

Influence of chitosan on hematological and histopathological changes in mice infected with *Brucella melitensis* immunized with Rev-1 vaccine

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Abstract

This study aimed to assess the changes of blood cells constitutions and study the histopathological sections of liver and spleen in dual sexes mice infected with *brucella melitensis* and the role of chitosan supplement with and without Rev-1 vaccine in enhancing the inflammatory proses, two experiments were undertaken on 100 albino mice, aged 8-10 weeks for 60 days, first experiment done on 50 males and second one done on 50 females. Results of hematological analysis showed that there are significant increase at $P<0.001$ of white blood cells count WBC ($\times 10^3$), red blood cells count RBC ($\times 10^6$), hematocrit (HCT), mean corpuscular value (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), lymphocyte (LY%), granulocyte (GR) and lymphocytes m/mm^3 (LYM) in males treated groups comparing with females, the highest values were in immunized infected mice with chitosan diet supplement compared to infected animals. The histopathological lesions recorded there is presence of chronic inflammatory reaction characterized by mononuclear cell infiltration with presence of granulomatous lesion in the liver of immunized animals, presence of mild to moderate lesions characterized by hyperplasia of lymphoid tissue in spleen and small granulomatous lesions in liver of immunized animals fed diet with chitosan supplement.

Keywords: Chitosan, Histopathological examination, Rev-1 vaccine, Hematological analysis, *Brucella melitensis*

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تأثير الكيتوسان على التغيرات في الصفات الدموية والنسجية في الفئران الملقحة بلقاح Rev-1 والمصابة بالبروسيلة المالطية

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الخلاصة

هذه الدراسة هدفت الى التحري عن التغيرات في الصفات الدموية ودراسة المقاطع النسيجية المأخوذة من كبد وطحال الفئران من كلا الجنسين الملقحة بلقاح Rev-1 والمصابة بجرثومة البروسيلة المالطية *Brucella melitensis* ودور الكيتوسان كمتعم غذائي مع أو بدون اللقاح في تحسين العملية الالتهابية، لتحقيق هذه الأهداف أجريت تجربتين على ١٠٠ فار ابيض بعمر ٨-١٠ أسابيع. أجريت التجربة الأولى على ٥٠ فار ذكر والثانية على ٥٠ فار أنثى. أظهرت نتائج التحليل الدموي وجود فرق معنوي $P<0.05$ في أعداد خلايا الدم البيضاء WBC ($\times 10^3$)، خلايا الدم الحمراء RBC ($\times 10^6$)، حجم الخلايا المكدسة (HCT)، الحجم الكروي الوسطي لخلايا الدم الحمراء (MCV)، حجم الهيموغلوبين الوسطي (MCH)، النسبة المئوية للخلايا اللمفية (LY%) وعدد الخلايا الحبيبية (GR) بين الذكور والإناث الزيادة الأكثر كانت في مجموعة الذكور الملقحة المصابة والمغذاة على الكيتوسان في العلف. كما كشفت المقاطع النسيجية وجود تفاعل التهابي مزمن في الأعضاء المفحوصة تميز بوجود ارتشاح خلوية وحيدة النواة مع وجود آفة من الورم الحبيبي داخل كبد الذكور الملقحة والمصابة، بينما كشفت الدراسة وجود آفات مرضية معتدلة الى متوسطة تتميز بوجود فرط تنسج للأنسجة اللمفية بالطحال وأورام حبيبية صغيرة في الكبد في الحيوانات المصابة الملقحة والمغذاة على الكيتوسان في العلف.

