Pathological changes inducing by Brucella mellitensis in mice immunized with Culture Filtrate Brucella mellitensis Antigens (SCFAgs) and chitosan

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Abstract

In order to determine the influence of Culture Filtrate Brucella mellitensis Antigens (CFAgs) on B. mellitensis infection in chitosan immunostimulater mice, sixty for white mice, both sex, 7-8 weeks age were divided randomly into for groups. 1st group (n=16) was immunized with 0.4ml of CFAgs B. mellitensis (concentration of protein(4.2mg/ml), i/p two doses, 2 weeks intervals. 2nd group (n=16) was feed on diet supplement with chitosan, (1mg/kg B.W) 4 weeks. group 3rd group (n=1) was inoculated with (0.4ml) I/P with 1X10² CFU/ML of viable virulent B. mellitensis and was served as control positive group. 4th group (n=16) was inoculated with 0.5ml sterile normal saline. Cellular and humoral immune response were recorded at 28-30 day post immunization, skin test and passive heamagglutination test respectively, then all animals of immunized and feed chitosan groups were challenge with B. mellitensis as control positive group. The results explained that dead for animals in cotral positive during 15 days post inoculation with virulent viable B. mellitensi with very heavy bacterial isolation, from animal of control positive group post infection The results revealed that immunization with CFAgs elicited both humoral and cellular immune responses, the level values of both arms of immune response also result reveal that immunization with CFSAg + chitosan elicited both humoral and cellular immune responses higher than other group. Severe pathological lesions were seen in examined organs of control positive group but these lesions are mild or few in animal immunization with CFSAg + chitosan. The main lesions in examined organs of these animals are suppurative inflammation, small granuloma. We conclusion that immunization with CFSAg + chitosan can improve the immune responses in the animals that are suffering from Brucella mellitensis infection.

KEYWORDS B. mellitensis.. CFB.MAgs. Chatosan

Introduction

Brucellosis is an important, highly contagious, economic, widespread zoonotic disease which is caused by the genus of Brucella(1). Brucella melitensis and Brucella abortus, a facultative intracellular gram-negative coccobacilli, are the two most common causative agents of Brucellosis in both human, Ovine and cattle. The disease causes by these organisms characterized by undulant fever, chronic fatigue, arthritis, endocarditis, meningitis and orchitis in humans and the infection become chronic if not treated, in addition the symptoms may recur years after the original infection(2). Chitosan is a modified natural carbohydrate polymer derived from chitin, which occurs principally in Arthropod which produce commercially by deacetylation of chitin which is the structure element in the exoskeleton of crustaceans (such as crabs, pandalus borealis, shrimp) and cell wall of fungi(3). Chitosan play role in stimulated immunity both humeral and cellular immunity(4). In the present study, we an attempt to improve the immunogenicity of the culture filtrate B.melitensis antigens in immunized animals fed diet supplement with chitosan(5).
Materials and Methods

Spicies of baccteria take from pathological branch from veterinary medicine collage of Baghadeed and confirm biochemical examination of bacteria and examination virulence of Brucella meillitensis

Preparation of Brucella meillitensis:
Culture filtrated Brucella meillitensis antigens (CFSAs):
Brucella meillitensis was cultured on 15 Tryptic soya agar plates and incubated at 37c° for 24-48 hrs then harvested by PBS 7.2, and the bacterial suspension was centrifuged at 3000 rpm 4 °C /30 minutes. The supernatant was taken in sterile method and filtrated by Millipore filter. The supernatant fluid was examined by G stain and culturing on blood agar to confirm sterility of these antigen.

- The total protein concentration of this antigen was measured according to Buiret procedure( 4.2 mg/ml) bacteria consider as(CFBAgs). Than part of this supernatant solution was cold centrifuged at 23000rpm for (30) minutes, the supernatant was consider as soluble culture filtrate Brucella melitensis antigen (SCFBAgs). The supernatant fluid was examined by gram stain and culturing on blood agar to confirm sterility of these antigen.

