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Clinico-Pathologic Evaluation of the Canine Heartworm Infestation

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Abstract: In this study, attempts were made to study the clinico-pathologic findings in dogs with *D. immitis* infestation in order to more accurately diagnose the infestation. One hundred and twenty two stray dogs in Garmsar area (central part of Iran) from March to November 2006 were examined for infection to *D. immitis*. Blood microfilaria were observed in 18 samples (14.75%): microfilaria of *Dirifilaria immitis* in 15 samples (12.29%), microfilaria of *Dipetalonema reconditum* in 2 samples (1.64%) and mixed infection in 1 sample (0.82%). Then, five dogs with dirofilariasis and five other dogs with negative results for parasite were selected as control group. Hematological and serum biochemical values were assessed in these dogs. Necropsy of infected dogs was also performed and tissue samples were taken from the heart, lung, liver and kidney for histopathological examination. The assessment of hematological parameters showed a mild anemia in dogs with *D. immitis* infection, with a significant decrease in the number of red blood cells and an increase in number of eosinophil. However, there was no significant difference between infected and non-infected dogs. The infected dogs showed a higher level of aspartate aminotransferase (AST) activity. In necropsy, many pathological lesions were found in sampled tissues, but those are not pathogonomic for dirofilariasis.

Key words: *Dirofilaria immitis*, dirofilariasis, histopathology, hematology, serum biochemistry, dog

INTRODUCTION

Dirofilaria immitis lives in the right ventricle, pulmonary artery and posterior vena cava and its microfilariae found in the peripheral circulation (Eslami, 2007). Infection with this parasite in carnivores, especially dogs is worldwide and has been reported from different regions of the world, in Brazil (Reifur *et al.*, 2004), Slovakia (Miterpáková *et al.*, 2009), Spain (Montoya *et al.*, 1998), Turkey (Agaoglu *et al.*, 2000) and other countries (Chen and Liang,

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1993; Rosa *et al.*, 2002). Epidemiological studies on heartworm disease in Iran showed that there was an incidence of *D. immitis* in canine of different parts of country (Bokai *et al.*, 1998; Meshgi and Eslami, 2001; Meshgi *et al.*, 2002; Ranjbar-Bahadori *et al.*, 2005; Razmi, 1999). Dirofilariasis is not only a veterinary problem but also a zoonosis in many regions of the world (Flieder and Moran, 1999; Hirano *et al.*, 2002; Salahi-Moghadam *et al.*, 2000). *Dirifilaria immitis* has been also reported to cause anemia by developing a reduction in the Red Blood Cells (RBC), Hemoglobin concentration (Hb) and Packed Cell Volume (PCV) (Atwell and Buoro, 1983). Meanwhile, leukocytosis has been reported in dogs with dirofilariasis. Alterations in Blood Urea Nitrogen (BUN), creatinine, Alkaline Phosphatase (ALP), Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT) and Gamma-Glutamyltransferase (GGT) have been reported (Sodicoff, 1995). So, Blood and serum biochemical findings have been playing an important role in the identification of the host-parasite relationship during the infection of *D. immitis* (Sevimli *et al.*, 2007). In the present study, attempts were made to study the clinico-pathological findings in dogs with *D. immitis* infestation in order to more accurately diagnose the infestation especially in animals with occult infection.

MATERIALS AND METHODS

Preparation of Animals

One hundred and twenty two stray dogs in Garmsar area (central part of Iran) from March to November 2006 were trapped and examined for *D. immitis* using modified Knott's method. Then, five dogs with dirofilariasis were selected for study. Also five other dogs with negative results for parasite were selected as the control group. Serological diagnosis by using a commercial heartworm antigen test kit (WITNESS CHW II kit[®], Japan) were used for diagnosis of the occult infection and confirmation of the result of Knott test for dogs with positive and negative results. Cases were maintained in same situations and similar feed for a month. The dogs ranged in age from 3 to 8 years and all of them were male. Dogs were necropsied and up to six heartworms were present in the right side of heart in infected dogs.

Blood Sample Collection

Blood samples were collected from cephalic vein in a standard manner. One milliliter of blood was added to 9 mL of formaldehyde 2% for parasitological examination and remaining was divided into EDTA containing tube for hematological measurements and plane tube for harvesting of serum for biochemical determinations. Serum samples were stored at -20°C, until use in the biochemical measurements.