Introduction

Brucellosis is an infectious zoonotic disease causes important economic and public health problem in human and animals, particularly abortion and infertility in natural host (1). Brucellosis are endemic in different countries worldwide including Iraq in addition, Middle East, Mediterranean area, Asia, Africa and South America (2). These bacteria cause 95.6% of the economic losses in cattle and buffalo in livestock (3) and over 500,000 infections every year in human (4), and less than 5% death human however patient remain asymptotically carrier for years (5). There are several routes of brucella infection include oro- digestive tract, aerosol, conjunctiva (6) Human infection with *Brucella* occur either through contact with infected animals, through one person to the other (7) and from consumption of infected animal products (8). *Brucella melitensis* is persistable in the blood of intraperitoneally infected mice for several weeks (9). Extreme hematological complications and abnormalities with mild anemia and leukopenia have been frequently associated with acute brucellosis, but pancytopenia and thrombocytopenia are less frequently encountered (10). The pathogenesis of pancytopenia in brucellosis has not been clearly understood, it seems to be multifactorial. Several possible mechanisms have been suggested for pancytopenia caused by brucellosis, such as hemophagocytosis, hypersplenism, bone marrow granulomas, bone marrow hypoplasia, and immune destruction (11). Chitosan is biocompatible, biodegradable, nontoxic, renewable biopolymer produced by alkali treatment of chitin, the second most abundant natural polysaccharide used in a biomedical material, as it exhibits a wide variety of biological activities, such as antitumor activities (12), immunostimulating, antiallergic effects (13), hemostatic agent and anticoagulant effects (14), anti- inflammatory activities (15), bacteriostatic as anti-biofilm-associated infections effects (16) wound-healing (17), and anti-fungal activities (18).

Material and methods

Experimental design

First Experiments (male experiment)

Fifty albino male mice, were divided randomly into five groups 10 each and treated as following; 1st group was vaccinated with Rev 1 vaccine (S/C), two doses, each dose 0.2 ml, repeated after two weeks (G1). 2nd group was vaccinated as 1st group and supplemented with chitosan 1 g/kg diet (19) daily (G2). 3rd group was supplemented with chitosan 1g/kg diet to the end of the experiment (G3). 4th group was infected with 0.3 ml virulent *B. melitensis* 1×10^{-8} CFU I/P (20) and considered positive control group (G4). 5th group was orally administrated with PBS served as negative control group (G5).

Second experiment (female experiment)

Done as first experiment but on females. In both experiments, 30 days post immunization, animals of 1st, 2nd, 3rd and 4th groups were injected I/P with 0.3 ml of bacterial suspension containing 1×10^{-8} CFU of viable virulence *B. melitensis*, at 60 days post injection, all animals were sacrificed, blood samples were taken for hematological examination and tissues from liver and spleen for histopathological examination.

Lio-vac rev-1 vaccine

It is a commercial vaccine against Brucellosis in ewes, lambs and kids aged 3 - 6 months which is composed of *Brucella melitensis* Rev-1 strain $1-2 \times 10^{-9}$ CFU per dose (manufactured by syvasa, Spain), manuf. date / 5. 2017 Exp. date 5.2018. the vaccine obtained from Al-Nahdha Veterinary Laboratories / Baghdad.

Bacterial isolate

The virulent *Brucella melitensis* isolate obtained from Al-Nahdha Veterinary Laboratories /Baghdad, growth and biochemical tests were performed for isolation, diagnosis, confirmation according to (20), the cultural characteristics were studied depending on colonies morphology (color, size, consistency and density) on growth media and other biochemical tests. Culture media: Tryptic Soya Agar (TSA) and Tryptic Soya Broth (TSB): (Rashmi diagnostics / India). Blood Agar and Peptone Water Media: (Oxoid /USA). Catalase test was performed according to (20); the babbles production indicated a positive reaction. Oxidase test was performed according to (20). Confirming the diagnosis of the isolate was performed using VITAK system. Preparation of chitosan diet: The pellets were grinded by food grinder and weighed, then 1gm of chitosan (fatsorb®) added to each kilogram of assorted commercial pellets, mixed well, converted to paste by passing through meat grinder to mould the paste into the original pellets form, then left exposed to air drying in room temperature (21).

Determination of challenge dose of *B. melitensis*

Activation of bacteria and bacterial counting done according to (20). The challenge dose was 1×10^{-8} CFU/ml (22).

Histopathological examination

Histological sections done according to (23).