- Whole Brucella Sonicated Ag. (WSBAg):
It was prepared as follow (Mitove et al., 1992):
- Brucella mellitensis cultured on Tryptic soya agar, incubated at 37 °C for 24-48 hrs. and harvested by PBS 7.2, centrifuged at 3000 rpm 4 °C /30 minutes then washed the precipitate three times with PBS, and the precipitate was re-suspended with PBS and put in the universal tube.
- Sonication: the universal tube that contained Brucella mellitensis suspension was placed in the ultrasonicator (type Karl Klob – Germany) at 12 Peak with 2 minutes intervals between them, for 30 minutes in cold environment (ice).
- The sonicated suspension was centrifuged at 23000 rpm for 30 minutes.
- The supernatant fluid was examined by gram stain and culturing on blood agar to confirm sterility of these antigen.
- The total protein concentration of this antigen, which measured according to Buret procedure 16 mg/ml and it was diluted to become 0.5 mg/ml this antigens was considered as soluble sonicated Brucella antigens(SBMAgs)

Determination of the virulent and Challenge Dose S.aureus:

Brucella Mellitensis cultured on a Tryptic soya agar nd incubated at 37 °C for 24 -48 hrs. Two mice were inoculated I/P with 0.2 ml of bacterial growth ,the animals were scarified at 72 hrs post inoculated and pieces from internal organs were culture on the blood agar for 24-48 hrs at 37c° and this process was recurrent until the inoculated animals were dead during hrs. 12 mice both sex were divided into three equal group and they were inoculated with 0.2 ml of bacterial suspension containing 1x10⁸ ,1x10⁹ and 1x10¹⁰ CFU of virulent Brucella Mellitensis respectively and we recorded the number of dead animal during 48-72 hrs post inoculation. The dose which killed half number of inoculated animal was consider as a challenge dose (1x10⁹ CFU/ML) (5). The preparation of the bacterial suspension of the counting was made using (7).

Preperetion of chatosan Diet
Commercial assorted pellets were grinded by food grinder and weighed, 1 gm of Chitosan was added to each kilogram of grinded pellets mixed well and converted into paste which passed through meat grinder to mould the paste into the original pellets from, left exposed to dry in room temperature (8).

Experimental Design:
One seventy four mice, both sex, 7-8 weeks old were divided randomly into (5) groups and treated as the following:

1. 1st group (n=16) was immunized with 0.4ml of Brucella mellitensis CFSAgS (concentration of protein 4.2mg/ml) i/p two doses, 2 weeks intervals.

2. 2nd group (n=16) was immunized with CFSAgs as 1st group and feed on diet supplement with chatosan (1g/kg) for week 3.

3. 3rd group (n=16) was inoculated with (0.4ml) I/P with 1X10^9 CFU/ML of viable virulent Brucella mellitensis and was served as control positive group.

4. 4th group (n=16) was inoculated with 0.5ml sterile normal saline.

Cellular immune response was detected at 28 days post immunization with skin test and at day 30 post immunization, 6 animals from 1st, 2nd, 3rd, 4th groups were sacrificed for collection of blood and to determine the homural immune response, then remain animals of 1st, 2nd, 3rd, 4th groups were challenge I/P with 1X10^9 CFU/ML of viable virulent Brucella mellitensis. Five animals from each group were sacrificed at, 30 days post challenge and post-mortem examination was done, pieces from internal organs were taken for bacterial isolation and other pieces were fixed in 10% neutrals buffer formaldehyde (72 hrs) for histopathological examination.

Plan of study:

Delayed Type Hypersensitivity Test (DTH):
The test was conducted according to (8).

Passive Hemagglutination Test (PHA Test)
The test was conducted according to (9).

Results and Discussion

Immunization:

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<tr>
<th>group</th>
<th>Mean skin thickness</th>
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<tr>
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<td>Against SCFBMAs</td>
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<tr>
<td>1</td>
<td>0.7 ± 0.17</td>
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<td>0.7 ± 0.17</td>
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At 24hr post testing, The results showed that the mean values of skin thickness against SCFSAgS and against SSSAgS (0.7 ± 0.7, 0.28 ± 0.64) were lower in 1st group as compared with 2 group (1.9 ± 0.57, 1.32 ± 0.25) respectively. At 48hr post examination, the mean values of DTH against both SCFSAgS and SSSAgS were decline in 1st group (0.52 ± 0.09, 0.22 ± 0.53) and in 2 group (1.58 ± 0.17, 0.61 ± 0.24) respectively in (table:1). The result of passive haemagglutination examination revealed the serum Abs titers in 1st group (108.8 ± 219.2) lower than in 4 group which consider (245.4 ± 74.63) (table: 2).
Passive Hemagglutination test (table 2).

