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Evaluation of Crude Anthrax Protective Antigen as an Adjutant For *Brucella Abortus* Vaccine In Mice

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ABSTRACT

Anthrax crude protective antigen (PA) was used in this study as an adjutant with *Brucella abortus* S19 strain vaccine in a ratio of 1:1 to immunize mice in dose of 0.1 ml s/c in order to decrease side effects of S19 vaccine and also to enhance its immunogenicity. Three groups of mice were immunized with the mixture (composed of anthrax PA +S19, S19 alone, and sterile PBS solution as a control. They were compared concerning cellular and humeral immunity. The group treated with the mixture was the best regarding skin test, the difference of thickness was 1.51± 0.103 mm after 24 h and 1.14± 0.103 mm and 0.89± 0.100 mm after 48 and 72 h, respectively. As well as that group revealed the highest antibody titer, which reported 0.7153± 0.08 nm as compared with 0.3743± 0.06 nm in S19 group. These findings were verified by studying the histopathological changes.

Keywords: *Brucella abortus*, vaccine, Anthrax, Protective antigen, adjuvant.

تقييم المستضد الحامي الخام لجرثومة الجمرة الخبيثة كعامل مساعد مع لقاح البروسيللا في الفئران

الخلاصة

أستخدمت بهذه الدراسة المستضد الحامي(PA) الخام لذيفان الجمرة الخبيثة كعامل مساعد يمزج مع لقاح جرثومة البروسيللا المالطية عترة S19 بنسبة 1:1 لتمنيع الفئران بجرعة 0.1 مل تحت الجلد وذلك من أجل تقليل التأثيرات الجانبية للقاح S19 وكذلك لزيادة كفاءته التمنيعية. منعت ثلاثة مجاميع من الفئران بالمزيج يتكون من S19+ PA، ولقاح S19 لوحده، ومحلول PBS المعقم كسيطرة. جرت مقارنة بين المجاميع فيما يتعلق بالمناعة الخلوية والخلطية. كانت مجموعة الخليط الأفضل بالفحص الجلدي، اذ سجلت فرق تثخن0.103 ± 1.51 ملم بعد 24 ساعة و 0.103 ± 1.14 ملم و 0.100 ±0.89 ملم بعد 48 و 72 ساعة، بالترتيب. فضلا عن ان هذه المجموعة أظهرت أعلى معيار حجمي للأجسام المضادة بتقنية الاليزا، اذ بلغ 0.08 ±0.7153 نانوميتر مقارنة بـ 0.06 ±0.3743 نانوميترفي مجموعة S19. تم التأكد من هذه النتائج بدراسة التغيرات المرضية النسيجية.

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INTRODUCTION

Brucellosis is one of the most common zoonotic diseases (1). Four species being recognized as causing infection in humans: *Brucella abortus* (cattle), *Brucella melitensis* (goats, sheep and camels), *Brucella suis* (pigs) and *Brucella canis* (dogs) (2). *Brucella* species are one of several bacteria that are viewed as potential agents of biological warfare and bioterrorist activity (3). The disease is acquired from handling of infected animals or consuming contaminated milk or milk products, exposure is frequently occupational and the disease is common in the Mediterranean and Arabian Gulf regions, Latin America, and parts of Asia (4).

The virulence of *Brucella* varies considerably according to species, strain and the size of infecting inoculum, and host susceptibility is also variable and is associated with the reproductive status (5). Brucellosis is treatable by antibiotics but, due to the intracellular nature of the infectious process, treatment usually requires combination therapy over along duration, there is no available human vaccine for brucellosis (6). Historically, 2% of untreated *B. melitensis*- infected patients die and *Brucella*- induced deaths are still reported. Therefore, to eradicate human brucellosis, the control of brucellosis in agricultural animals is crucial because of the zoonotic aspects of this infectious disease (7).