Hematological examination

Blood was collected immediately after anesthesia, directly from the heart using insulin syringes 1 ml 60 days post infection, samples analyzed by an automated blood counter (Abacus junior Vet Diatron Hematology Analyzer, Hungary). The total white blood cells (WBC), red blood

cell (RBC), hemoglobin (HGB), platelets (PLT) and hematocrit (HCT) levels were measured.

Statistical Analysis

The statistical analysis was carried out for data by using factorial experiment in (CRD) method according to (24,25).

Results

Hematological analysis

The results of whole white blood cells count (Table 1) show high significant differences between treated groups ($P \leq 0.01$) at 60 days post infection with *B.melitensis* compared to non-infected control group in both male and female although higher value obtained in immunized group

compared to groups with chitosan supplement with or without immunization. There is a decrease in red blood cells (RBCs) count in infected groups in both sexes compared to treated groups while the highest was in immunized animals fed diet supplement of chitosan in both sexes.

Also, the results revealed an increasing level in granulocytes (GR m/mm^3) in the immunized groups with or without chitosan (G2 and 3) compared to infected group with diet supplement of chitosan only (G1) in both sexes, the highest values in males. The MCH, MCHC and MCV values show an increase in infected groups (G5) in both sexes comparing with non-infected groups (G4) and the higher values in immunized groups with and without chitosan (G1, 2 and 3) in both sexes (Table 1 and 2).

Table 1: Hematological profile of infected male groups with different treatment 60 days post infection

Parameters	G1	G2	G3	G4	G5
WBC ($\times 10^3$)**	5.19 \pm 0.01 d	9.530 \pm .04 a	8.20 \pm 0.02 b	7.60 \pm 0.02 c	4.06 \pm 0.141 e
RBC ($\times 10^6$)*	7.25 \pm 0.05 c	7.63 \pm 0.01 a	7.61 \pm 0.42 e	6.12 \pm 0.01 d	7.85 \pm 0.07 b
HCT**	31.34 \pm 0.04 bc	32.98 \pm 0.05 b	30.54 \pm 0.01 cd	37.27 \pm 1.86 a	30.33 \pm 0.39 d
MCV**	40.00 \pm 1.00 a	49.50 \pm 0.06 a	43.00 \pm 0.04 a	42.00 \pm 0.01 a	19.50 \pm 1.45 b
MCH**	13.70 \pm 0.60 c	13.50 \pm 0.40 c	16.80 \pm 0.01 a	16.60 \pm 0.30 a	13.50 \pm 0.10 c
MCHC**	318.00 \pm 2.00 d	339.00 \pm 0.61 b	322.00 \pm 1.0 c	333.00 \pm 1.09 dc	349.00 \pm 3.50 a
LY %**	60.30 \pm 0.01 d	82.20 \pm 0.10 ab	79.05 \pm 0.05 b	54.75 \pm 0.95 d	36.60 \pm 3.80 e
GR**	29.60 \pm 0.10d e	57.35 \pm 7.95 a	38.90 \pm 0.06 c	48.25 \pm 2.75 ac	16.70 \pm 0.02 e
LYM**	6.13 \pm 0.04 c	6.74 \pm 0.01 b	3.12 \pm 0.01 f	1.50 \pm 0.20 h	5.22 \pm 0.11 d

Different letters indicate significant differences between treated groups at * $P < 0.05$ and ** $P < 0.01$.

Table 2: Hematological profile of infected female groups with different treatment 60 days post infection

