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STUDY OF SOME PATHOGENESIS ASPECTS IN PUPPIES AFTER EXPERIMENTAL INFECTION OF PUPPIES WITH *GIARDIA DUODENALIS*

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ABSTRACT : The purpose of this study was to investigate some pathogenesis aspects in puppies after experimental infection with *G. duodenalis* of 10 local breed dogs, age 4 months as experimental infected dogs and 5 dogs as control. A clinical signs of experimental infection of puppies showed mild increased in the temperature and respiratory rate after one week of infection. The results of biochemical tests showed significant increas in the levels of blood sugar and decreased in the insulin and amylase level; significant decreased in the levels of serum cholesterol, triglycerides, VLDL, LDL and HDL, while alkaline phosphatase showed significant increased.The measurement of antibodies concentration revealed that the significant decreased in the concentration of the IgA was found in the experimental infected dogs compared with control dogs, while no significant differences was found in the concentration of the IgG.The measurement of antioxidant concentration demonstrated a significant decreased in the levels of glutathione and superoxide dismutase in the duodenum, liver, gall bladder and pancreas in infected dogs compared with non infected dogs.A different histopathological changes was found induodenum, Pancreas, gall baldder and liver. Internal tissue (duodenum, pancreas, liver and gall bladde) were Immunohistchemically evaluated for two receptors , which were tumor nectosis factor – alpha (TNF-alpha) and interluken- 6 (IL-6). Tumor nectosis factor – alpha receptor was positive in 80% of doudenum , 60% of pancreas, 50% of gall bladder and 30% of liver of the expermintal infected dogs. Duodenum postive (70%) for IL-6 more frequent than of the panceas, liver and gall bladder (60%, 20% and 60%, respectivly).

Key words : TNF-alpha, IL-6, glutathione, superoxide dismutase, *Giardia*, dog.

INTRODUCTION

Giardia duodenalis is a neglected parasites disease. The WHO has reported more than 200 million of human infection each year. Intraepithelial giardiasis is a rare entity, there are only five reported showing invasive giardiasis (Martinez-Gordillo *et al*, 2014). *G. duodenalis* is intestinal parasite that causes water borne diarrhea in humans and animals especially pet animals (dogs and cats) (Swadi, 2008, 2011). Giardiasis is may by asymptomatic or symptomatic with foul smelling diarrhea with steatorrhea, abdomen pain, anorexia and weight loss due to malabsorption (Hill, 1993). *G. duodenalis* has been divided into genotypes. Genotypes A (subgenotypes AI and AII) and B infect human and animals. C and D restricted to infected dogs, and isolated in dogs in Iraq (Swadi and Zenad, 2019), E infect cattle, F in feline, G was isolated from rats (Read *et al*, 2004) and H found in marine mammal (Lasek-Nesselquist *et al*, 2010).

The pathophysiologic mechanism involves trophozoite adhesion to epithelial cell by the ventral suckling disc, using nonspecific mechanical forces (Adam, 2001). The interaction between *Giardia* suckling disc and enterocytes lead to a lesion on microvillus border of epithelium (Erlandsen *et al*, 1988) and increase in intestinal permeability (Troeger *et al*, 2007). In recent studies reported that *G. duodenalis* is have been ability to invading host tissue (Cotton *et al*, 2015). In sever giardiasis, impairment of the exocrine of pancreatic function can occur (Nakano *et al*, 1995) and present of fat in the feces is result from insufficient exocrine function of pancreas (Nelson *et al*, 2003). Inflammatory lesion in hepatic tissue represent as chronic hepatitis was accompanying infection by *Giardia* (Sotto and Gra, 1985). In giardiasis, some studies recorded significant decreased in serum cholesterol and triglycerides (Salman *et al*, 2007) and elevation of serum alkaline phosphatase (Al-Jebory, 2005). After *Giardia* antigen recognized by the host is lead to increase

production of IL-6, which elevated total IgE as well as local and systemic stimulates, differentiation of B-cell and regulates of IgA production (Jimenez *et al*, 2004). The elevation in production of cytokine tumor necrosis factor alpha (TNF-α) during giardiasis, contributes in protection against *Giardia* parasite, especially in the early control of disease (Saghaug *et al*, 2016). Other function for TNF-α is increase permeability of small intestine epithelium (Gibson, 2004), therefore TNF-α is an essential cytokine for determining duration and burden of *Giardia* infection in animals and deficiency in TNF-α lead to persistence *Giardia* infection (Zhou *et al*, 2007).