In this study I have tried an adjuvant (crude anthrax PA) combined with reduction of S19 vaccine dose. It was reported that PA is able to potentiate protective immunity against a heterologous antigen, demonstrating the potential of *B. anthracis* recombinant strains for use as live vaccine vehicles (8). Researchers (9) have made use of the anthrax toxin system to deliver cholera toxin subunit A (CT-A) into the cytosol of mammalian cells, thereby directing CT-A to alternative receptors (of PA), which are universally present on all mammalian cells. Their result suggested that anthrax lethal factor (LF) - CT-A is internalized into the cytosol through PA. Unlike many protein vaccines, the PA of *B. anthracis* possesses a receptor expressed by mammalian cells that may influence the type of immune response induced, as well as the presence of PA receptor (ATR) in virtually all mammalian cells would increase its immunogenicity (10). In the present study crude PA was mixed with *Brucella* vaccine S19 and compared with S19 alone regarding cellular and humeral immunity.

MATERIALS AND METHODS

1- *Bacillus anthracis* Sterne stain (34F2): was obtained from Al-Kendi Company for production of veterinary vaccines and drugs, Abu-Ghraib / Baghdad. Bacterial suspension was prepared by growing this bacterium on blood agar medium for 48 h and harvesting it by sterilized phosphate buffered saline (PBS) with glass rod. The suspension was put in sterile test tubes and washed three times with PBS in centrifuge. It was compared with McFarland tube no.2 which contains 6×10^8 CFU/ ml. It was used to inoculate modified Casamino acid medium to prepare anthrax toxin.

2- Virulent *Brucella melitensis* was obtained from Zoonotic Diseases Unit, college of Veterinary Medicine, Baghdad University. It was used in challenge experiments.

3- *B. abortus* S19 strain vaccine was taken from Zoonotic Diseases Unit, as above. It was used to vaccinate animals.
4- Anthrax PA: anthrax toxin was prepared from *B. anthracis* strain 34F2 by using Casamino Acid medium according to (11) with some modifications that include: using mixture of 5.13 gm of Bacto peptone, 2 gm of sodium chloride and 1.6 gm of yeast extract in solution no. 6 instead of (tryptophan 2.6 gm, cystine 0.6 gm and 0.750 gm glycine) in the previous original method.

Casamino acid was added in weight of 4.16 gm instead of 3.6 gm. The other modifications included the addition of 0.35 gm of multivitamins to 460 ml of the prepared medium, then sterilized at 121 ºC for 15 min. in autoclave. 20% glucose solution sterilized by filtration was added to the cooled medium. 2 ml of bacterial suspension (prepared above) was used to inoculate 460 ml of Casamino acid medium and incubated at 37 ºC. After 4 h of incubation, 60 ml of 9% sodium bicarbonate (instead of 55 ml solution in the same concentration) sterilized by filtration was added to the medium and the incubation period continued to 24 h. Gram stain was applied to confirm purity of the cultured medium from any other contaminated bacteria.

The cultured medium was centrifuged at 9000 RPM for 20 min. using cold centrifuge to get ride of bacterial cells, the supernatant was taken and the PH was adjusted by HCl to be 7.2. Horse serum was not added to the toxin medium which was filtered by Seitz filter using 0.22 µ filter paper in order to obtain PA or what was called previously (filter factor). Protein concentration was measured by commercial kit (Biolabo, France kit).
5- Brucellin: *B. melitensis* Rev.1 strain (2×10^8 CFU) was applied to prepare brucellin according to the method of (12). Dilutions were done to make the protein equal to 100 µg / 0.1 ml. 0.1 ml of brucellin was injected intradermally (I. D.) in the foot pad of mice. Reading of thickness was recorded before injection, 24, 48 and 72 h after injection.
6- Laboratory animals: 15 white mice *Mus musculus* strain Balb/c aged 3-4 weeks from both sex weighted 25-35 gm were used in this study. They were purchased from the National Center for Farmaceutical Researches/ Ministery of Health, Baghdad, Iraq. They were divided randomly into 3 equal groups.
Immunization: The vaccinated animals included the following groups:
A- Mixture group: five mice were injected subcutaneously (s/c) with 0.1 ml of the mixture containing PA of anthrax toxin in the concentration of 2 mg/ ml and *Brucella* S19 vaccine in the same concentration. The proportion of PA to S19 was 1:1.
B- S19 group: five mice were injected with S19 alone in the same route and dose.
C- Control group: five mice were injected with sterile PBS as above.