Parameters	G1	G2	G3	G4	G5
WBC ($\times 10^3$)**	6.18 \pm 0.03 e	9.28 \pm 0.02 a	6.27 \pm 0.01 c	6.80 \pm 0.01 b	4.25 \pm 0.06 d
RBC ($\times 10^6$)*	7.37 \pm 0.04 bc	7.37 \pm 0.14 bc	7.98 \pm 0.01 a	6.41 \pm 0.05 bc	7.37 \pm 0.01bc
HCT**	32.75 \pm 0.19 bc	35.27 \pm 0.73 a	36.18 \pm 0.81 a	3.73 \pm 0.04c d	31.58 \pm 0.02 bc
MCV**	44.00 \pm 0.01 a	48.00 \pm 0.44 a	46.50 \pm 0.06 a	41.00 \pm 0.04 a	43.50 \pm 0.02 a
MCH**	15.75 \pm 0.60 b	16.70 \pm 0.10 a	15.10 \pm 0.09 b	15.50 \pm 0.35 b	15.40 \pm 0.05 b
MCHC**	355.00 \pm 4 bc	349.00 \pm 3 c	326.50 \pm 0.5 d	374.00 \pm 3.00 a	360.00 \pm 0.05 b
LY %**	35.05 \pm 1.75 e	53.55 \pm 6.35 d	69.00 \pm 0.10 c	84.80 \pm 1.01 ab	87.70 \pm 0.01 a
GR**	30.60 \pm 0.01e	55.25 \pm 5.7 ab	43.85 \pm 4.65 c	39.20 \pm 0.30 d	15.80 \pm 0.03 e
LYM**	4.32 \pm 0.04 e	8.16 \pm 0.04 a	2.26 \pm 0.30 g	0.76 \pm 0.04 i	5.77 \pm 0.03 c

Different letters indicate significant differences between treated groups at * $P < 0.05$ and ** $P < 0.01$.

Histopathological examination

Results of histopathological changes of infected animal with *B. melitensis* revealed presence of coagulative necrosis in liver parenchyma, congestion with neutrophils infiltration of central vein and sinusoids (Fig1: A,B) other sections of liver showed fibrosis of portal area with infiltration of inflammatory cells (Fig1: C) while spleen section of the same group showed fibrin network deposition and neutrophils infiltration in red pulp with depletion of

white pulp (Fig1: D), however histopathological section of immunized animal at 60 days post infected with *B. melitensis* showed aggregation of mononuclear cells around blood vessels with proliferation of Kupffer cells in the liver (Fig2: E, F). Histological section of immunized animal fed diet supplement with chitosan at 2 months post infected with *B. melitensis* showed severe infiltration of mononuclear cells around central vein and bile duct in liver portal area with hyperplasia of white pulp (Fig3: G,I, J).