MATERIALS AND METHODS

Experimental animals

Two groups (10 dogs for infected and 5 dogs for control) of local breed dogs age four months, free from infected by *Giardia duodenalis*. Dogs were breeding at the Al-Furat Al-Awsat Technical University, Institute of Mussaib for the period from March 2017 until the end of the experiment.

Infection

Giardia duodenalis (assemblage C) cysts were obtained from feces of diarrheic household dog, infected dogs by oral administration of 25000 *Giardia duodenalis* cysts through an esophageal tube (Ali *et al*, 2014). Clinical sings and blood from radial vein for different examination was done before infection and weekly for three weeks after appearance of cysts in the infected animals together with control animals. At end of experimental study euthanized infected and control dogs (Collins *et al*, 1987). After sacrification of all animals, two parts of the duodenum, liver, gall bladder and pancreas from each animal were taken, one part immersed in concentration 10% of buffered formalin solution for histopathological examination (Bancroft *et al*, 1996) and other part immersed in phosphate buffered solution (pH7) for measurement the antioxidant level in these tissues.

Immunohistochemistry

Detection of canine TNF-α receptors in the different tissues

TNF-alpha receptors were detected as protocol manufacture instructions by antibodies-online® GmbH/ Germany kit, which is polyclonal antibodies utilizing for IHC assay to identify TNF-alpha expression in tissues.

Detection of canine IL-6 receptors in the different tissues

Canine IL-6 receptor was detected as protocol manufacture instructions by antibodies-online® GmbH/ Germany kit, which is polyclonal antibodies it has been ability to recognize IL-6 in immunohistological staining and identify IL-6 expression in the tissues.

Determination of IgA and IgG proteins by radial immuno-diffusion plate and examination of biochemical, Amylase, Insulin, Glutathione (GSH) and Superoxide dismutase was done as protocol manufacture instructions.

Statistical analysis

The Statistical Analysis System- SAS (2012) program was used to effect of difference factors in study parameters. LSD test to significant compare between means in this study (SAS, 2012).

RESULTS

The results of biochemical tests

Some pancreas function tests

The results showed significant increas in the the levals of blood sugar and decreased in the insuline and amylase level in the exparemintal infected dogs compared with control non infected dogs (Table 1).

The lipid profile and alkaline phosphat result

The resulrs showed signficant decreased in the levals of serum cholestrol, triglycerides, VLDL, LDL and HDL, wiohle alkilne phosphatease showed signficant increased

Table 1 : Some pancreas function tests of the dogs in the experimental infection study.

Parameter	Week								LSD value
	0		1		2		3		
	Control	Infected	Control	Infected	Control	Infected	Control	Infected	
Blood sugar (mg/dl)	95±4.68	95.2±4.74	95.6±5.46	128.4±8.21	95.4±4.62	136±6.50	94.4±4.02	147.8±9.39	25.71 *
Insulin concentration (μIU/ml)	28.15±1.52	42±1.84	35.85±1.25	19.69±1.07	25.86±1.44	14.31±0.72	36.29±1.37	10.57±0.46	22.68 *
Amylase concentration (U/L)	384.2±14.62	405.4±17.51	301±11.86	294.2±8.30	366.4±14.05	211.7±10.24	355.2±13.20	201±7.52	83.74 *

* ($P<0.05$).

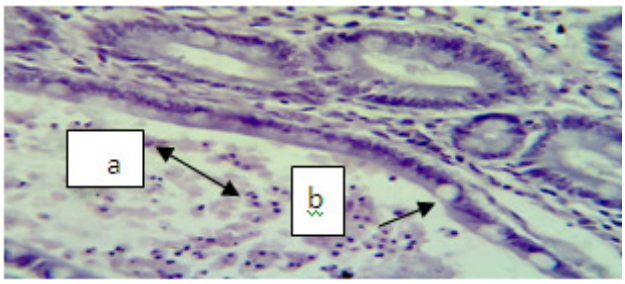

Fig. 1 : Section in the duodenum shows mucus and inflammatory cells in in the dilated mucosal gland (a) with hyperatrophy of goblet cells (b) (H and E stain 400X)

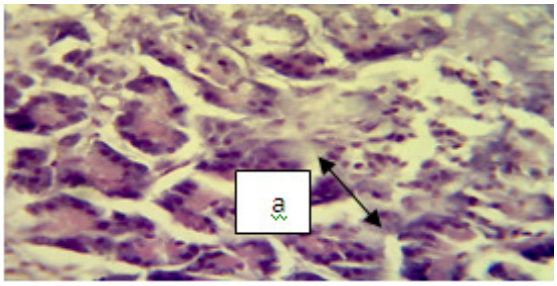

Fig. 2 : Section in the pancrease shows depletion of islets cells (a) (H and E stain 400X).