Mice in each group were boosted for two times by 2 weekly s/c with mixture, S19 alone and sterile PBS, respectively.
Skin test: It was done 11 days after the second booster, mice were injected I.D. in the right foot pad with the dose of 0.03 ml brucellin by using disposable insulin needle, while the left foot pad was injected with sterile PBS as a control. The results of skin test were read 24, 48 and 72 h post-injection by applying caliper.

Protection experiment: Mice in all groups were challenged by s/c injection of 1×10^6 CFU of virulent *B. melitensis* in dose of 0.1 ml 60 days post vaccination.

10 days after challenge, all the infected mice were anesthetized with inhaled ether, and then were sacrificed. Blood was collected directly from heart without anticoagulant to obtain serum used for ELISA (13).

Bacteria isolation: Few drops of blood and pieces of organs (spleen, liver, kidney and lung) were removed aseptically for bacterial culturing on blood agar, Trypticase soya broth and *Brucella* agar and broth (14).

Histopathology: Parts of tissues (1 cm^3 thickness) taken from spleen, liver and kidney were kept in Neutral buffer Formalin 10% for studying histopathological changes (15).

ELISA

Before applying the technique, the complement was inactivated by putting the serum containing tube in water bath at 56 ºC for 30 min. The titer of serum immunoglobulin G (IgG) was determined by using French commercial kit called SERELISA™ *Brucella* OCB Abs Mono Indirect manufactured by Synbiotics Corporation. This kit contains anti- LPS for *Brucella*. ELISA reader at a wave length of 450 nm was used. ELISA values were calculated according to the following equation:

ELISA result = 0,50 × (OD reading – 0.6 × OD P)

In which OD P means Optical Density of control positive.

Or ELISA results can be obtained by analyzing Optical Densities in which the result is positive if OD ≤ (0.6× OD P), while in negative result OD < (0.6 × OD P) according to the instructions of the manufacturer.

RESULTS

Protein content of PA: the concentration of protein in PA was 312 µg/ ml.

Bacterial culture

- Vaccinated animals: there were no bacteria isolated from liver, spleen, lung, kidney and blood taken from both immunized groups.
- Control animals: bacteria were isolated from blood and all internal organs of non-vaccinated animals.

Skin test

- Control group: negative results were noticed in foot pads of non-immunized mice injected with brucellin and the left foot pad of immunized animals injected with PBS.
- Mixture group: mice immunized with the mixture recorded mean difference of thickness 1.51± 0.103 mm, 1.14± 0.103 mm and 0.89± 0.100 mm after 24, 48 and 72 h, respectively (table 1).
- S19 group: the mean difference of thickness was 1.54± 0.289 mm after 24 h and 1.06± 0.141 mm and 0.8± 0.127 mm after 48 and 72 h, respectively.

Elisa

It was recorded the following:

OD P= 0.934 nm, the average OD of mixture= 1.991 nm, and the average OD of S19= 1.309 nm. By application of the equation above, both groups were positive, in which the average of ELISA readings was 0.7153± 0.08 nm in mice

vaccinated with mixture, while it was 0.3743± 0.06 nm in those immunized with S19 only.

Histopathological Changes

- The mixture group: the liver showed accumulation of mononuclear cells in parenchyma. In spleen, there was lymphocytic hyperplasia in the periarteriolar sheath and proliferation of mononuclear cells around the sinusoids.
- S19 group: the liver revealed accumulation of mononuclear cells around central veins in liver parenchyma, as well as proliferation of kupffer cells. There was moderate hyperplasia in the white pulb of spleen with proliferation of mononuclear cells around sinusoids forming cord- like structure. In kidney, there were no important pathological changes except aggregation of few lymphocytes and macrophages between renal tubules.
- Control group: the liver of non-immunized animals revealed degenerative changes represented by vaculation of nuclei of hepatocytes with presence of granulomas distributed in liver parenchyma, composed of macrophage and lymphocyte aggregation. The histopathological changes in spleen of control mice included congestion of red pulb with hyperplasia of white pulb.

