days of the first injection of rats I/P by *A. simplex* crude antigen and these clinical signs continued till the end of the experiment. These clinical signs were observed also in animals inoculated orally by *A. simplex* larvae (freshly collected and frozen), obviously noticed in these groups after a second exposure.

Determination of Some Clinical Chemistry Tests: Results of analysis in Table 1 after 7 days from I/P injection of *A. simplex* crude Ag and oral inoculation of *A. simplex* larvae (freshly collected and frozen) in different rats groups revealed that non significant change in serum ALT and AST activities in all experimental groups except rats group which injected I/P by *A. simplex* crude Ag which showed highly significant increase in the activity of these enzymes in comparison with the control group. Serum ALP activity showed highly significant increase in all experimental groups in comparison with the control group, the highest value was observed in rats group inoculated orally with *A. simplex* larvae (frozen).Serum total proteins concentration showed highly significant increase in all experimental groups in comparison with control group, the highest value was observed in the group injected I/P by *A. simplex* crude Ag. Serum albumin concentration showed highly significant increase in rats groups inoculated orally by *A. simplex* larvae (freshly collected and frozen) and highly significant decrease in rats group injected I/P by *A. simplex* crude Ag in compare with the control group. Serum globulins concentration showed non significant change in rats group inoculated orally by *A. simplex* larvae (freshly collected) and a highly significant increase in rats groups inoculated orally by *A. simplex* larvae (frozen) and injected I/P by *A. simplex* crude Ag, where the highest value was observed in this group in comparison with the control group. Serum creatinine concentration showed non statistical significant change in all experimental groups, while serum urea concentration showed highly significant increase in rats groups inoculated orally by *A. simplex* larvae (freshly collected and frozen) and non significant change in rats group injected I/P by *A. simplex* crude Ag in comparing with the control group.

Given the results of the Table 2 after 14 days from I/P injection of *A. simplex* crude Ag and oral inoculation of *A. simplex* larvae (freshly collected and frozen) in different rats groups, we found that a highly significant increase in serum ALT, AST and ALP activities in all experimental groups in comparison with the control group, the highest values of serum ALT and ALP activities were observed in rats group inoculated orally by *A. simplex* larvae (frozen).

Table 1: Some clinical chemistry tests in serum of rats after 7 days from I/P injection of *A.simplex* crude antigen and oral inoculation of *A. simplex* larvae (freshly collected and frozen)

	Experimental groups					
Parameters	Gp.(1)	Gp.(2)	Gp.(3)	Gp.(4)	SEM[1]	p-value
ALT (U/L)	21.27^{b}	24.67^{a}	21.84^{b}	22.07^{b}	0.36	0.001
AST (U/L)	48.72^{bc}	73.87^{a}	50.97^{b}	45.58^{c}	2.59	0.000
ALP (U/L)	330.78^{c}	348.96^{b}	344.08^{b}	391.51^{a}	5.26	0.000
Total proteins (g /dl)	6.57^{d}	7.11^{a}	6.77^{c}	6.93^{b}	0.04	0.000
Albumin (g /dl)	3.27^{b}	3.09^{c}	3.42^{a}	3.41^{a}	0.03	0.000
Globulins (g /dl)	3.30^{c}	4.02^{a}	3.35^{bc}	3.52^{b}	0.06	0.000
Creatinine (mg/dl)	0.59	0.55	0.62	0.61	0.01	0.319
Urea (mg/dl)	26.09^{c}	27.15^{c}	32.68^{a}	30.18^{b}	0.61	0.000

[1]SEM: Standard error of the mean

Means bearing different superscripts within the same row are significantly different (P<0.05)

Gp. (1) Control group, Gp. (2) Injected I/P with *A. simplex* crude Ag, Gp. (3) Inoculated orally by *A. simplex* larvae (freshly collected), Gp. (4) Inoculated orally by *A. simplex* larvae (frozen) , ALT= Alanine aminotransferase, AST=Aspartate aminotransferase, ALP=Alkaline phosphatase