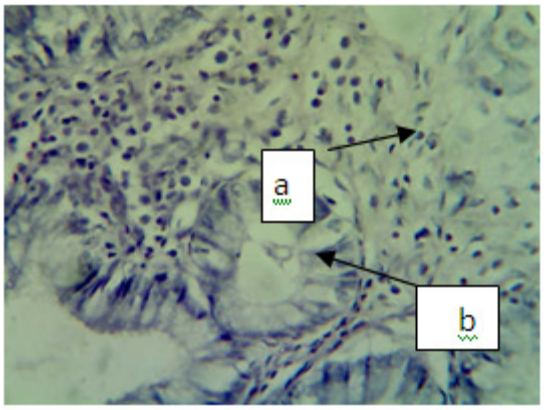

Fig. 3 : Section in the gall bladder shows inflammatory cells particularly mononuclear cells infiltration in subepithelial layer (a) and erosion of epithelial cells (b) (H and E stain 400X).

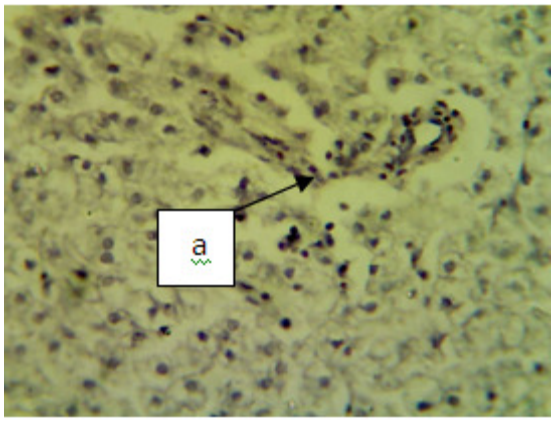

Fig. 4 : Section in the liver shows vacuolation of hepatocytes with few inflammatory cells aggregation in portal area (a) (H and E stain 400X).

Table 2 : The results of the lipid profile and alkaline phosphat tests of the dogs in the experimental infection study.

Parameter	Week								LSD value
	0		1		2		3		
	Control	Infected	Control	Infected	Control	Infected	Control	Infected	
Serum cholesterol (mg/dl)	166.4±9.53	164.7±11.67	164±8.39	155.2±8.48	168.2±12.36	137.5±7.42	167±9.68	115.6±6.32	35.82 *
Triglycerides (mg/dl)	95±4.78	100.5±7.51	103.4±5.66	91.6±4.06	95.4±4.93	62.4±2.46	98.4±4.72	60.2±2.53	27.74 *
VLDL(mg/dl)	19±1.05	20.1±0.84	20.68±0.79	18.32±0.88	19.08±1.17	12.48± 0.57	19.68±0.82	12.4±0.48	5.33 *
LDL(mg/dl)	35.4±1.58	24.6±1.07	36.3±2.48	32.88±1.03	33.12±1.85	30.02±1.58	31.32±2.37	16.56±0.66	8.21 *
HDL(mg/dl)	112±5.47	120±7.42	107±5.81	104±4.77	116±6.31	95±2.85	116±6.24	87±4.64	19.57 *
Alkaline phosphatase (U/L)	14.74±0.41	36.67±1.64	15.22±0.66	39.17±2.15	14.80±0.42	41.04±2.36	16.30±0.54	42.18±1.92	8.49 *

* ($P<0.05$).

in the exparemantal infected dogs compared with control dogs (Table 2).

Measurement of antibodies concentration

The significant decreased in the concentration of the IgA was found in the experimental infected dogs compared with control dogs, while no significant differences were found in the concentration of the IgG (Table 3).

Measurement of antioxidant concentration

The concentration of the antioxidant concentration (Table 4).