DISCUSSION

Vaccination is the only practical means of controlling the disease when its prevalence is high (16). The attenuated vaccine contains all the immunogenic components that can be involved in protection, making this vaccine type more efficient (7). The first effective *Brucella* vaccine was based on live *B. abortus* strain 19, a laboratory derived strain attenuated by an unknown process during subculture (17). This vaccine induces reasonable protection against *B. abortus* (18). But the vaccine has the disadvantages of causing abortion, and of being pathogenic for human. However, its main disadvantage is the induction of O-polysaccharide (O-PS) specific antibodies that interfere with the widely used serological tests which employ S-LPS as antigen (19), so that *B. abortus* S19 vaccine was used in this study, but in a reduced dose in combination with anthrax PA as an adjuvant. A reduction of the vaccine dose induces a shorter and less intense antibody response following vaccination (5). We used the mixture in a ratio of 1:1 to immunize mice in dose of 0.1 ml s/c in order to decrease side effects of S19 vaccine.

Brucellae produce a high- molecular- weight protein factor that specifically inhibits expression of tumor necrosis factor α (TNF- α) in activated human macrophages. This inhibition likely contributes to the ability of *Brucella* species to evade host defenses and maintain an intracellular existense. Intracellular ribosomal protein antigens designated L7/L12 has been found to stimulate protective cell- mediated immune responses along with delayed-type hypersensitivity (20). In our study, the results of brucellin skin test were 1.51 ± 0.103 mm, 1.14 ± 0.103 mm and 0.89± 0.100 mm after 24, 48 and 72 h; respectively when immunize mice with the mixture. S19 group showed approximately similar results of 1.54 ± 0.289 mm after 24 h, 1.06 ± 0.141 mm and 0.80 ± 0.127 mm after 48 and 72 h, respectively. The live vaccine has the great advantage that entery of the challenge inoculum into the tissues induces an accelerated recall of the earlier cell- mediated immunity which is able to control the further growth of the organism (21).

Cytokines produced by activated macrophages including TNF-α, TNF-γ and interleukin-1 (IL-1) and IL-2 are known to influence macrophage- mediated anti-*Brucella* activity during induction of cell- mediated immune response (22). Adaptive immune responses are critical for providing the memory function that is the key player in vaccination. Adaptive immune response functions in brucellosis can be classified into three mechanisms: first, IFN-γ produced by $CD4^{+,}$ $CD8^{+}$ and γδ T cells activates the bactericidal activity of the macrophages hampering the intracellular survival of *Brucella*. Second, cytotoxicity of $CD8^{+}$ and γδ T cells kills the infected macrophages. Third, Th1- type antibody isotypes such as IgG2a and IgG3 opsonize the pathogen to facilitate phagocytosis (7, 23).

It was concluded that $CD8^{+}$ T cells could be the major component in immunity against brucellosis. Therefore, in this study we have used a vehicle containing a nontoxic component of the anthrax toxin: the crude PA in order to activate cytotoxic T- lymphocyte (CTL) (23). For activation CTLs, the presented protein antigen must be translocated into the cytosol and presented to class I MHC molecules. The macrophages infected with *Brucella*, can be agood target for CTLs, if CTLs are induced, and T cell receptor (TCR) complexes recognizing MHC-I molecules, alongside with *Brucella* CTL- peptides, are produced at their surface. In order to activate CTL researchers (24) have used a vehicle containing nontoxic components of the anthrax toxin: PA and associative domain of the LF.
Because PA is able to potentiate a humoral response against heterologous antigens, so that the mixture group (containing crude PA) gave the highest antibody titer of 0.7153± 0.08 nm as compared with 0.3743± 0.06 nm of S19 group in ELISA. Crude PA of anthrax toxin used in the present study may contain some amounts of the other toxin components (like LF). PA binds to ubiquitous receptor on the surface of mammalian cells and is cleaved by furin-like proteases. This processing result in the release of 20 KDa amino-terminal fragment, and the cell-associated 63 KDa fragment interacts with LF. The PA63- LF complex is then internalized by receptor-mediated endocytosis. LF is translocated into the cytosol. The amino-terminal part of LF (LF254) binds to PA63. Fusion proteins consisting of LF254 and heterologous antigens have been shown to be successfully delivered to cells via PA (8).