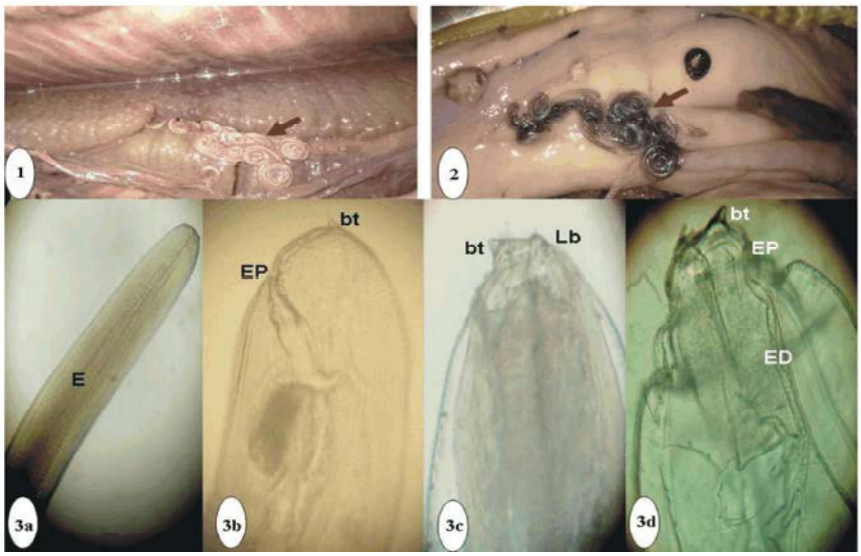
Table 2: Some clinical chemistry tests in serum of rats after 14 days from I/P injection of *A.simplex* crude antigen and oral inoculation of *A. simplex* larvae (freshly collected and frozen)

	Experimental groups					
Parameters	Gp.(1)	Gp.(2)	Gp.(3)	Gp.(4)	SEM[1]	p-value
ALT (U/L)	18.44^{d}	22.49^{b}	20.72^{c}	24.37^{a}	0.53	0.000
AST (U/L)	49.36^{c}	87.44^{b}	102.90^{a}	100.61^{a}	4.94	0.000
ALP (U/L)	293.60^{d}	452.87^{b}	408.71^{c}	488.39^{a}	16.85	0.000
Total proteins (g /dl)	6.23	6.02	6.05	6.28	0.04	0.058
Albumin (g /dl)	3.25^{a}	3.16^{b}	3.29^{a}	3.09^{c}	0.01	0.000
Globulins (g /dl)	2.98	2.86	2.76	3.18	0.152	0.807
Creatinine (mg/dl)	0.61	0.52	0.64	0.59	0.02	0.251
Urea (mg/dl)	27.78^{c}	29.84^{c}	39.65^{b}	43.08^{a}	1.49	0.000

[1]SEM: Standard error of the mean

Means bearing different superscripts within the same row are significantly different (P<0.05)

Gp. (1) Control group, Gp. (2) Injected I/P with *A. simplex* crude Ag, Gp. (3) Inoculated orally by *A. simplex* larvae (freshly collected), Gp. (4) Inoculated orally by *A. simplex* larvae (frozen), ALT= Alanine aminotransferase, AST=Aspartate aminotransferase, ALP=Alkaline phosphatase



Figs. 1&2: Photographs showing heavy infestation with *A. simplex* larvae in peritoneum of infected fish (arrows)

Fig. 3: Photomicrographs showing anterior end of *A. simplex larvae* (a -e) with esopaghus (E), boring tooth (bt), labia papillae (Lb), excretory pore (EP) and excretory duct (ED) and ventriculus (V)

Fig. 4: Photomicrographs showing posterior end of *A. simplex larvae* (a&b) with mucron (M), anus (An), anal glands (Ag) and transverse striations of cuticle (Ts).