Histopathological results

Duodenum (Fig. 1), pancreas (Fig. 2), gall bladder (Fig. 3) and liver (Fig. 4).

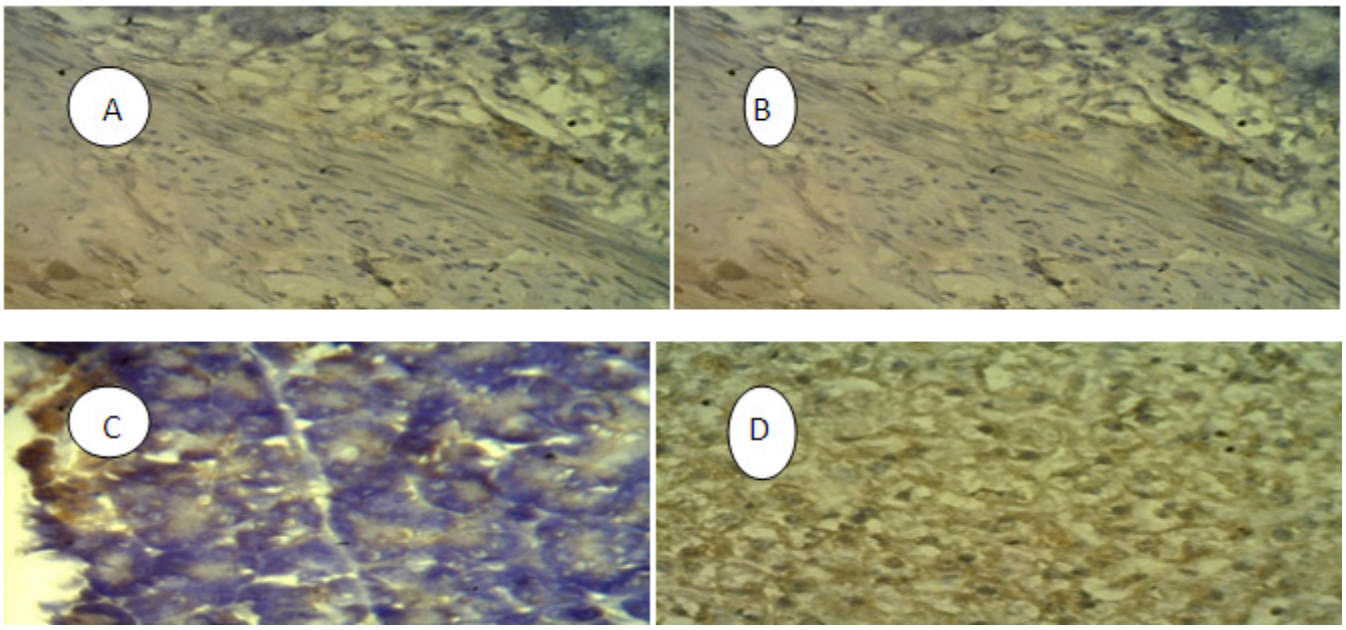

Fig. 5 : Positive immunohistochemical expression of Tumor Necrosis Factor (A: duodenum; B: liver;C: gall bladder; D: pancreas).

Table 3 : The concentration of the IgA and IgG of the dogs in the experimental infection study.

Parameter	Week								LSD value
	0		1		2		3		
	Control	Infected	Control	Infected	Control	Infected	Control	Infected	
IgG (mg/dl)	1039.1 ± 46.55	1016.06± 51.09	9693.06± 43.94	1054.62± 61.68	1008.62± 48.02	1089.77± 39.73	1046.7± 45.25	1129.33± 55.62	174.37 NS
IgA (mg/dl)	95.1±3.61	95.07±3.58	96.4±4.36	85.8±3.07	93.74±3.63	79.32±2.85	96.4±3.73	72.99±3.02	14.65 *

* (P<0.05). NS: Non-Significant.

Table 4 : The concentration of antioxidant of the dogs in the experimental infection study.

Parameter	Week								LSD value
	0		1		2		3		
	Control	Infected	Control	Infected	Control	Infected	Control	Infected	
Glutathione (mmol/g)	0.302± 0.006	0.053± 0.001	0.309± 0.004	0.049± 0.002	0.313± 0.005	0.051± 0.001	0.281± 0.006	0.038± 0.002	0.093 *
Superoxide dismutase (U/g)	76.04±3.47	28.61±1.38	52.43±2.07	24.39±0.94	62.96±1.86	26.99±1.52	68.40±2.38	25.93±1.04	11.52 *

* (P<0.05).

