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IMMUNOHISTOCHEMICAL STUDY OF TUMOR NECROSIS FACTOR-ALPHA (TNF- α) EXPRESSION IN LUNG, LIVER, AND SPLEEN DURING BRUCELLOSIS INFECTION

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ABSTRACT
The present study was conducted to investigate the level of TNF-α in the liver, spleen, lung and brain tissue at 9 weeks. Fifteen adult Swiss Albino mice at the age of two months were divided into five groups The1st group (G1) consist of 10 mice immunized with (CFBAgs), two doses for two weeks intervals (protein concentration 4.2mg\ml)). The 2nd group consist of 10 mice, dosed orally daily with diazinon (83. 7 mg/k.g B.W) for 9 weeks. The 3rd group consists of 10 mice that were immunized as in the 1st group and were administrated with diazinon daily for 9 weeks as in 2nd group. The 4th group consist of 10 mice that was not given anything but they injected I/P with 0.4 ml of bacterial suspension containing 1 x 10^9 CFU/ ml of viable virulent *B. abortus* after one month and served as control positive group. The 5th group consists of 10 mice that were administrated orally with 0.3 ml of normal saline and served as control negative group. At the end of the experiments (9 weeks), all animals were sacrificed and pieces of liver, spleen, lung and brain tissue were fixed in 10% normal buffer formalin for 72 hrs for Immunohistohistochemistry examination .The present study showed that the diazinon significantly decreased the TNF level as compared with the control and other groups.

KEYWORDS: TNF-α, Swiss Albino mice, diazinon.

INTRODUCTION

Brucellosis is caused by Brucella species, which infect a wide range of mammalian hosts. The World Health Organization considers brucellosis as one of the seven neglected zoonoses that contributes to the perpetuation of poverty[1]. Cytokines play two critical roles in immune responses of brucellosis: (i) to mediate innate and adaptive immunity and (ii) to direct the immune response among immune-associated cells. Cytokines, regarded as key players in brucellosis, are IL-12, IFN-γ, and TNF-α, IL-12 is a key cytokine produced by B cells, NK cells and macrophages and leads Th1 immune responses in the host that will ultimately induce the secretion of IFN-γ from T cells[2]. Tumor Necrosis Factor-alpha (TNF-α) is important pro-inflammatory cytokines that is produced simultaneously and share a common spectrum of biologic activities and plays a major role in host defense and regulation of the immune response [3]. TNF-a play a role in eliciting immune response that both tumor necrosis factor (TNF)- alpha and CD8 T cells were involved in controlling bacterial numbers. The role of TNF-alpha may depend upon the presence of interferon-gamma early in the infection since when TNF-alpha was neutralized in interferon-gamma gene knockout mice there was a marked increase in splenic macrophages, NK cells and neutrophils but not a significant increase in colony-forming units[5], showed Pathology has been largely determined by the recovery of Brucella colony-forming units (CFU) in well-studied organs such as liver and spleen. Immunohistochemistry is one of the several methods used to diagnose Brucella spp., and it has been used to detect Brucella spp.antigens in formalin-fixed, paraffin-embedded tissues in cows[6]. This technique allows us to decisively in dicate the presence of metabolically active bacteria in deep tissues of mice[7]. Immunohistochemical technique could be a complementary tool to serology and bacteriology for the diagnosis of brucellosis [8].

MATERIALS & METHODS

Experimental design

Fifteen adult Swiss Albino mice at the age of two months were divided into five groups The1st group (G1) consist of 10 mice immunized with (CFBAgs), two doses for two weeks intervals (protein concentration 4.2mg\ml). The 2nd group consist of 10 mice, dosed orally daily with diazinon (83.7 mg/k.g B.W) for 9 weeks. The 3rd group consists of 10 mice that were immunized as in the 1st group and was administrated with the diazinon daily for 9 weeks as in 2nd group. The 4th group consist of 10 mice that was not given anything but they were injected I/P with 0.4 ml of bacterial suspension containing 1 x 10^9 CFU/ ml of viable virulent *B. abortus* after one month and served as control positive group. The 5th group consists of 10 mice that were administrated orally with 0.3 ml of normal saline and served as control negative group.