Table 3: Some hematological parameters of rats after 7 days from I/P injection of *A. simplex* crude antigen and oral inoculation of *A. simplex* larvae (freshly collected and frozen)

	Experimental groups					
Parameters	Gp.(1)	Gp.(2)	Gp.(3)	Gp.(4)	SEM[1]	p-value
RBCs ($\times10^6/\mu l$)	5.75^c	6.62^a	6.24^b	6.71^a	0.09	0.000
PVC (%)	34.97^c	39.17^a	36.57^b	36.55^b	0.37	0.000
Hb (g%)	11.41^c	12.92^a	12.25^b	12.29^b	0.13	0.000
T.L.C ($\times10^3/\mu l$)	12.27^a	10.24^b	12.64^a	11.96^a	0.22	0.000
Neutrophils ($\times10^3/\mu l$)	1.49^c	2.00^{ab}	2.24^a	1.77^{bc}	0.07	0.001
Lymphocytes ($\times10^3/\mu l$)	10.56^a	8.16^b	10.25^a	10.12^a	0.22	0.000
Monocytes ($\times10^3/\mu l$)	0.15	0.02	0.04	0.05	0.02	0.258
Eosinophils ($\times10^3/\mu l$)	0.07	0.06	0.11	0.02	0.01	0.482

[1]SEM: Standard error of the mean

Means bearing different superscripts within the same row are significantly different (P<0.05)

Gp. (1) Control group, Gp. (2) Injected I/P with *A. simplex* crude Ag, Gp. (3) Inoculated orally by *A. simplex* larvae (freshly collected), Gp. (4) Inoculated orally by *A. simplex* larvae (frozen), RBCs=Red blood corpuscles, Hb=Hemoglobin, PCV=Packed cell volume, T.L.C.=Total leucocytic count

Table 4: Some hematological parameters of rats after 14 days from I/P injection of *A. simplex* crude antigen and oral inoculation of *A. simplex* larvae (freshly collected and frozen).

	Experimental groups					
Parameters	Gp.(1)	Gp.(2)	Gp.(3)	Gp.(4)	SEM[1]	p-value
RBCs ($\times10^6/\mu l$)	5.69^c	6.73^a	6.40^b	6.53^{ab}	0.09	0.000
PVC (%)	34.84^c	37.38^a	36.52^b	36.36^b	0.21	0.000
Hb (g%)	11.40^d	13.60^a	12.58^b	12.29^c	0.18	0.000
T.L.C ($\times10^3/\mu l$)	12.21^c	15.73^b	18.69^a	17.75^a	0.58	0.000
Neutrophils ($\times10^3/\mu l$)	1.48^c	2.40^{ab}	2.64^a	2.24^b	0.10	0.000
Lymphocytes ($\times10^3/\mu l$)	10.62^c	13.24^b	15.95^a	15.46^a	0.50	0.000
Monocytes ($\times10^3/\mu l$)	0.08	0.00	0.05	0.05	0.01	0.382
Eosinophils ($\times10^3/\mu l$)	0.03	0.08	0.05	0.00	0.01	0.210

[1]SEM: Standard error of the mean

Means bearing different superscripts within the same row are significantly different (P<0.05)

Gp. (1) Control group, Gp. (2) Injected I/P with *A. simplex* crude Ag, Gp. (3) Inoculated orally by *A. simplex* larvae (freshly collected), Gp. (4) Inoculated orally by *A. simplex* larvae (frozen), RBCs=Red blood corpuscles, Hb=Hemoglobin, PCV=Packed cell volume, T.L.C.=Total leucocytic count

Table 5: Total serum IgE levels of rats after 7 days from I/P injection of *A. simplex* crude antigen and oral inoculation of *A. simplex* larvae (freshly collected and frozen)

	Experimental groups					
Parameters	Gp.(1)	Gp.(2)	Gp.(3)	Gp.(4)	SEM[1]	p-value
IgE (ng/ml)	0.10^{d}	0.35^{b}	0.26^{c}	0.50^{a}	0.04	0.000

[1]SEM: Standard error of the mean

Means bearing different superscripts within the same row are significantly different (P<0.05)

Gp. (1) Control group, Gp. (2) Injected I/P with *A. simplex* crude Ag, Gp. (3) Inoculated orally by *A. simplex* larvae (freshly collected), Gp. (4) Inoculated orally by *A. simplex* larvae (frozen), IgE= Immunoglobulin E.