Immunohistchemical evalution of tissues

Internal tissue (duodenum, pancreas, liver and gall bladde) were Immunohistchemically evaluated for two receptors, which were TNF-α and IL-6.

TNF-α receptor

Eight of 10 doudenum of the expermintal infected dogs (80%), 60% of pancreas, 50% of gall bladder and 30% of liver were positive for TNF-alpha. The positive cases of of doudenum were distributed as 2 weak and 6 strong (25% and 75%, respectively) (Table 5, Fig. 5).

Interluken-6 receptor

Duodenum tissue postive (70%) for IL-6 more frequent than othe panceas, liver and gall bladder (60%, 20% and 60%, respectivly) (Table 6, Fig. 6).

DISCUSSION

Giardiasis a common enteric protozoan among human and animals in developing countries including Iraq, the prevalence of stool positivity may range from 1% to 40% depending on the geographic area and age group surveyed, it is higher in poor sanitations area (Benenson, 1995). In developed countries, infection rates varied from 2-5% (Farthing, 1996). Depending on the previous many

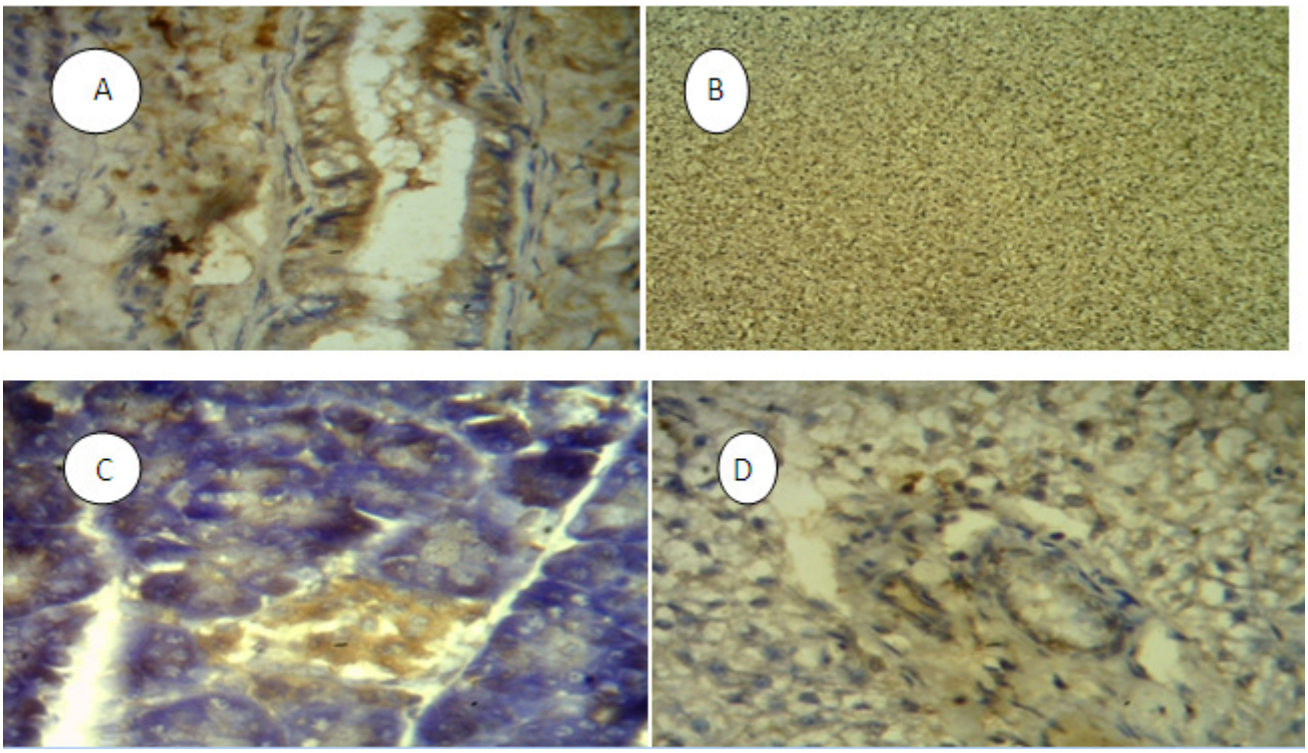

Fig. 6 : Positive immunohistochemical expression of Intrluken-6 (A: duodenum; B: liver; c: gall bladder; d: pancreas).