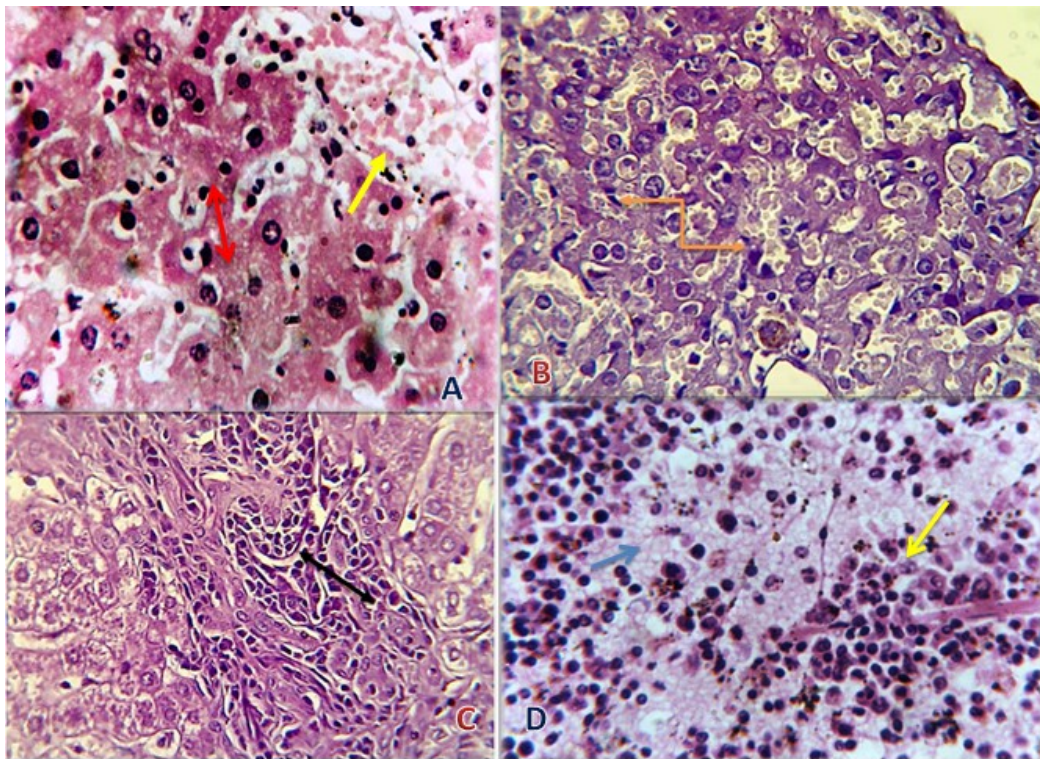


Figure 1: Histopathological section of infected animal with *B. melitensis* 60 days post infection. A: liver, congestion of central vein with neutrophils in its lumen (yellow arrow), with coagulative necrosis (red arrow) through liver parenchyma, H&E, 132x. B: liver, severe congested of dilated sinusoids containing inflammatory cells (orange tow head arrow), H&E stain, 400x. C: liver, inflammatory cells in fibrosis portal area (black arrow), H&E stain, 400x. D: spleen, deposition of fibrin network (blue arrow) and neutrophils infiltration in red pulp which lead to depletion of white pulp (yellow arrow), H&E, 132x.

Discussion

Hematological analysis

The results of WBCs agree with (27) who mentioned that white blood cell count increased significantly in vaccinated animals after vaccination up to 90th day post challenge in comparison with initial decrease, circulating antibodies induced by vaccination intensely reduce persistence of bacteria in the blood following challenge. The *B. melitensis* bacteria is lasts for at least 2 weeks in the blood of infected mice after intraperitoneally injection. They are located primarily in extracellular then found inside erythrocytes (9). This theory explains the decrease in red blood cells (RBCs) count in infected groups in both sexes comparing with treated groups while the highest value in immunized animals fed diet supplement of chitosan in both sexes. Also results revealed that there is an increasing level in granulocytes (GR m/mm³) in the immunized groups with or without chitosan comparing with infected group fed with diet supplemented with chitosan only in both sexes, the highest in males this agrees with (28) who explained that

neutrophil phagocytic activity increased about threefold in infected groups with brucellosis probably due to it is the consider first immune associated cells in the host defense against *Brucella* invasion and replication in phagocytic cells, such as macrophages and dendritic cells (29), moreover chitosan act as vaccine adjuvant the effect of chitosan was first demonstrated by (30). The mechanism of bacterial growth inhibition is thought to be that the cationically charged amino-group may combine with anionic components such as N-acetylmuramic acid, sialic acid and neuraminic acid, on the cell surface, and may suppress bacterial growth by impairing the exchanges with the medium, chelating transition meal ions and inhibiting enzymes (31). The MCH, MCHC and MCV values were in infected groups comparing with non-infected groups and the higher values in immunized groups with and without chitosan in both sexes this agrees with (32) who suggested that anemia was the most common hematologic sign of brucellosis. Anemia prevalence in patients with and without occupational exposure history.