Table 6: Total serum IgE levels of rats after 14 days from I/P injection of *A. simplex* crude antigen and oral inoculation of *A. simplex* larvae (freshly collected and frozen)

	Experimental groups					
Parameters	Gp.(1)	Gp.(2)	Gp.(3)	Gp.(4)	SEM1	p-value
IgE (ng/ml)	0.09^{c}	0.28^{a}	0.18^{b}	0.09^{c}	0.02	0.000

[1]SEM: Standard error of the mean

Means bearing different superscripts within the same row are significantly different (P<0.05)

Gp. (1) Control group, Gp. (2) Injected I/P with *A. simplex* crude Ag, Gp. (3) Inoculated orally by A. simplex larvae (freshly collected), Gp. (4) Inoculated orally by A. simplex larvae (frozen), IgE= Immunoglobulin E

Serum total proteins concentration showed non statistical significant change in all experimental groups. Serum albumin concentration showed a highly significant decrease in rats groups injected I/P by *A. simplex* crude Ag and inoculated orally with *A. simplex* larvae (frozen) and non significant change in rats group inoculated orally by *A. simplex* larvae (freshly collected) in comparison with the control group, the lowest value was observed in rats group which injected by crude Ag. Serum globulins and creatinine concentrations showed non statistical significant change in all experimental groups.

Serum urea concentration showed a highly significant increase in rats groups inoculated orally by *A. simplex* larvae (freshly collected and frozen) and non significant change in rats group injected I/P by *A. simplex* crude Ag in compare with the control group, the highest value was observed in rats group inoculated by frozen *A. simplex* larvae.

Some Hematological Parameters: The obtained results (Tables 3 and 4) after 7 and 14 days from I/P injection of *A. simplex* crude Ag and oral inoculation of *A. simplex* larvae (freshly collected and frozen) in different rats groups indicated that a highly significant increase in the total erythrocytic count, packed cell volume (PCV) value and hemoglobin (Hb) concentration in all experimental groups in comparison with the control group.

According to the results (Table 3) after 7 days of I/P injection of *A. simplex* crude Ag and oral inoculation of *A. simplex* larvae (freshly collected and frozen) in different rats groups, a highly significant decrease was observed in total leucocytes and lymphocytes counts in rats group injected I/P by *A. simplex* crude Ag and non significant change in other groups in comparison with the control group. Neutrophils count showed a highly significant increase in all experimental groups except rats group which orally inoculated by *A. simplex* larvae (frozen) which showed non significant change in comparison with the control group. Monocytes and eosinophils counts showed non statistical significant change in all experimental groups.

According to the results (Table 4) after 14 days from I/P injection of *A. simplex* crude Ag and oral inoculation of *A. simplex* larvae (freshly collected and frozen) in different rats groups, a highly significant increase was observed in total leucocytes, neutrophils and lymphocytes counts in all experimental groups in comparison with the control group. Monocytes and eosinophils counts showed non statistical significant change in all experimental groups.

Total Serum IgE Levels: According to the results (Table 5) after 7 days of I/P injection of *A. simplex* crude Ag and oral inoculation of *A. simplex* larvae (freshly collected and frozen) in different rats groups, a highly significant increase was observed in total serum IgE levels in all experimental groups in comparison with the control group, the highest value was observed in rats group inoculated orally with *A. simplex* larvae (frozen). After 14 days from I/P injection of *A. simplex* crude Ag and oral inoculation of *A. simplex* larvae (freshly collected and frozen) in different rats groups, a highly significant