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<th>Mean values of antibodies titers at 30 days post-immunization, (Mean ± S.E)</th>
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<tr>
<td>1</td>
<td>108.8 ± 19.2</td>
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<tr>
<td>2</td>
<td>245.4 ± 74.63</td>
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The results of Delayed Type Hypersensitivity (DTH) in the present study may indicated that the CFBAs elicited cell mediated immune response in immunized animals, since DTH is the essential type of CMT and it is mediated by CD4+Tcells and CD8+Tcell cytokines production, these evidence was supported idea that mentioned by (8,9), who reported that Candida CFAs and Candida CFAs were stimulated CMI. The induction DTH reaction in animals immunized with CFBAs in the present study may be due to the protein nature of extracellular secretion of B.mellitensis which is considered a good stimulator of cell mediated immune responses, these observation was supported the idea that recorded by (10) who explained that CFAs of S.aureus stimulated cellular and humoral immunity. The differences between mean values of the skin thickness against CFAs and SSAs in the present study may be due to antigen specificity and protein concentration in both antigens which may be high in the SCFAs, these observations were in consistence with (11), who explained that the protein antigens were a better stimulator of APCs and T cells that produced INF-y and TNF-alpha which play important role in expression of DTH. and humoral immune responses, these result may be indicated that these type of Ags elicited both subsets of Th1 which responsible for CMI and Th2 which responsible for proliferation and differentiation of B-lymphocytes to plasma produsing antibodies, these suggestion was supported by idea of (12) who found that immunized mice with soluble Brucella antigens stimulated spleen cells of these animals to generate Th2 response which play mainly role in stimulated humeral immunity. Our observation revealed that animals immunized either with CFBMAgs fed diet supplement with chitosan expressed high level of DTH and antibody titers, these finding may indicated that chitosan augment both arms of immune response, these idea was agreement with (6) who explained that immunized mice with viscous chitosan solution stimulated cellular and humoral immunity. also the present study found that immunized animals with CFBMAgs + chitosan expressed high values of DTH and Abs titers as compare with other groups, these result may be indicated that chitosan strength the immune response induced by CFBMAgs these idea was agreed with observation of (13), who said that the Chitosan has been used as an immunostimulant for protection against bacterial disease in fish, and as a diet supplement.

Clinical signs and bacterial isolation:

There is clear clinical symptoms noticed on non-immunized infected animals particularly during the first month post-infection, and these clinical symptoms characterized by loss appapitate, losse movement, and 4 animals died during first
15 days post-infection while no clear clinical symptoms noticed on immunized infected animals during the course of the study. Bacterial isolation were variable according to protocol of immunization and the period of sacrifice but the levels bacterial growth in non-immunized infected animals were high during 15-35 days post infection as compare with immunized animals. Our finding was agreement with (14) who said that the responses of mice for virulent brucellae are more severe as compare with immunostimulated mice.

**Pathological examination:**

**Gross examination:**

Infected The Gross examination of the internal organs of control challenged died mice during the first 15 days post challenge demonstrated severe congestion of those organs, while no clear gross lesions were reported in examined organs of immunized challenged animals.

**Histopathological examination:**

**Non-immunized infected animals at day 30 post-infection**

**Lung**

The lung showed hyperplasia of the epithelial lining cells of bronchiol more extensive than those noticed at (30) in addition to congestion blood vessels with neutrophils in their lumen (Fig: 23)

**Liver**

Histopathological examination revealed multigranulomatous lesions in the liver parenchyma consisting from aggregation of macrophages (Fig: 15)

Histopathological examination revealed to dilated of the sinusoids with mononuclear cells in their lumen (Fig: 17)

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Fig: 17. Histopathological section in the liver of animal at 30 days post-infection shows focal aggregation of mononuclear cells in the liver parenchyma with present of megakaryocyte (H&E stain 40X)
Immunized animals with CFBAs at 30 days

Liver
Multiple granulomatous lesions consisting from activated macrophage and lymphocytes were seen in the liver parenchyma and around the central veins (Fig: 35, 36).

Kidney
also mononuclear cells particularly lymphocytic cells aggregation in the interstitial tissue of the kidney more intensity than that recorded in day 15 post-infection (Fig: 38).
Fig: 35. Histological section liver of immunized animal with CFBMAgs at 30 days post-infection shows multiple granulomatous lesions with Kupffer cells (H&E stain 40X).

Fig: 36. Histological section in the liver of immunized animal with CFBMAgs at 30 days post-infection shows granulomatous lesions in one side of central veins with proliferation of Kupffer cells (H&E stain 40X).
Immunized animals with CFBAs+fed on diet supplement with chitosan
At day 30 post-infection
liver
The no clear lesions in the liver except proliferation of kupffer cells(Fig:80),
Spleen
the spleen showed marked hyperplasia of white pulp and proliferation of mononuclear cells around the sinus of red pulp(Fig:81).
Lung
hyperplasia of the epithelial lining cells of bronchiol with hyperplasia of lymphoid tissue in the wall of the airways(82),
Fig: 81. Histopathological section in the spleen of immunized animal with CFBAgs+chitosan at day 30 post infection shows marked proliferation of lymphocytes in the periarteriolar sheath (H&E stain 40X).