Treatment

Median lethal dose (LD_{50}) of diazinon**:**-"Up-and-down" method was performed according to Dixon [9]**.**

Brucellin preparation: This antigen was prepared according to Mitov [10]**.**

Culture filtrated *B. abortus* antigen (CFB Ags) This Ag was used for immunization animals.

1. *B. abortus* was cultured on TSA plate and incubated at 37°C for 7days.Microscopic examination by Gram stain to insure pure the purity of culture.
2. The culture was harvested by adding PBS PH 7.2 after 10min.
3. The suspension was centrifuged at (30000 rpm /4 °C /30 min.) by cold centrifuge.
4. The supernatant was filtered by Millipore filter 0.22nm.
5. The filtrated fluid was examined by Gram Stain and cultured in blood agar to confirm sterility of this antigen.
6. The total protein concentration of this antigen was measured according to Biuret procedure.

the detection of TNF- α antigen in paraffin embedded sections

A- Paraffin- embedded sections from each specimen was cut at 4μm, mounted on glass and dried overnight at 37°C.

B-To determine the signal specificity, negative and positive control slides were included for each immunohistochemical run.

1-Immunohistochemistry procedure

Slides were dipped sequentially as followed:

- The tissue sections were deparaffinized in xylene (2×10min) and Re-dehydrated through graded alcohol as follow:
- Two changes of xylene were used for 15 minutes.
- Two changes of Absolute ethanol were used for 5 minutes.
- Ethanol 95% for 5 minutes.
- Ethanol 70% for 5 minutes.
- Immersion in distilled water for 5 minutes.
- - Endogenous peroxidase activity was blocked, using H2O2 (1.5 %) (1.5ml). H2O2+ 98.5 ml D.W.) for 5-10 minutes
- Immersion in distilled water for 5 minutes.
- Immersion in PBS for 5 minutes.
- Immersion in Retrival solution (citric buffer 1ml + 99 ml D.W) for 10 minutes at 100^0C.
- Immersion in D.W. for 5minutes
- Immersion in PBS for 5 minutes
- Excess buffer was taped and wiped around section
- Enough blocking serum was applied (0.8 ml PBS +10 µl Blocking serum) for 1 hour at 37 °C.
- Immersion in D.W + PBS each 5 minutes

Immunohistochemical analysis for

- Enough blocking serum (0.8 ml PBS +12 µl Blocking serum) + 16µl primary AB (primary antibody 1 ul: 50 µl Blocking serum) were applied over night at 37 37 ° C.
- Immersion in DW +PBS for 5 minutes each, then wiped.
- Application of secondary Ab (Biotinylated)(0.8 ml PBS +10 µl Blocking serum +10 µl Biotinylated Ab) at 37 ° C for 1.5 -2 hours.
- Immersion in D.W+PBS (5minutes), then wiped.
- Application of AB enzyme (o.75 ml PBS+ 15µl solution A +15µl solution B) for 30 minutes at 37C°. Immersion in D.W+PBS for 5 min, and wiped as mentioned above.
- Application of substrate –chromagen solution (DAB) {(0.75 ml +5 drops substrate+ 1drop DAB +1 drop proxidase)} for 30min or until the brown color appeared.
- Immersion in D.W. for 5 minutes.
- Washing in tap water for 2 minutes.
- Counter staining with hematoxylin for 15 seconds.
- Washing in Tap water.

Dehydration:-

- Immersion in Ethanol 70%for.2 mint.
- Immersion in Ethanol 95%for 2 mints.
- Immersion in Ethanol 100%for 2 mint.(two times)
- Immersion in Xylene 100% for2 mint. (two times)
- Cover slipping and mounting.