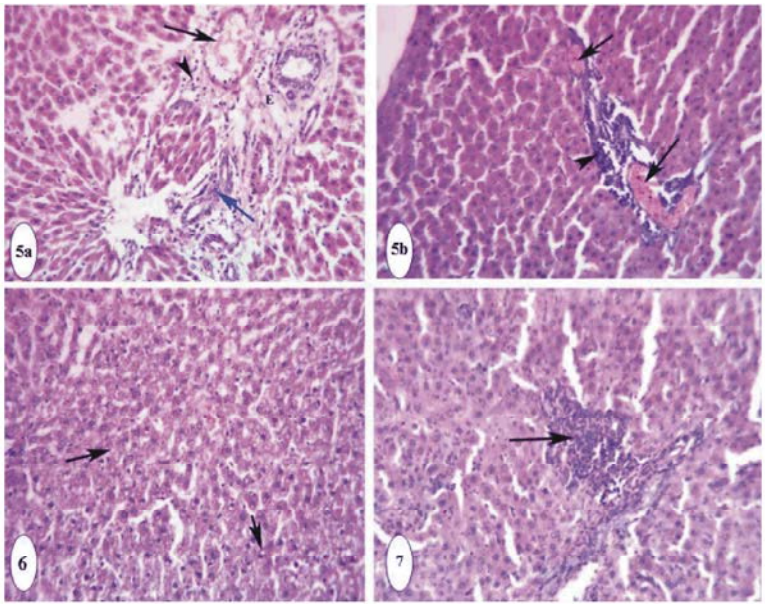


Fig. 5: Photomicrograph of H&E stained section of rat liver in group injected I/P by *A.simplex* crude antigen at 7th day of the experiment (a) showing portal congestion (black arrow), edema (E), few mononuclear cell infiltration (arrowhead) and proliferated bile ductules (blue arrow), X400 and at 14th day of experiment (b) showing portal congestion (arrows) and mononuclear cell infiltration (arrowhead), X400.

Fig. 6: Photomicrograph of H&E stained section of rat liver at 14th day of the experiment in group inoculated orally by freshly collected *A.simplex* larvae showing vacuolation of hepatocytes (arrows), X400.

Fig. 7: Photomicrograph of H&E stained section of rat liver at 14th day of the experiment in group inoculated orally by frozen *A. simplex* larvae showing focal necrotic area replaced by lymphocytic aggregation (arrow), X400.

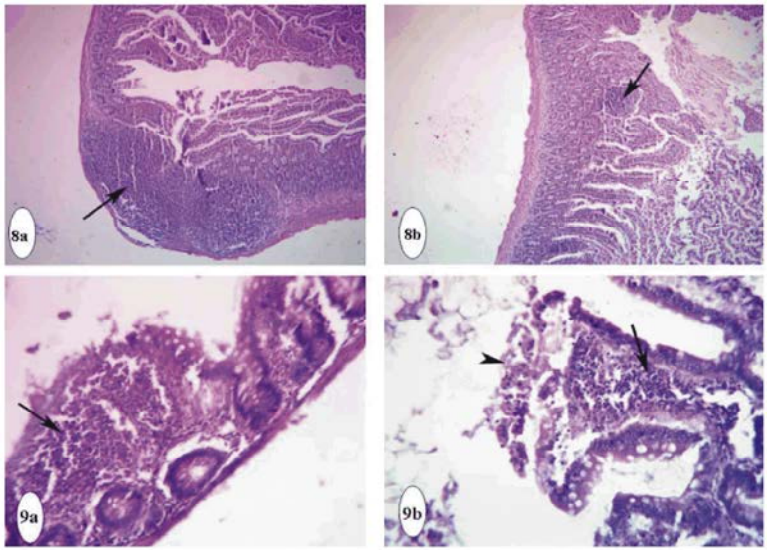


Fig. 8: Photomicrograph of H&E stained section of rat intestine in group inoculated orally by freshly collected *A. simplex* larvae at 7th day of the experiment (a) showing lymphoid hyperplasia in the Peyer's patches (arrow), X100 and at 14th day of experiment (b) showing intraepithelial focal mononuclear cell aggregation (arrow), X100.

Fig. 9: Photomicrograph of H&E stained section of rat intestine in group inoculated orally by frozen *A. simplex* larvae at 7th day of the experiment (a) showing leucocytic infiltration with expansion of the intestinal villi (arrow), X400 and at 14th day of experiment (b) showing leucocytic infiltration mostly of eosinophils in the intestinal villi (arrow) with desquamation of the tip of the villus (arrowhead), X400.

increase was observed in total serum IgE levels in all experimental groups except rats group inoculated orally by *A. simplex* larvae (frozen), which showed non significant change in comparison with the control group, the highest value was observed in rats group injected I/P by *A. simplex* crude Ag.