Parasitological Method

Modified Knott's method (Eslami, 2007) was used for diagnosis of blood parasite and calculation of microfilariae in each milliliter of peripheral blood circulation. In Knott's method, the sediments from prepared samples were searched for microfilariae; identification of them was performed using the key presented by Etinger and Feldman (2000) based on their morphology. Then number of microfilariae in each mL of the blood sample was calculated.

Hematological and Biochemical Measurements

Complete Blood Count (CBC) was performed using standard manual methods. The concentrations of Albumin (Alb), Total Protein (TP), Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), ALP, Blood Urea Nitrogen (BUN), Creatinine (Cr), Glucose (Glu), Cholesterol (Chl), crud and real fibrinogen were measured using commercial kits (Zist Chimi Co, Tehran, Iran) and a spectrophotometer (Unico Uv 2100, New Jersey, USA).

Pathological Procedure

Necropsy of five infected dogs was performed and tissue samples were taken from the heart, lung, liver and kidney. Samples were kept in 10% buffered formaldehyde. Sections were prepared in 4-5 μm thickness by routine procedures. They were stained with hematoxylin and eosin (H and E) and they were examined with light microscope.

Statistical Analysis

Statistical analysis was conducted using SPSS for Windows (release 16, SPSS Inc., Chicago, USA) and with parametric independent t-test. Correlations between measured parameters and number of microfilariae in each mL of blood were determined using Pearson test.

RESULTS AND DISCUSSION

In the present study, blood microfilaria were observed in 18 samples (14.75%): microfilaria of *Dirifilaria immitis* in 15 samples (12.29%), microfilaria of *Dipetalonema reconditum* in 2 samples (1.64%) and mixed infection in 1 sample (0.82%). In parasitological exam of five dogs with *D. immitis* infestation, numbers of microfilariae in peripheral blood circulation were 880, 4300, 3500, 4550 and 3120 microfilariae in each milliliter of the blood. The average number of microfilariae per mL of blood in five infected dogs was 3270 ± 1456.60 . After necropsy, in three dogs were isolated, three adult worms (two males and one female), four (three adult male and one female) and six worms (four adult female and two male), respectively.

Hematology and Serum Biochemistry

The assessment of hematological parameters demonstrated a mild anemia in dogs with *D. immitis*, infestation with a significant decrease ($p < 0.05$) in the number of red blood cells, compared to the non-infected dogs. Data analysis also showed an increase in the number of eosinophil. However, there was no significant difference between infected and non-infected dogs ($p > 0.05$). Significant increase in AST activity was seen in infected dogs in comparison with non-infected ones ($p < 0.05$). There was an increase in alkaline phosphatase (ALP) activity, but the difference was not significant between two groups of animals ($p > 0.05$) (Table 1). Significant negative correlations were seen between HCT ($r = -0.895$, $p = 0.04$) and albumin ($r = -0.913$, $p = 0.03$) with number of microfilariae in blood.

Pathological Findings

In macroscopic examination, no significant lesion, except congestion of organs, was found. Signs of petechial hemorrhage in subepicardium and scattered necrotic foci of liver (nutmeg liver) were observed. In microscopic examination of renal tissue, severe congestion in medullary and cortical juxtamedullary portions along with inflammatory cell infiltration of macrophage, lymphocyte and plasma cell types (focal mononuclear interstitial nephritis) were noticed. Many glomeruli were swollen and showed hypercellularity along with increased number of mononuclear cells in mesangial matrix. In some rare cases they appeared with increased mesangial matrix as well as presence of eosinophilic amyloid like substances. In certain glomeruli; signs of protein loss in urine were observed as hyaline droplet formation in urinary spaces and renal tubules (Fig. 1, 2). In cardiac tissue, there was vascular engorgement and interfibrillar accumulation of edematous fluid in myocardial muscle fibers which appeared as separation of connective tissue fibers and edematous dilatation of lymphatic vessels.