. Additionally, the number of macrophage was counted in the fields. All immune stained sections were examined by the same two observers with a ×400 objective under the light microscope for evaluating TNF alpha expressions in the uterus specimens TNF alpha expression in macrophages was evaluated by counting 100 cells of each section. TNF-α expression was quantitatively assessed as 0 (no stained cells), score 1 (from 1-25 positive cells), score 2 (from 26-50 positive cells), score3 (from 51-75 positive cells) and score 4(from 75 and over). The intensity was scored as 0 (nil), (low), (moderate), or (high). The pattern and intensity of staining in the different cell types of liver, spleen, kidney, brain, and lung samples were evaluated by

Scoring

When counting the number of positive cells in the staining tissues samples, at least 10 high-power fields were chosen randomly on each section two independent observers using a light microscope at a magnification of 200 X (20 x objective and 10 x ocular). The degree of staining in each cell type was graduated as described by Zenclussen [11].

RESULTS

The result of immunohistochemistry showed that the score and intensity of tissue TNF-α in the G1,G4 group was significantly ($P<0.05$) increased more than those values in the G2 group which significantly decreased and in the G3 group as compared with the negative control G5 group (Table1).

TABLE 1: The results of immunuohistochmistry of TNF-α ratio of different groups at 4weeks

G	Organ	(Mean Error)	Std.	Score	Intensity
G1	Lung	75.50 ± 9.48	C	3	High
	Liver	61.34 ± 4.72	D	3	Moderate
	Spleen	61.98 ± 5.72	D	3	Moderate

	Kidney	51.64 ± 4.72 D	3	Moderate
	Brain	25. 02 ± 4.72 D	1	Low
G2	Lung	49.44 ± 0. 70 E	2	Low
	Liver	47.34 ± 0. 05 E	2	Low
	Spleen	45.14 ± 0. 66 E	2	Low
	Kidney	40.22 ± 0. 51 E	2	Low
	Brain	20.12 ± 0. 85 E	1	Low
G3	Lung	48.20 ± 0. 16 E	2	Moderate
	Liver	39.96 ± 5.02 F	2	Moderate
	Spleen	29.83 ± 1.25 F	2	Moderate
	Kidney	27.23 ± 2.05 F	2	Low
	Brain	20.75 ± 1.05 F	1	Low
G4	Lung	100.89 ± 1.37 A	4	High
	Liver	87.37 ± 0. 62 B	4	High
	Spleen	84.50 ± 4.46 B	4	High
	Kidney	80.11 ± 6.2 B	4	Moderate
	Brain	30.26 ± 2.01 F	4	High
G5	Lung	38.16 ± 5.009 F	2	Moderate
	Liver	35.21 ± 6.05 F	2	Low
	Spleen	30.86 ± 1.5 F	2	Moderate
	Kidney	28.77 ± 3.4 F	2	Moderate
	Brain	25.23 ± 7.9 F	1	Low

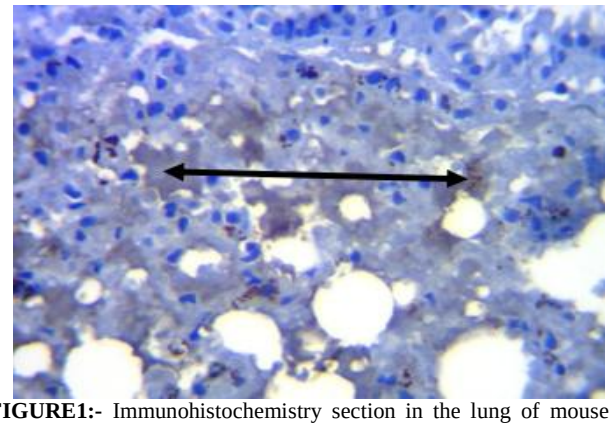

FIGURE1:- Immunohistochemistry section in the lung of mouse G1 group showed inflamatory cell interalveolar septa filled with intracytoplasmic TNF expression score 3,high intensity Stained by(DAB-chromogen ⟷ (Brown color immunostaining X 40).