Histopathological Studies: Regarding the histopathological results, hepatic tissues of the group injected I/P by *A. simplex* crude Ag at 7th day of experiment displayed portal congestion, edema, few mononuclear cell infiltration and proliferated bile ductules (Figure 5a), while the changes in hepatic tissue in this group at the 14th day of the experiment appeared as portal congestion and mononuclear cell infiltration (Figure 5b). On the other hand, the histopathological changes in the hepatic tissue of group inoculated orally by freshly collected *A. simplex* larvae appeared as vacuolation of hepatocytes (Figure 6), while the changes in the hepatic tissue of group inoculated orally by frozen *A. simplex* larvae appeared as focal necrotic area replaced by lymphocytic aggregation (Figure 7), the changes in these groups appeared at the 14th day of the experiment. The histopathological changes in the intestine of the group inoculated orally by freshly collected *A. simplex* larvae appeared as lymphoid hyperplasia in the Peyer's patches at 7th day of the experiment (Figure 8a) and intraepithelial focal mononuclear cell aggregation at 14th day of experiment (Figure 8b). On the other hand, the histopathological changes in the intestine of the group orally inoculated by frozen *A. simplex* larvae appeared as leucocytic infiltration with the expansion of the intestinal villi at 7th day of the experiment (Figure 9a) and leucocytic infiltration mostly of eosinophils in the intestinal villi with desquamation of the tip of the villus at 14th day of experiment (Figure 9b). There are no obvious histopathological changes appeared in the kidney in all experimental groups in different experimental periods. The observed histopathological changes in the liver and intestine of different experimental groups were in compare with the normal control group.

DISCUSSION

Several studies illustrated that *Anisakid* larvae are found mainly in fish muscles, while the present study revealed that they are encapsulated in viscera and body cavities mainly. The morphological features in the present study resemble that described by Rocka, Koinari *et al.* Chen *et al.* and Abo-Rahma *et al.* [12-15].

In our study, highly significant increase in serum transaminases (ALT and AST) activities occurred in rats group injected I/P by *A. simplex* crude Ag at 7th and 14th days of the experiment which may be related to the inducing effects of *A. simplex* antigen to the immunological environment within the liver which resulting pathological inflammatory response and disrupted hepatic tissue homeostasis and lasting injury to the hepatic tissue and releasing enzymes into circulation [16]. Also, increasing serum AST activity may be due to soft-tissue damage and /or necrosis [17]. The hepatic injury appeared early after first exposure to parasitic antigen in this group in comparison with other groups inoculated orally with *A. simplex* larvae may be due to the I/P route can attain very rapid absorption to injectable substance as it directly delivered to liver without being subjected to intestinal first pass metabolism when compared with the oral route [18]. The liver histopathological changes in this group in the current study confirmed these results.

The raising of serum transaminases activities in groups inoculated orally by *A. simplex* larvae after the second exposure to parasitic antigen (at 14th day of experiment) probably due to that repeated exposures of the animal body to same antigen may lead to more serious reactions because the animal became sensitized which mean even a limited exposure to small amount of antigen (allergen) can trigger a sever reaction [19]. The liver histopathological changes in these groups in the current study confirmed our results.

According to our study, highly significant increase in serum ALP activity in rats group injected I/P by *A. simplex* crude Ag at 7th and 14th days of the experiment may be due to rising level of the liver isoform of the serum alkaline phosphatase which have been associated with inflammatory and cholestatic hepatic disease conditions [20]. The increasing in the activity of same enzyme in rats groups inoculated orally by *A. simplex* larvae (freshly collected and frozen) at 7th and 14th days of experiment may be due to rising level of the intestinal isoform of the serum alkaline phosphatase which have been associated with gastrointestinal disorder mainly after first exposure to *Anisakis* antigen, adding to that increasing level of the liver isoform of the serum alkaline phosphatase after second exposure to antigen [21]. The intestine histopathological changes in these groups in the current study confirmed these results.