In this study, *Brucellae* were isolated from non- immunized mice 10 days after infection from blood, liver and spleen in large numbers. It has been mentioned that the increase of *Brucella* numbers in the host is mainly due to their ability to avoid the killing mechanisms and proliferate within macrophages. Few numbers were isolated from kidney (7); our findings are similar to those of (25). There were no bacteria isolated from vaccinated animals either in the mixture or in S19 group.

To confirm our results, histological sections have been taken from spleen and liver of immunized and control animals to study the histopathological changes. The group immunized with the mixture was better than that vaccinated with S19 alone; concerning the spleen of the first group there was proliferation of mononuclear cells around the sinusoids in addition to lymphocytic hyperplasia in the periarteriolar sheath. While the spleen of the S19 group showed moderate hyperplasia in the white pulb with proliferation of mononuclear cells around sinusoids forming cord-like structure. In control animals, there were severe

histopathological changes in which liver showed severe aggregation of mononuclear cells with proliferation of kupffer cells in addition to degeneration of hepatocytes, necrosis and granuloma. The changes in spleen included congestion of red pulb with hyperplasia of white pulb. These severe changes refer to highly virulent strain that was applied in this research. The cell envelopes of *Brucella* play a critical role in virulence due to some unusual physicochemical properties linked to peculiar lipids (i.e. phospholipids, ornithine lipids, Lipid A and lipoproteins) and to the properties of periplasmic cyclic β- glucans (26, 27). Finally, our instructions imply studying the immunization efficacy of the mixture in farm animals and compare it with other *Brucella* vaccines.

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Table (1): The difference of thickness in foot pad of mice vaccinated with the mixture

Animal No.	Difference of Thickness (mm) after		
	24 h	48 h	72 h
1	1.4	0.9	0.7
2	1.8	1.3	0.8
3	1.25	1.2	0.8
4	1.4	0.9	0.9
5	1.7	1.4	1.25
Mean± Standard Error	1.51± 0.103	1.14± 0.103	0.89± 0.100

Table (2): The difference of thickness in foot pad of mice immunized with S19

Animal No.	Difference of Thickness (mm) after		
	24 h	48 h	72 h
1	2.4	1.4	0.9
2	1	1	0.5
3	1.8	1	0.8
4	0.8	0.6	0.6
5	1.7	1.3	1.2
Mean± Standard Error	1.54± 0.289	1.06± 0.141	0.8± 0.127

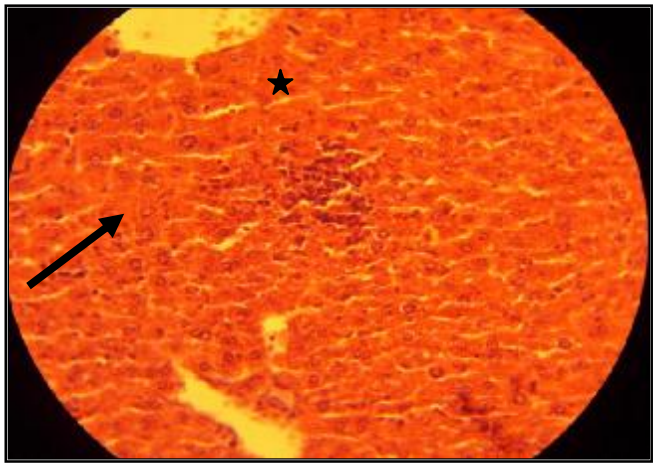

Figure (1) Histological section in the liver of an animal after 70 days post immunization (35 days from the second booster dose) with S19 &PA, showed aggregation of mononuclear cells in the liver parenchyma with proliferation of kupffer cells . (H & E 40×)