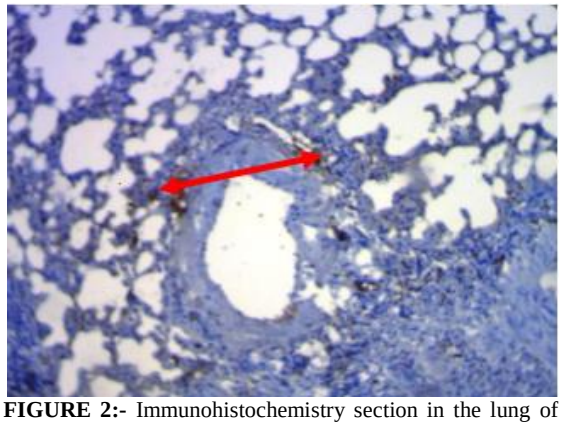

FIGURE 2:- Immunohistochemistry section in the lung of mouse G4 group showed inflamatory cell in the bronchiol wall and interalveolar septa filled with intracytoplasmic TNF expression score 4,high intensity ⟷ Stained by(DAB-chromogen (Brown color immunostaining,X10).

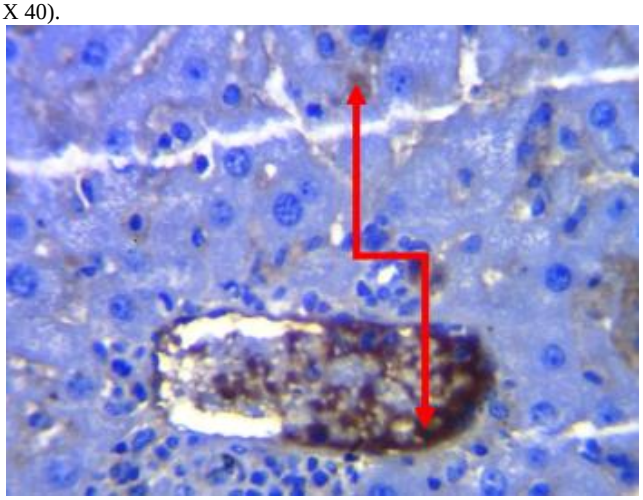

FIGURE 3:-Immunohistochemistry section in the liver of mouse G1 group showed inflamatory cell in the central vein and liver parenchyma filled with intracytoplasmic TNF expression score 3,modreat intensity ⟷ Stained by (DAB-chromogen (Brown color)immunostaining,X40).

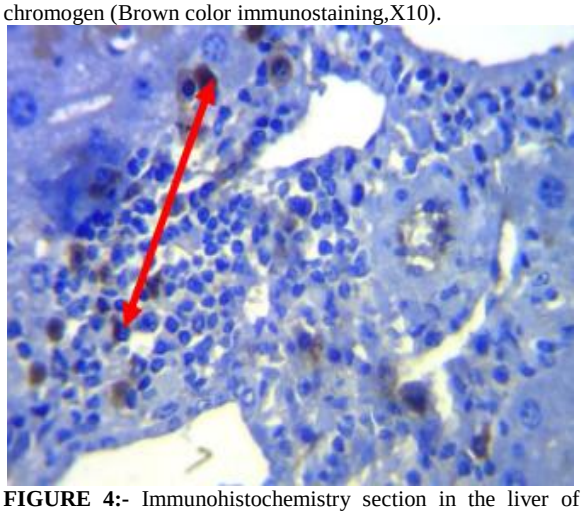

FIGURE 4:- Immunohistochemistry section in the liver of mouse G4 group showed macrophages in the sinusoids and liver paranchyma. infiltrates and Kupffer cells filled with intracytoplasmic TNF expression score 3, modreat intensity ⟷ Stained by (DAB-chromogen (Brown color) immunostaining, X40).

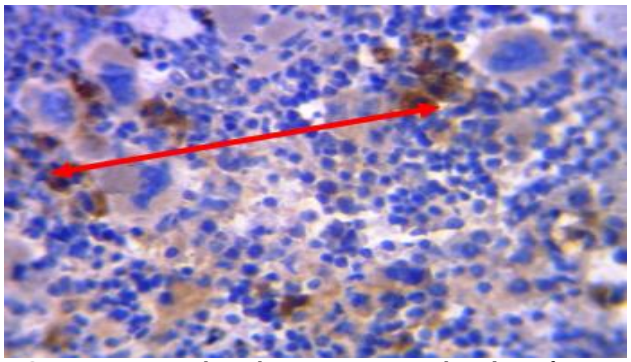

FIGURE 5:- Immunohistochemistry section in the spleen of mouse G2 group showed macrophages in the splenic sinusoids and red pulp filled with intracytoplasmic TNF expression score 2,low intensity ↔ Stained by (DAB-chromogen (Brown color) immunostaining, X 40).