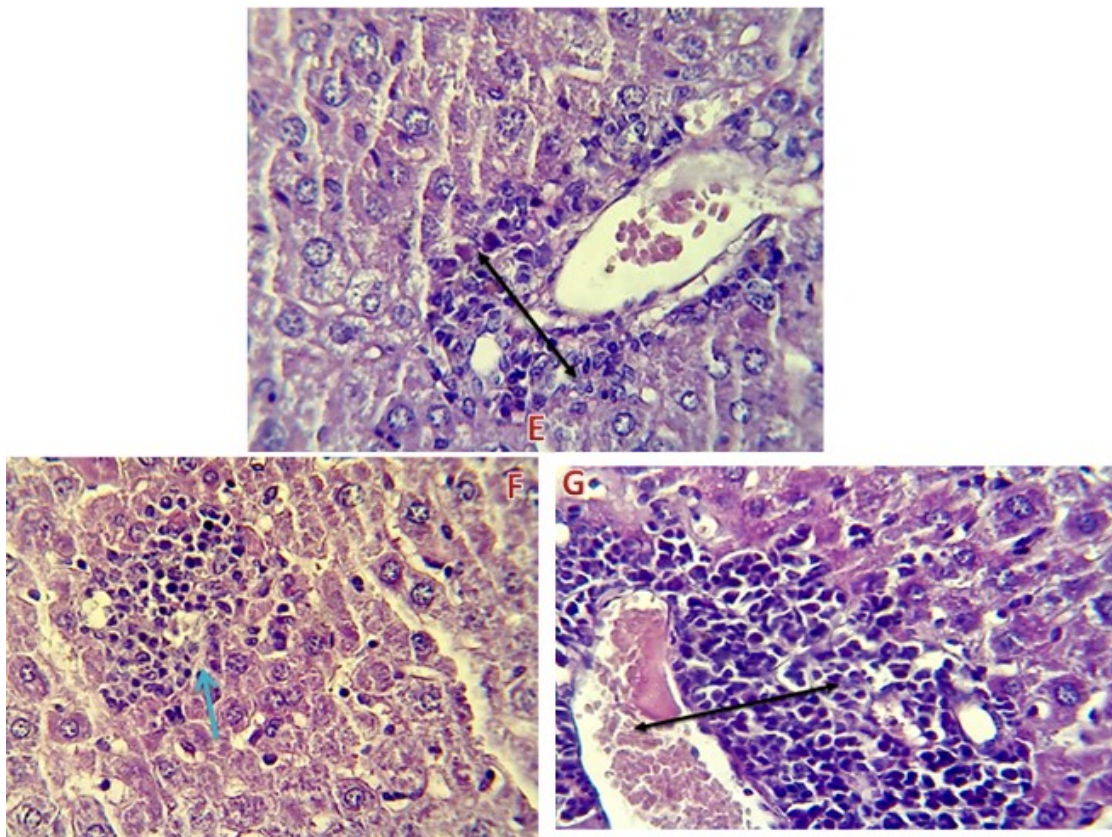


Figure 2: Histopathological section of immunized animal at 60 days post infected with *B. melitensis* (H&E stain, 400x). E: liver, mononuclear cells aggregation around blood vessels with proliferation of Kupffer cells (black arrow). F: liver, mononuclear inflammatory cells aggregation around central vein in parenchyma (blue arrow). G: liver, of immunized animal fed diet supplement with chitosan at 2 months post infected with *B. melitensis* shows mononuclear cells aggregation around congested central vein (black arrow).

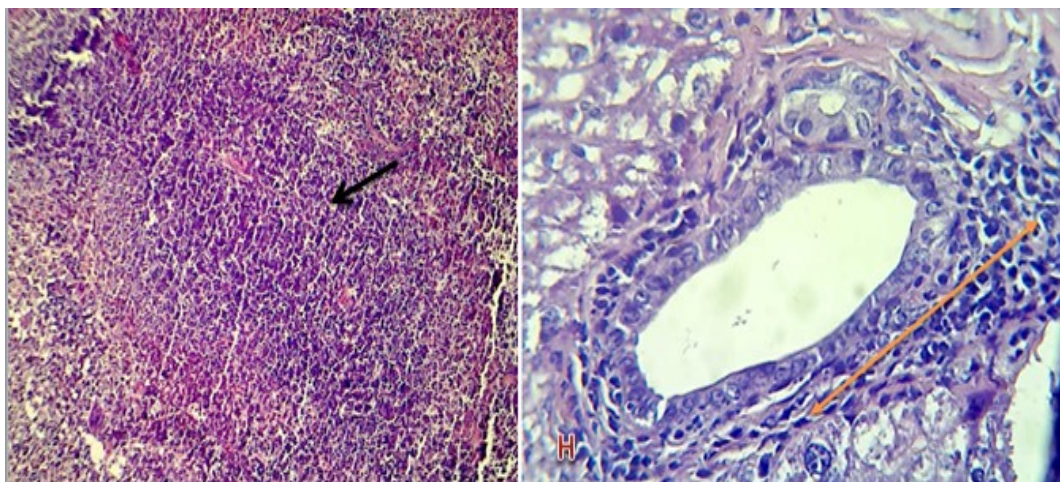


Figure 3: Histological section of immunized animal fed diet supplement with chitosan at 2 months post infected with *B. melitensis*. H: liver, shows severe mononuclear cells infiltrated around bile duct in portal area, H&E stain, 400X. (red arrow). I: hyperplasia of white pulp (blue arrow), H&E stain, 100X.