Table 1: Hematology and serum biochemistry results from infected to heartworm and non-infected dogs

Selected values	Non-infected dogs	Infected to heartworm dogs	p-value
	----- (Mean±SD) -----		
RBC ($\times 10^6$ cells μL^{-1})	5.67±0.61	6.14±1.40	0.009
WBC ($\times 10^3$ cells μL^{-1})	6.31±1.03	6.10±1.91	NS
Differentiation (%)			
Neu	69.20±4.82	69.40±5.37	NS
Lym	25.80±4.15	18.60±5.77	NS
Eos	2.20±0.84	10.20±1.92	NS
Mon	2.00±1	1.00±0.71	NS
Bas	0.20±0.45	0.40±0.55	NS
PCV (%)	42.00±6.71	47.4±2.07	NS
Hb (g dL^{-1})	14.80±2.95	18.00±1.58	NS
Albumin (g dL^{-1})	3.00±0.84	3.58±0.76	NS
ALT (U L^{-1})	36.40±24.59	41.60±11.41	NS
AST (U L^{-1})	19.00±0.46	80.80±54.66	0.007
Urea (mg dL^{-1})	11.00±5.52	22.20±8.81	NS
BUN (mg dL^{-1})	5.16±2.58	9.10±2.88	NS
Cr (mg dL^{-1})	0.28±0.16	0.78±0.19	NS
ALP (U L^{-1})	66.00±16.66	76.20±72.85	NS
Cholesterol (mg dL^{-1})	147.80±42.72	185.80±90.48	NS
Fibrinogen (mg dL^{-1})	124.80±24.35	119.80±18.13	NS
Glucose (mg dL^{-1})	83.00±8.15	106.20±3.03	NS
Total Protein (g dL^{-1})	5.50±0.34	5.34±0.59	NS
Microfilariae (No mL^{-1} of blood)	-	3270.00±1456.60	-

RBC: Red blood cell; WBC: White blood cell; PCV: Packed cell volume; Hb: Hemoglobin concentration; Alb: Albumin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; BUN: Blood urea nitrogen; TP: Total protein; Glu: Glucose; Chl: Cholesterol; Cr: Creatinine; NS: Non significant

Small and occasionally disseminated hemorrhages in epicardial fat and myocardial fibrillar bundles were observed (Fig. 3). In pulmonary tissue, emphysematous foci as well as areas of interstitial pneumonia were found. In these areas inside the alveoli was empty. Moreover, due to proliferation of smooth muscle cells, fibroblasts and increased number of connective tissue collagenous fibers, the interalveolar walls were thicker than normal (Fig. 4). There were scattered foci of coagulative necrosis in hepatic tissue. These foci were small and had no particular histological pattern. There were also scattered and limited areas of mononuclear infiltration. Mild to moderate fibrosis of portal area was noticed, although fibrosis of the central vein was so severe that its wall was thickened. The fibrosis (centrilobular) was severe and distributed throughout the hepatic sections (Fig. 5, 6).

Heartworm or *Dirofilaria immitis* is a parasite of cardiovascular of domestic and wild carnivores especially dog. In the present study, hematology, serum biochemistry and histopathological findings of five mixed-breed stray dogs with *D. immitis* were evaluated in this study and we thought, that these findings could be used for diagnosis of this helminth infestation. The assessment of hematological parameters demonstrated a mild anemia in dogs with *D. immitis* infestation with a significant decrease in the number of red blood cells, compared to the non-infected group. Data analysis also showed an increase in number of eosinophilia. However, there was no significant difference between infected and non-infected dogs. Niwetpathomwat *et al.* (2007) reported that, a major hematological finding in dogs infested with *D. immitis* showed a mild to moderate anemia, mild to severe thrombocytopenia, marked leukocytosis, moderate to marked neutrophilia, eosinophilia and monocytosis. Decrease in RBC, Hb and PCV levels were also reported by Atwell and Buoro (1983) and Sevimli *et al.* (2007). Present results concerning to RBC parameters were in agree with the mentioned reports. In the present study, there was a mild eosinophilia in infested dogs. It is in agreement with the results of Niwetpathomwat *et al.* (2007) that reported a general leukocytosis, eosinophilia and neutrophilia in dogs with dirofilariasis.