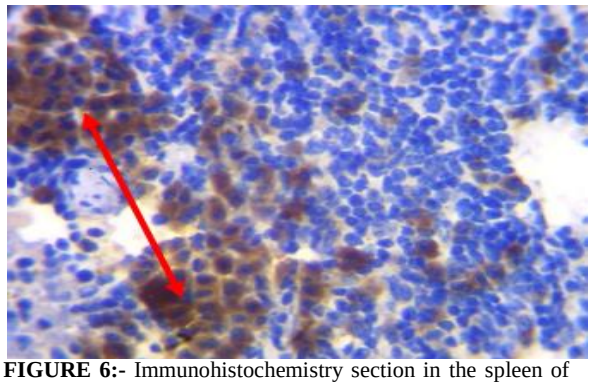

FIGURE 6:- Immunohistochemistry section in the spleen of mouse G4 group showed macrophages in the splenic sinusoids and red pulp filled with intracytoplasmic TNF expression score 4, high intensity ↔ Stained by (DAB -chromogen (Brown color) immunostaining, X40).

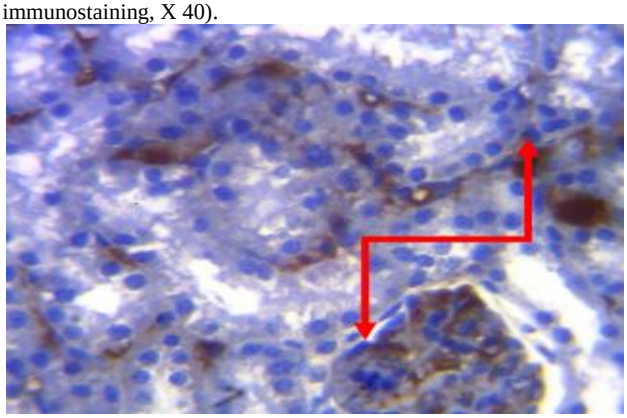

FIGURE 7:- Immunohistochemistry section in the kidney of mouse G4 group showed macrophages in the interstial tissue filled with intracytoplasmic TNF expression score 4,modreat intensity Stained by ↔ (DAB-chromogen (Brown color immunostaining X40).

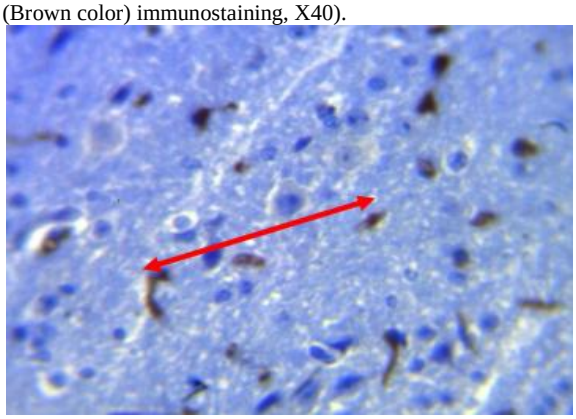

FIGURE 8:- Immunohistochemistry section in the brain of mouse G1 group showed macrophages in brain glial cell filled with intracytoplasmic TNF expression score 1,low intensity Stained by (DAB- chromogen (Brown color ↔ immunostaining, X40).