Hyperproteinemia in rats groups inoculated orally by *A. simplex* larvae (freshly collected and frozen) at the 7th day of the experiment resulted mainly from

hyperalbuminemia which may occur in these groups due to hemoconcentration which leads to greater concentration of albumin in the blood, which occurred as a result of increased vascular permeability and extravasation of plasma, resulting in a decrease in plasma volume intravascular under the influence of histamine which released as a result of response of rats to inoculated *Anisakis* antigens [22-24]. Also, hyperglobulinemia in rats group inoculated orally by *A. simplex* larvae (frozen) and non significant increase in this parameter in group inoculated by fresh *A. simplex* larvae probably resulted from host response to antigenic stimulation of dead parasites [25]. Rats group injected I/P by *A. simplex* crude Ag showed also hyperproteinemia at the 7th day of the experiment which occurred concurrent to hyperglobulinemia which was observed clearly in this group may be due to an acute inflammatory condition which caused by parental injection of parasitic crude Ag which triggered synthesis of positive acute phase proteins beside production of different types of immunoglobulins as a result of antigenic stimulation. At the same time, hypoalbuminemia in this group may be occurred due to decrease albumin production by hepatocytes during acute inflammation (albumin as negative acute phase protein) and / or hepatic dysfunction [25, 23]. Observed results after the second exposure to *A. simplex* larvae antigen at the 14th day of experiment revealed that non significant change in total proteins concentration in all experimental groups resulted from parallel changes in albumin and globulins fractions. Hypoalbuminemia with a normal globulins concentration, which estimated in rats groups injected I/P by *A. simplex* crude Ag and inoculated orally by *A. simplex* larvae (frozen) may be due to hepatic insufficiency, which leads to insufficient production of albumin and some fractions of globulins such as (α and β) but immunoglobulins which composed most of globulins fraction are not affected so the serum globulins were observed normal [26]. Non significant change in these parameters in rats groups inoculated orally by *A. simplex* larvae (freshly collected) not mean the normal condition, but it may indicate the interaction between histamine action which previously mentioned and hepatic insufficiency which appeared with lower degree in this group.

In our study, there is no statistical change in serum creatinine level in all experimental groups at 7th and 14th days from starting of experiment which mean there is no effect of *A. simplex* which administrated in different forms and routes on renal functions.

Highly significant increase in serum urea concentration in rats groups inoculated orally by *A. simplex* larvae (freshly collected and frozen) was observed at 7th and 14th days of the experiment may be due to prerenal retention of nitrogenous wastes which associated with hemoconcentration [27]. Non significant change in serum urea l evel in rat group injected I/P by *A. simplex* crude Ag at 7th and 14th days of experiment not mean the normal condition, but may be indicate a degree of reducing the urea level as a result of hepatic insufficiency [17], accompanied by increasing in its concentration as a result of hemoconcentration, so finally it was observed in normal level (falsely).

In our study, we observed highly significant increase in the total erythrocyte count, packed cell volume value and hemoglobin concentration (relative polycythemia) as a result of hemoconcentration [28] in all experimental groups at 7th and 14th days of the experiment.

By linking the results of biochemical and hematological parameters we can observe that, changes in extracellular fluid volume affect both the interstitial and intravascular volumes and appear in the results of measurements PCV and total proteins. In some cases as (anemia and hypoproteinemia) PCV and total proteins measurements may not reflect the real ECF hydration status [27].

Generally, all helminthes liberate relatively large amounts of antigenic materials and this huge production may change immune responses or even deplete immune potency even dead *Anisakis* larvae release internal antigens which are capable of generating a host immune response [29, 30]. Leucopenia in rats group injected I/P with *A. simplex* crude Ag at 7th day of the experiment may be due to a temporary lymphopenia which resulted from redistribution of lymphocytes from the blood and lymphopoietic organs to other tissues where prepare to antibody formation after exposure to antigenic stimulation. Also, extravascular movement of lymphocytes occurs after inflammatory stimuli by antigen injection. The formation of antigen–antibody complexes and inflammatory condition after injection of parasitic crude Ag lead to neutrophilia in this group [31, 32]. Neutrophilia in rats groups inoculated orally by (freshly collected) *A. simplex* larvae at the same stage of the experiment may be due to the same reasons of previous group.