Table 5 : Internal tissue distrbuted by the expreession of tumer nectosis factor-alpha.

Tissue	Total	Tumernectosis factor-alpha reaction	Total positive		Weak		Intermetiate		Strong	
			No.	%	No.	%	No.	%	No.	%
Dodenum	10	Positive	8	80	2	25.00	0	0.00	6	75.00
		Negative	2	20	-	-	-	-	-	-
Pancreas	10	Positive	6	60	3	50.00	0	0.00	3	50.00
		Negative	4	40	-	-	-	-	-	-
Liver	10	Positive	3	30	1	33.33	2	66.67	0	0.00
		Negative	7	70	-	-	-	-	-	-
Gall bladder	10	Positive	5	50	2	40.00	1	20.00	2	40.00
		Negative	5	50	-	-	-	-	-	-

Table 6 : Internal tissue distrbuted by the expreession of IL-6

Tissue	Total	Tumernectosis factor-alpha reaction	Total positive		Weak		Intermetiate		Strong	
			No.	%	No.	%	No.	%	No.	%
Dodenum	10	Positive	7	70.00	2	28.57	2	28.57	3	42.86
		Negative	2	30.00	-	-	-	-	-	-
Pancreas	10	Positive	6	60.00	1	16.67	3	50.00	2	33.33
		Negative	4	40.00	-	-	-	-	-	-
Liver	10	Positive	2	20.00	1	50.00	1	50.00	0	0.00
		Negative	8	80.00	-	-	-	-	-	-
Gall bladder	10	Positive	6	60.00	3	50.00	-	-	3	50.00
		Negative	4	40.00	-	-	-	-	-	-

surveys studies performed in different regions in Iraq by old and new technical methods to detection of *Giardia duodenalis* among peoples, the prevalence of infection ranges from 1.77% to 38.5%.

Giardia duodenalis is one of the most common protozoal infections in different animal species in Iraq, which cause diarrhea or may be asymptomatic infection and remains as reservoir causing transmission of this infection to human. In many different surveys performed in animals, the highest infection rate was 76.4% in asymptomatic cattle (Ahmed and A'aiz, 2015) and the lowest was 2.5% in cats (Hadi and Faraj, 2014), some factors such as geographical location, status of animal ownership, sampling protocols, demographic factors, anthelmintic usage and diagnostic techniques are responsible for the wide range of parasite prevalence. In 2000, the first detection of *Giardia* sp. in dogs in Baghdad with infection rate7.05% (Swadi, 2000).

Experimental infection study

Clinical signs

The time between experimental infected dogs and the appearance of *Giardia* cysts in the feces was two to three weeks, this agrees with other study of Jokipii *et al* (1985). The most prominent symptoms of all infected dogs were diarrhea and only two infected dogs showed vomiting, those signs recorded in many studies (Hurber *et al*, 2005). There was slight increase in respiratory rates and temperature in the first week after infection in infected group, this hyperthermia and increased respiratory rate may be due to acute phase of infection and the effected of the protozoan on internal tissues, this agree with other study which showed fever was present at the beginning of infection with giardiasis (Ortega and Adam, 1997).

Biochemical examination

Some pancreas function tests

The results showed significant increas in the the levals of blood sugar and decreased in the insuline and amylase level in the exparemintal infected dogs compared with control non infected dogs table, this result disagree with Gupta and Mehta (1973) were found, in study of pancreatic function in children infection with giardiasis, a significant reduction in lipase and trypsin activity, but none in amylase in the duodenum juice.