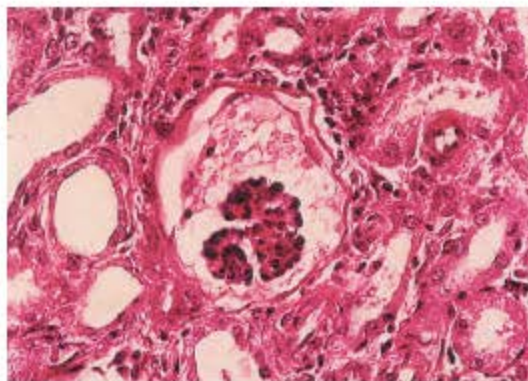


Fig. 1: Renal tissue, atrophy of glomeruli, presence of eosinophilic proteinaceous substances in urinary space and fibrosis of periglomerular area (H and E, x401/5)

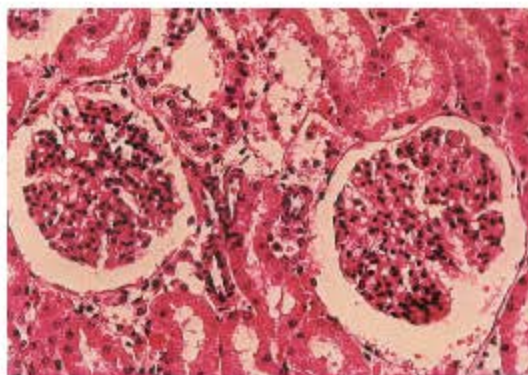


Fig. 2: Renal tissue, glomerular hypercellularity with increased number of mesangial matrix, mild infiltration of mononuclear and multinuclear cells which might be indication of mild membrano-proliferative glomerulonephritis (H and E, x343)

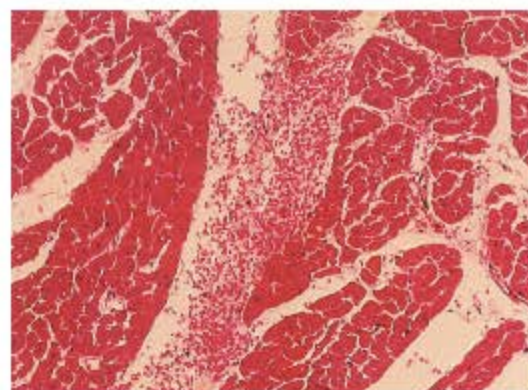


Fig. 3: Cardiac tissue, massive interfascicular hemorrhage of heart (H and E, x224)

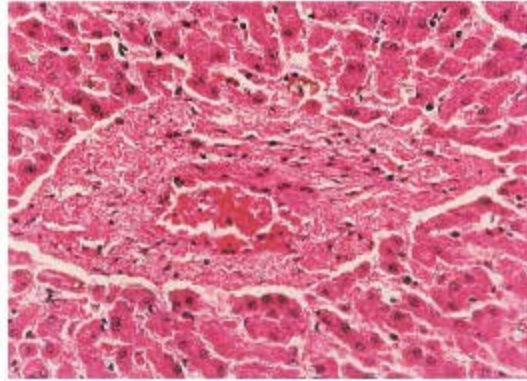


Fig. 4: Pulmonary tissue, alveoli are empty and interalveolar wall is thickened due to proliferation of connective tissue and smooth muscle indicating interalveolar fibrosis (H and E, x105)

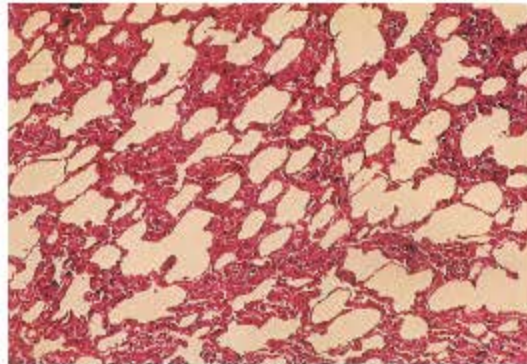


Fig. 5: Hepatic tissue, focal coagulative necrosis of liver having no particular pattern (H and E, x235)