Histopathological examination

The histopathological changes in examined organs showed moderate inflammatory cells particularly neutrophils and mononuclear cells recorded in examined organs, these lesions may indicate that the strain of *B. melitensis* cause suppurative reaction due to pathogen associated molecular pattern (PAMP) of these pathogen attached with toll-like receptor 4,5 and 9 that lead to activate events end with activation of nuclear factor kappa-beta (NF- κ B) which activates kappa gene in the nuclei of macrophage to stimulate expression of pro-inflammatory genes that encoded pro-inflammatory cytokines such as IL-1, TNF- α and IL-12 as well as chemokines particularly IL-8 a potential attracted factor for neutrophils leading to suppurative reaction, this idea was correlated with (33) who previously observed that both innate and acquired immune responses used in the protective host against brucellosis and innate immune response consist of neutrophils, macrophage/ monocyte, dendritic cells as well as natural killer cells, these cells have pattern recognition protein (PRR) that recognizes PAMPs leading to production of pro-inflammatory cytokines, chemokines and type I and II IFN which are important factors in host defense, clearance infection occur by activated T and B cells by chemokines and cytokines in addition to inflammatory reaction that initiated by pro inflammatory cytokines (34).

The present findings recorded necrosis in the liver and spleen, these two organs are the targets of reticuloendothelial system of *Brucella* in which the pathogen can survive and replicate in these organs as well as disseminated to other organs of the body, necrosis maybe due to oxidative stress induced by brucellosis that causes damage of protein, carbohydrate, cell membrane and DNA (35).

Granulomatous lesions are found in immunized animals with or without chitosan may indicate that the body attempt be localized and destroy the pathogen due to granulomatous reaction is consider as a strong body defense against virulent pathogens, as well as *Brucella* infection is usually associated with development of granulomatous reaction (36) these reaction occur as a result of *Brucella* antigens can stimulated pro inflammatory cytokines such as IL-1, IL-12 which play role in development of cell mediated immune response associated with granulomatous lesions development.

However, in the present results recorded hepatitis and splenitis in infected animals, these lesion may indicated hematogenous spread of virulent strain of *B. melitensis*, hepatitis is a common lesion in human brucellosis but not in animals, also (37) recorded lymphoplasmacytic infiltration in the renal interstitial tissue of swine infected with *B. abortus* and multifocal lymphoplasmacytic infiltration in the liver with pyogranuloma and necrosis, the less degree of lesions in the immunized animal with or without chitosan

may indicated that chitosan do seem to have essential role against oxidative damage scavenging or dimensioning activity of ROS, these result was agreed with (38), who demonstrated that antioxidant such as vitamin C and vitamin E can inhibited activity of NF- κ B and depress cytokine production.

The mild to moderate pathological lesions in immunized animals with or without chitosan supplement diet may be due to good immune response provided by Rev -1 vaccine and chitosan that killed most of the pathogen at site of inoculation and few of them disseminated to internal organs that killed by activated macrophages that form granulomatous lesions, these idea was agreement with (39), who recorded that chitosan can activated both cellular and humoral immune response also (40) recorded high antibodies titers and activated CD4 T cells in animals fed diet supplement with chitosan.

Conclusion

Overall, chronic infection with *brucella melitensis* causes anemia, chitosan as food supplement acts as immune stimulator with Rev-1 vaccination against infection with *brucella melitensis* and enhancing inflammatory proses by elevation of WBCs, GR, MCH, MCHC and MCV, hyperplasia of lymphoid tissue and formation of granulomatous reaction in spleen and liver respectively.

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