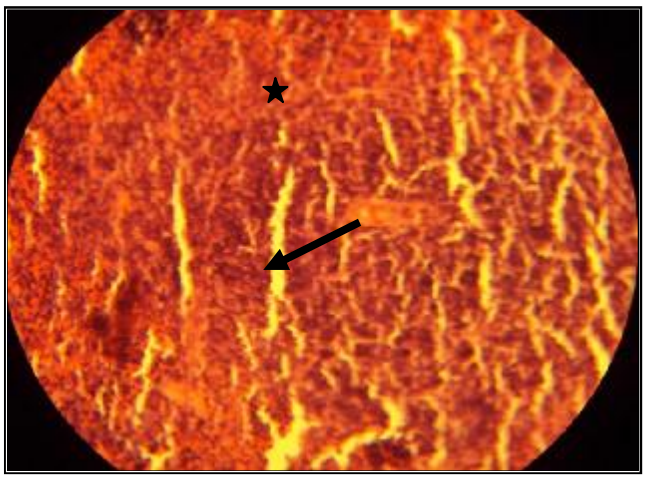

Figure (2) Histological section in spleen of an animal after 10 days after challenge, immunized with (S19 &PA) showed lymphocytic hyperplasia in the periarteriolar sheath and proliferation of mononuclear cells around the sinusoid . (H & E 40×)

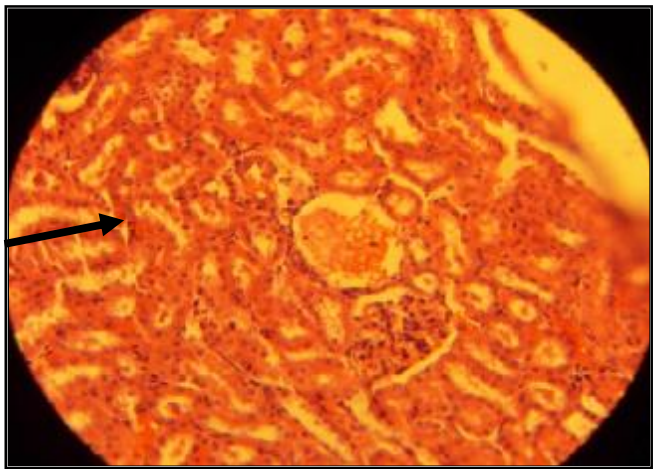

Figure (3) Histopathological section in kidney of a mouse immunized with S19 only. There are no pathological lesions except few lymphocytes and aggregation between renal tubules . (H & E 40×)

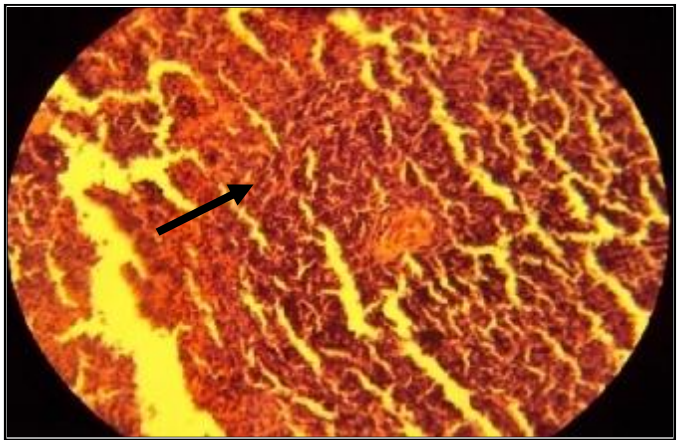

Figure (4) Histopathological changes in the spleen of one of the animals immunized with S19, showed moderate white pulb hyperplasia (H & E 40×)

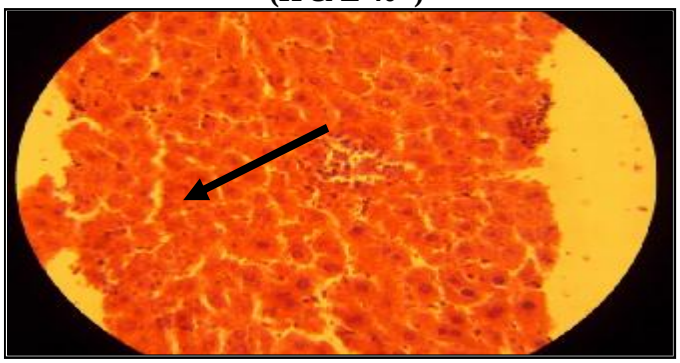

Figure (5) Histopathological section in liver after immunization with *Brucella* S19, showed congestion of sinusoid with mononuclear cells their lumen as well as in the liver parenchyma (H & E 40×)