Leucocytosis which occurred in all experimental groups at the 14th day of experiment resulted mainly from neutrophilia and lymphocytosis, which probably occurred due to exposure the experimental animals to

second antigenic stimulation which induced reactive lymphocytosis and strong chemotactic properties which attract more neutrophils [31, 32]. From these results, it is possible to suggest that second exposure to antigenic stimuli may induce clear and strong leucocytic response than first exposure. For the group which inoculated orally by *A. simplex* larvae (frozen) there is the graded leucocytic response which can't be detected after the first exposure to antigen in other words, sub-clinical reaction but after re-exposure to the antigen the response appears clearly. Also, the concentration of exposed antigen is a main determinant factor of body immune response [33, 34].

Non statistical significant change in eosinophils count was observed in all experimental groups along the experimental periods may be due to eosinophils predominantly tissue-dwelling cells have a short half-life with diurnal variation and accelerate rate of removal from the blood circulation after the early stage of the body response so sometimes occurs inability to determine any changes in eosinophils count or inability of chemical fractions or metabolic products of the parasite homogenate or dead parasites to stimulate the occurrence of eosinophilia , in another word the load of parasites may be light to stimulate the eosinophils [35-37].

Immunoglobulin E (IgE) normally appears at very low levels in circulation. Parasitic infection and allergy may lead to increase its level [38]. Given the results of this study, serum total IgE levels showed highly significant increase in all experimental groups at 7^{th} day of experiment which may be related mainly to generating antibody response to cuticular and somatic (SA) antigens that released from dead larvae and the considerable amount of excretion-secretion (ES) antigen in addition to somatic antigen as a result of injection of *A. simplex* crude Ag intraperitoneal [39]. Elevation of total serum IgE levels may occur in some cases before expression of any clinical signs or reactions and that's what happened in rats groups inoculated orally by *A. simplex* larvae [38]. Also, route of administration of antigen strongly influence IgE synthesis so we found total serum IgE levels were lower in the group injected I/P by *A. simplex* crude Ag than the group inoculated orally by *A. simplex* larvae (frozen) [40]. A normal total serum IgE levels does not exclude the possible sensitization, but in some cases, low to normal concentrations of total IgE levels may be combined with a high levels of antigen-specific IgE (sIgE). As total IgE was not completely caused by the accumulation of sIgE [41]. This may explain non significant change in total serum Ige levels in group inoculated orally with *A. simplex* larvae (frozen) at 14^{th} day of the experiment.

By observing the results of the most measured parameters, especially at 14^{th} day of experiment, the group inoculated orally by *A. simplex* larvae (frozen) showed significant changes in compare with the group inoculated orally with *A. simplex* larvae (freshly collected) may be due to the effect of freezing on the permeability and microstructure of the larval cuticle which lead to release more of somatic and cuticular antigens from dead larvae with minimal quantities of ES antigens beside the internal components of larvae and consequently an additional body response to these different antigens, which mainly are resistant to stomach digestive enzymes and acids [42].The nematodes mainly characterise by their thick cuticle [43].Several studies recorded that, freeze-thawing procedure has the ability to disturb the morphological structures of different parasites and release most of its internal soluble material [44].

CONCLUSION

In summary, the detected changes in biochemical and hematological analytes and total serum Ige levels in the different experimental groups reflected the body reaction against dead *A. simplex* larvae and its antigen. Peak of changes were faster and higher after the second exposure mostly more than the primary exposure. Freezing considered a good precaution against infection by a living parasite, has a larvicidal effect could be attained in a shorter time, but it might not be adequate to destruct the antigenic structure which has health impact. So, the consumption of parasitized fish with *A. simplex* larvae even in dead condition and after exposure to freezing for a long time can cause human health disturbance. The method of handling and the extent of fish processing (heading, gutting and trimming), besides the quality of the fish source can all contribute to the control of the risks posed by helminths. It may be important to consider more sweeping measures than the ones present currently upon consumption of fish to protect the public.

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