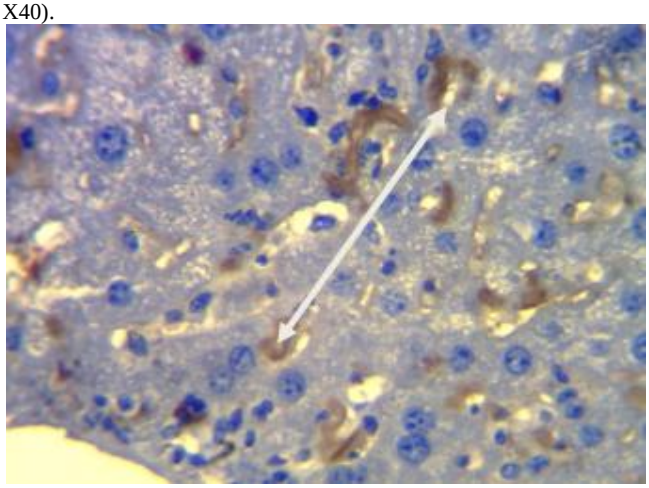

FIGURE 9:- Immunohistochemistry section in the liver of mouse G3 group showed macrophages in the sinusoids and liver paranchyma. infiltrates and Kupffer cells filled with intracytoplasmic TNF expression score 2,modreat intensity ↔ Stained by(DAB-chromogen (Brown

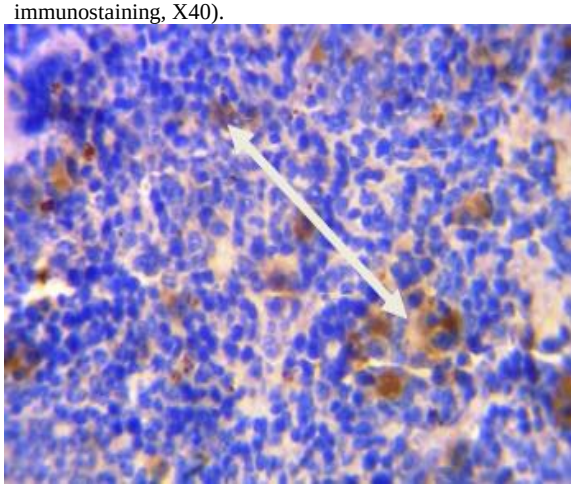

FIGURE 10:- Immunohistochemistry section in the spleen of mouse G3 group showed macrophages in the splenic sinusoids and red pulp filled with intracytoplasmic TNF expression score 2,moderat intensity ↔ Stained by (DAB-chromogen (Brown color) immunostaining, X40).

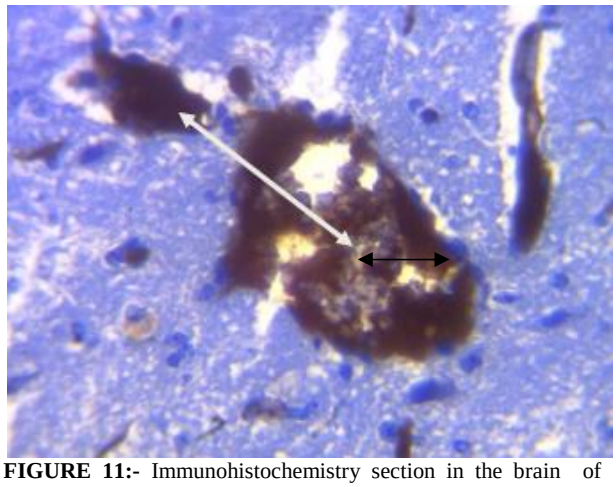

FIGURE 11:- Immunohistochemistry section in the brain of mouse G4 group showed macrophages in brain paranchyma and glial cell filled with intracytoplasmic TNF ⟷ expression score 4,high intensity Stained by(DAB-chromogen (Brown color immunostaining, X40).

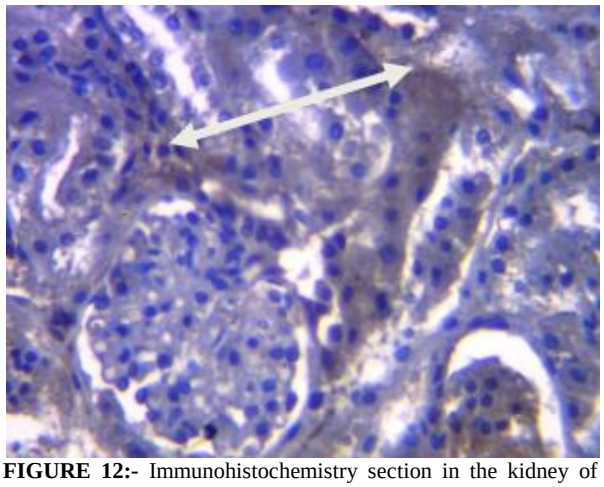

FIGURE 12:- Immunohistochemistry section in the kidney of mouse G3 group showed macrophages in the interstial tissue filled with intracytoplasmic TNF expression score 2,Low intensity ⟷Stained by (DAB- chromogen (Brown color immunostaining, X40).