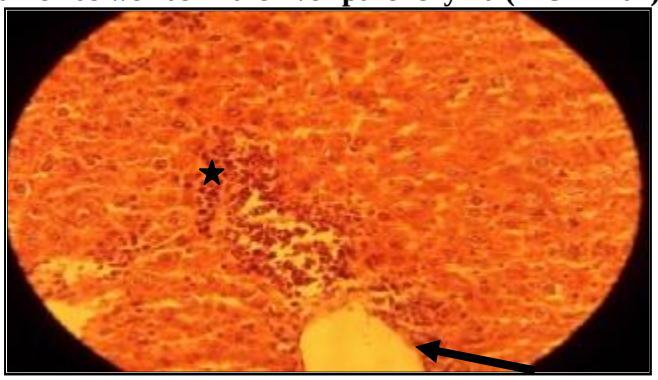

Figure (6) Histopathological changes in liver of control non-immunized animal injected with virulent *B. melitensis* after 10 days of infection, showed sever mononuclear cell a ggregation around central vein an d liver parenchyma together with proliferation of kupffer cells. (H & E 40×)

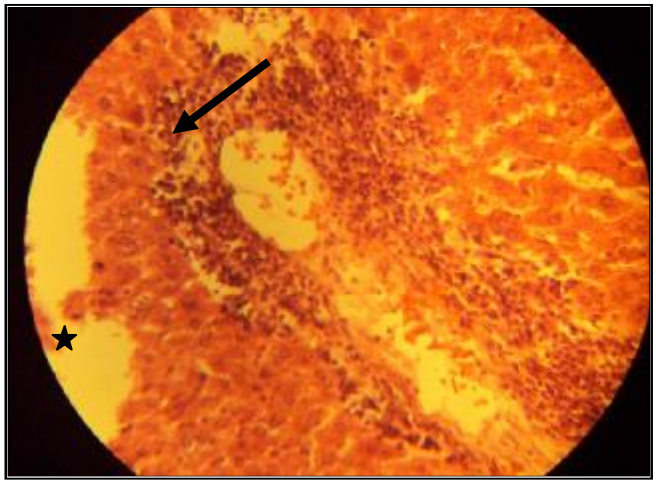

Figure (7) Histopathological section in liver of a non- immunized animal 10 days post inoculation with *B. melitensis*, showed sever inflammatory cell infiltration mainly macrophages around blood vessels and in liver parenchyma together acute cellular degeneration of hepatocytes and necrotic area . (H & E 40×)

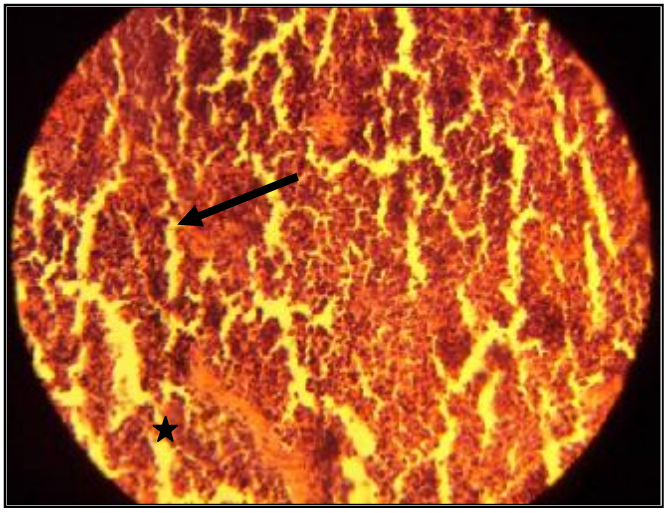

Figure (8) Histopathological section in spleen of one of unimmunized mice after 10 days post infection, showed depletion of white pulb with congestion of red pulb . (H & E 40×)