The lipid profile and alkaline phosphat result

The resulrs showed signficant decreased in the levals of serum cholestrol, triglycerides, VLDL, LDL and HDL, while alkilne phosphatease showed signficant increased in the exparemantal infected dogs compared with control dogs table. Studies of the lipid metabolism of *G. duodenalis* trophozoites have revealed no synthesis of cellular phospholipids or sterols, rather, fatty acids and cholestrol are incorporates from the growth medium into the lipid fraction of the trophozoite (Jarroll *et al*, 1981). This result was agree with many studies, such as Al-Shamari and Jabir (2013) that thay found decreased in cholestrol levels in giardiasis patients while triglycerids, high density lipoprotein, low dwnsity lipoprotein and very low density lipoprotein were normal in the same pateins, also, Alhuchaimi *et al* (2017) found significantly declined in the total serum cholestrol leval in *G. lamblia* infection among children with acute diarrhea in Al-Najaf province. This result agree with Muhsin and Daoud (2015) they showed signficant decreased in cholestrol and triglycerides in infected patients with giardiasis and the healthy persons group. *G. duodenalis* consumed the cholestrol of the host in the biosynthesis of the cell, because the parasite is unable to synthesize cholestrol by itself, *Giardia* may consumed only cholestrol and neglecct the other lipids, cholestrol starvation consider a trigger for trophozoite differentiatin into cyst (Katelarisal *et al*, 1991).

The importance of bile in the growth of *Giardia* was first suggested by the propensity of the organisms to colonize the duodenum and jejunum. The enhanced growth of trophozoites in the presence of biliary lipids suggest that bile may be important as a source of lipids (Farthing *et al*, 1985) and *Giardia* obtain cholesterol, which consider necessary for membrane biogenesis from the milieu of the upper small intestine, which particularly rich in biliary and dietary cholestrol (Thomson *et al*, 1993). Result of this study showed significant increase in serum alkaline phosphatase (ALP), this result agree with other study which found same elevation in serum ALP in all patients infected with giardiasis (Salman *et al*, 2007). The elevation of serum ALP during giardiasis is because of that giardiasis produce diarrhea and malabsorption (Behrman, 1996) and beccause of the important role of ALP in the process of transportation of phosphate, calcium, sodium and potassium, in addition to its imprtant role in the metabolic process, the increase level of ALP during diarehea due to loss of these mineral during this period (Lott and Wolf, 1986).

Measurement of antibodies concentration

It was well known that cell mediated and humeral immune response play a major role in protection of the body against the infection (Roberts-Thompson *et al*, 1980). The presence of Immunoglobulin in the serum and other body fluids consider being as a part of the immune system response to antigens presents. Antibodies include several immunoglobulin classes IgM, IgG, IgA and IgE, the detection of individual class or whole classes can be

done by serological tests depending on the test protocol (Woof and Burton, 2004). Each of this immunoglobulin is differing in function and significance: in infections, the antibody IgM, is usually detected first but decline within a few weeks. IgG often show arises a little later but usually lasts for longer, mainly found in the absence of detectable IgM and give the evidence for past infection, resent infection is usually indicating by the presence of IgM, with or without IgG. IgA is present in the serum and it secreted into the mucosal, it is particular association with parasitic and other infections in gut mucosa (Faubert, 2000). Study of antibodies are used to help characterize the pathogenesis and pathology, yield epidemiologically information that reflect the prevalence and incidence and in clinical diagnosis (Priest *et al*, 2005).

The significant decreased in the concentration of the IgA was found in the experimental infected dogs compared with control dogs, while no significant differences were found in the concentration of the IgG. This result was agreeing and disagrees with other studies. Significant decrease in immunoglobulin A and G in patients with chronic giardiasis in compared to healthy group were recorded by Al-Shebly and Shlash, 2016). In the present study, decreased level of immunoglobulin A in experimental infected dogs while other reports indicate increased total serum immunoglobulins in patients with persistent diarrhea and giardiasis (Char *et al*, 1993). Also, Al-Khayat *et al* (2016) found higher concentration of serum IgM, IgG and IgA in patients infected with *G. duodenalis* in Al-Karkh region/Baghdad by using Enzyme Linked Immunosorbent Assay.

Measurement of antioxidant concentration

The current results showed signficant decreased in the levals of glutathione (GSH) and superoxide dismutase (SOD) in the duodenum, liver, gall bladder and pancreas in infected dogs compared with non infected dogs. Same result was obtaining by Al-Hadraawy, 2014), which was showed significant decreased ($p<0.05$) in GSH in *G. lamblia* infected patients in compared to control group. The decrease of GSH and SOD levels in those tissues can give an idea on oxidative damage and indicated to important role of antioxidant in development of the disease. Moreover, lesions which are found in these organs after experimental infection by *G. duodenalis* may be occur due to oxidative stress is believed to be accomplished by a signficant decreased in the levals of glutathione and superoxide dismutase in the duodenum, liver, gall bladder and pancreas. It may be due to the resistant of parasite to phagocytosis by increasing the free radical and this leads to decreased in the level of GSH in tissues (Yazar *et al*, 2003). Those results were similar to the finding by Al-Mezwery, 2006), who observed significant decreased of GSH level in three tissue organs (liver, spleen and heart) of rats which are infected with *Myocardia asteroids* compared to the healthy control groups.