Fig. 6: Liver tissue, severe fibrosis of periportal (centrilobular) and central vein were seen. The wall of this vein is thickened and this lesion is called cardiac fibrosis indicating prolonged congestion due to right heart failure (H and E, x384)

The serum biochemical findings were different in five infected dogs. But there was a moderate and mild increase in activity of aspartate aminotransferase and alkaline phosphatase, respectively. Mild decrease in BUN concentration was also determined. Niwetpathomwat *et al.* (2007) showed marked increase in AST and ALP, but difference between infected animals and control groups was not significant. It has been reported that AST, ALP, ALT, BUN and creatinine may increase in serum of dogs with dirofilariasis (Sevimli *et al.*, 2007; Sodicoff, 1995). Also study on the hematological and biochemical values may be useful for determining blood component profiles. Variations in these results compared with reference values can give information related to the health status of animal (Niwetpathomwat *et al.*, 2007).

Meanwhile, five male dogs with spontaneous dirofilariasis were necropsied and tissue samples were taken from the heart, lung, liver and kidney for histopathology. Several lesions were observed in pathologic exams in these organs. Although, these findings were not pathognomonic for dirofilariasis but we can use histopathological exams accompanied with blood and biochemical parameters for diagnosis of *D. immitis*. Paes de Almeida *et al.* (2003) showed that in infected dog with *Dirofilaria immitis*, thickening of the Glomerular Basement Membrane (GBM), the presence of dense deposits in the GMB and foot process effacement were the most frequent lesion observed. If the infection period is longer, these lesions are more severe. The presence of dense deposits indicate that pathologic lesions in dirofilariasis caused by deposits of immune complexes in the membrane. Meanwhile, these findings indicate that immature worms, as well as microfilariae and even adult worms can cause glomerulonephropathy (Paes de Almeida *et al.*, 2003). In this study, focal mononuclear interstitial nephritis in the kidneys of dogs with *D. immitis* infestation was observed. A membranoproliferative glomerulonephritis was observed in dogs infested with *Dirofilaria immitis* (Grauer *et al.*, 1987). There was irregular thickening and dense deposits in the basement membranes of glomerular capillaries, along with a modest increase in mesangial cells and matrix. Also, in liver, there were numerous granulomas in the sinusoids which contained degenerated microfilariae (Simpson and Jackson, 1985). Therefore, several kinds of kidney damage are described in dogs infested with *D. immitis*: (1) immune-mediated glomerulonephropathy, (2) glomerulosclerosis, (3) chronic interstitial nephritis and (4) amyloidosis (Niwetpathomwat *et al.*, 2007). Lesions indicative of right-sided heart failure consisting of right ventricular dilation and chronic passive congestion of the liver were observed. An acute inflammatory reaction in lung characterized by aggregates of eosinophils, neutrophils, macrophages and lymphocytes was associated with dirofilariasis. Other lesions in interalveolar septa of dogs with chronic infection included endothelial degeneration and hypertrophy, perivascular aggregates of plasma cells, hypertrophy and hyperplasia of smooth muscle cells and interstitial fibrosis (Castleman and Wong, 1982).

Heartworm is not only of veterinary importance but also has zoonotic potential and their pulmonary nodule may discover on chest radiographs during a routine physical examination. Of course pulmonary dirofilariasis, caused by *D. immitis*, is not common in human (Hirano *et al.*, 2002). Radiographically, in human, most lesions as a single spherical nodule were found in the inferior or middle portion of the right lung. Peripheral eosinophilia was noted in only 10% of the patients. Pathologically, all cases featured a histiocyte-rimmed necrotic nodule containing fragments of a partially degenerated *D. immitis* (Flieder and Moran, 1999). Also, coagulation necrosis with fibrosis and granulation in the nodule edge, which contained inflammatory cells, were observed (Hirano *et al.*, 2002). Lesions caused by *D. immitis* may often be confused with the primary or metastatic lung carcinomas in humans and precise diagnosis is given as a result of histopathological examinations (Milanez de Campos *et al.*, 1997).

In conclusion, complete laboratory test, especially serological test accompany with history and clinical findings are better for diagnosis of *D. immitis* and it seems that some organs show non-specific lesions with heartworm infestation because of the circulating microfilariae.

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