Fig: 82. Histopathological section in the lung of immunized animal with CFBAgs+chitosan at day 30 post-infection shows marked proliferation of the epithelial lining cells of the bronchiol mononuclear cells aggregation in the wall of the blood vessels (H&E stain 40X).

In the present study, histopathological examination showed severe lesions in the examined organs of non-immunized infected animals particularly the liver and spleen, these results may be indicated that the Brucella strain used in the present study overcome the normal defense mechanism of these organs, these result in consistent with (12), acute inflammatory response against bacterial infection and starting of cell mediated immune response that induced granulomatous reaction. These investigations were in consistent with (15) who explained that the acute phase of brucellosis start from day three to 2nd and 3rd week and these stage characterized by rapid increase in number of bacteria in the target organs particularly spleen and liver. While immunized animal we recorded moderate
pathological lesion in the examined organs of immunized animals with CFBMAs at day 30 post-challenge with B. melitensis, these results may be indicated that these Ags provided a partial protection, these idea was supported by (Cassataro et al., 2005) who recoered that immunized mice with Omp31 stimulated a CD4+ Th1 response which provided partial protection against B. melitensis infection. Also, we recorded that the intensity of pathological lesions in immunized animals with C f B Ags and feed diet supplementing chitosan lower as comparing with those in non-immunized infected animals, immunized animals fed diet not supplement with chitosan, these results also supported out results of immunity and bacterial isolation and supported idea that chitosan activated and strength immune responses. These finding was agreement with (Asiad, 2012) who suggested that chitosan strength both cellular and humoral immune responses.

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التغيرات المرضية المحدثة بواسطة بكتريا البروسيلا في الفئران الممنعة

بمختصر الاستراتيجي لـ *Brucella* البروسيلا و المحفز المناعي الكيتوس

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الخليصة

من أجل تحديد تأثير فعالية مستضد الراشج أزرعلي بكتريا البروسيلا الضارية على الإصابة بالبروسيلا في الفئران التي تتغذى على عينف معاملة بالكيتوس. هذا الفراض استخدمت أربعة و ستون فارا من كل الجنسين تتراوح أعمارهم بين سبعة إلى ثمانية أسابيع سُميتها عبارة عن إمراء مجموع. أحتوت المجموعة الأولى تحتوي على 16 فارة داخل الخب جرعة (CFBMAgs) وملعنة (0.5) المجموعة الثانية: أحتوت على (16) فار والتي منعت مستضد الراشج البكتيري كما في المجموعة الأولى ولغة على الريق المعاملة بالكيتوس. المجموعة الثالثة: ضمت هذه المجموعة (16) فار والتي تعتبر كمجموعة سيطرة ما حقق 4 في المخلب بجرعة الت تقني 1×10^9 خلية/مل من بكتريا المكورات العنقودية الدهنية الضارية. المجموعات الرابعة: ضمت هذه المجموعة (16) فار حلت بجرعة (0.5) من المحلول الفيسيولوجي المعدل واعتبرت مجموعة سيطرة سالبة. فحص المناخة الخلوية والخلطية اجري في يوم 28-30 على الخبواح. بعد ذلك تم إصابة جميع الخبواح المتبقية (الممانعة والمعالجة) بمصيرة بالإصابة بالبروسيلا. أظهرت النتائج أن المجتمع مستضد الراشج البكتيري (CFBMAgs) في الفئة من المنخ phụcية والخلاصة، أحد قيم مستوى كل من طرفي المناخة (الخلاصة، الختام) أفضل في الخبواح الممانعة بواسطة اختبار تفاعل الوراثة و تقسيم الجملة. أما أيضاً أضرحت النتائج كان التغيرات المناعية في المحمية المعينة بالراشج البكتيري المتغير و المغذية على الكيتوس كانت أكثر تغيير مناعي بباقي المجموعات. وأيضاً اضرحت النتائج ان إلى موت أربع حيوانات من مجموعة السيطرة الموجهة بعد 15 يوم من بعد الحق الجرعة التوتاني. و اضرحت عزل بكتيري نادر من الأعشاب الحياتي و أضرحت النتائج خلال لحمة الخص في النسيج. و بعد فلته وسرعه كان أبرزها الإصابة الجيبة و النضجة القيحية في النسيج. لذلك استنتج أن المجتمع بالراشج البكتيري البروسيلا (CFBMAgs) الممانعة على مطقات الإصابة البيكتري البكتريا (CFBMAgs) *Brucella melitensis*