Histopathological results

Different organs of experimental infected dogs with *Giardia duodenalis* were subjected to histopathological examination to evaluate these tissues in terms of histopathological types.

Histopathological sections of duodenum revealed variable changes characterized by mucous and inflammatory cells in the dilated mucosal gland with hyperatrophy of goblet cells and showed erosion of their epithelial cells in addition of inflammatory cells particularly mononuclear cells infiltration between mucosal glands with atrophy of the villi characterized by mononuclear cells infiltration in dilated lamina properia and fused the villi together. The same picture was shown by many studies, which is showed trophozoites during the invasion process and goblet cells hyperplasia in samples from dogs, therefore, the epithelial invasion phenomenon may be play a role in the pathogenic mechanisms involved in the symptomatic giardiasis (William and Ramzy, 2008). Histological analysis of *Giardia muris* infections shows small intestinal mucosal tissues signs of significant inflammation, such as increases in intra-epithelial lymphocyte numbers and mast cell hyperplasia (Hardin *et al*, 1997).

A histopathological examination of the liver showed few histopathological changes was showed in the liver such as vacuolation of hepatocytes with few inflammatory cells aggregation in portal area with with inflammatory cells aggregation in one side of blood vessels. Same result recorded by Sotto and Gra (1985), they found inflammatory lesions hepatic tissue represented by chronic hepititis. Also, pancreas showed depletion of islets cells and congested blood vessels in the interstitial tissue. Results of this study revealed ability of *G. duodenalis* to produce variable lesions, it may be due to that *Giardia* known to contain or release a variety of potentially toxic substances, such as proteinase and these proteinase activate host protein receptors and have ability to localization hepatic tissue throw portal area to produce many change such as vacuolor degeneration, fatty change and apoptosis via the mucosal injury after attachment of large numbers to brush border, or may be due to decreased in activity of enzymes that localized in brush border (Scott *et al*, 2002).

The present study demonstrated that inflammatory

cells particularly mononuclear cells infiltration in subepithelial layer and erosion of epithelial cellsin the gall baldder, this result agree with stuy in cows in Turky (Degeri and Ozcelik, 2003), they found *Giardia* in the gall bladder with histopathological changes.

Immunohistchemical evalution of tissues

Tissue section of duodenum, pamcreas, liver and gall blader from expermintal infected dogs with *Gradia duodenalis* were immunohistochremicaly evaluted for two cytokines, which were TNF-α) and IL-6. TNF-α play an important role in the eary control of giardiasis and an important cytokine for determining the parasite burden and duration of *G. lamblia* infection and infected mice deficient in TNF-α with *G. lamblia* had much higher parasite numbers than controls during the firest two weeks of infection (Gibson, 2004). In the present study detection of TNF-α in the doudenum, pancreas, gall bladder and liver indected to induce immune respone during giardiasis.

IL-6 is important innumerous infection. IL-6 can promote T cell survival and differentiation toward Th 17 cell, as well as B cell proliferation and differentiation to plasma cells. IL-6 is required for control of this infection,but it is unclear what its role is or which cells are required to produce this cytokine to generate efficient immunity, therefore inability of IL-6 deficient mice to respond to *Giaridia* challenge (Kamda *et al*, 2012). Duodenum tissue postive (70%) for IL-6 more frequent than other panceas, liver and gall bladder (60%, 20% and 60%, respectivly) indected to concentated infection in the duodenum. The detection of IL-6 in different tissues in this study is agree with Matowicka-Karna *et al* (2008), they found in patients infected with *G. intestinalis*, the mean levels of IL-6 was statically signicantly higher as compared to healthy pateints ($p<0.001$).

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The research was performed independently, there is no funding, influence over study design, analyses, manuscript preparation, or scientific publication.

Ethical clearance

The project was approved by the local ethical committee (College of Veterinary Medicine, Baghdad University).

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