The result of immunohistochemical staining increased in immunized animals, this may due to that *Brucella* vaccine can persist in the host to stimulate Th1 immune responses and provide the antigens to stimulate B and T cells to produce TNF-α[12]. Also[13] are thought vaccination promote cytokines can include IL-2, TNF-α, IFN-γ and cytolytic enzymes perforin and granzyme. The current finding revealed that mean values, score and intensity of tissue TNF-α were low in animals treated with diazinon as compared with other groups, this result may indicate that organophespharous Ops which can cause structural or functional alterations in humoral or cell mechanisms (nonspecific or adaptive) of the immune response, there's an increase in the susceptibility to infections [14], as well as the results obtained by Girón-Pérez [15] revealed that the administration of diazinon induce an increase in the concentration of ACh, which significantly diminishes lympho proliferation in vitro. In addition, the damage in the lymphoid tissue is the result of the phosphorylation, oxidative damage, and/or altered neuronal function, induced by Ops[16]. Also Li[17] reported that the Ops a diminished the NK cell, LAK cell and cytotoxic activities. Das[18] reported that some Ops induce apoptosis and necrosis in culture human lymphocytes of peripheral blood. It was also reported that the OPs not only induce alteration in the number of cells, but also in the morphology and functionality of them. Hence, it was reported that diazinon [19]. It was investigated that mean values of TNF-α in immunized – diazinon treated animals were low but high in animal infected with *Brucella* as compared with these values in the animals treated with diazinon. This result may indicate that Overproduction of this chemical especially during parasitic infestation can lead to "immunosuppression[20]. In addition causes more damage the immune cells located near injured tissue often secrete Tumor Necrosis Factor (TNF), which at low levels assists in host defenses, but at higher levels "evidently has some means of inducing immunosuppression[21]. But *B. abortus* vaccine induce effective adaptive immune responses in both humoral and cellular immunity expression of NOS, TNF-α and IL-1β without induction of inflammatory reaction [22]. These results also contributes to oxidant resistance caused by immunization by affecting the secretion of antioxidant enzymes Sod B and Kat G. Similar results were obtained by Ragavan *et al.* [23]. The present study showed that infected animals with *B. abortus* expressed significant increased in mean values of tissue TNF-α and high intensity staining compared with control and other group this may indicate that *Brucella* infection stimulated over production of TNF-α in the tissue, and cytokine this result agreed with results obtained by Weiss *et al*.[24] who showed that *Brucella* induced the expression of proinflammatory cytokines such as TNF-α and IL-12 both in vivo and in vitro [24]. Morover Scian *et al.* [25], reported that the intra-articular injection of heat-killed *Brucella* further suggests that joint infection can induce a pro-inflammatory environment. However, the nature of the cellular and inflammatory responses in vivo following infection remains to be tested. In addition to bacterial persistence in the host lead to most inflammation- and fibroblast- monocytes elicit inflammatory mediators following infection [26]. The spread and dissemination of bacteria to multiple organs results in severe clinical manifestations in lung, liver, spleen, kidney brain, heart and osteoarticular tissues. Due to *Brucella* interaction with the host at these diverse infectious foci could explain important bacterial tissue-specific pathogenic mechanisms and niches that conceal bacteria and contribute to brucellosis-induced complications. These results are in agreement with Skyberg *et al.* [26].

From the results obtained, it can be concluded that TNF-α expression level was increased and related with the progress of infection and immunization with CFBAgs provide a good protection against *Brucella* infection and augment immune response may decrease toxic effects of diazinon.

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