The Cellular Immunoprotection of BALB/C mice vaccinated with Salt-Extractable *Brucella abortus* S19 antigens and Immunoadjuvant *β*eta-glucan challenged with *Brucella abortus* Virulent Strain

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Summary

The aim of this study was to evaluate the cellular immune responses of salt-Extractable *Brucella abortus* S19 antigens with immunoadjuvant soluble βeta-glucan in BALB/C mice later challenged with *B. abortus* virulent strain. The 0.72mg/ml of SEBA was used according to the results obtained from experiment to determine the macrophages Nitric oxide production and delayed type hypersensitivity test. One hundred BALB/C mice were divided into four groups. G1 were injected i.p with 0.2 ml of saline, G2 were vaccinated S.C with 0.1ml (10^8 CFU/mouse) of *B. abortus* S19, G3 were vaccinated i.p. with 0.2 ml of salt-Extractable *Brucella abortus* S19 antigens and G4 were vaccinated i.p. with 0.2 ml of salt-Extractable *Brucella abortus* S19 antigens and 0.2ml *β*eta glucan. At 27 days after immunization the delayed type hypersensitivity test was conducted with the significant ($P<0.05$) an increase in the foot pad thickness of the G4 as compared to G3, G2 and G1. At day 30 of immunization all remaining mice were i.p. challenged with 0.2ml of $1x10^4$ CFU/ml *B. abortus* virulent strain. The log^{10} CFU/spleen count was demonstrated at day 7 and 14 after challenge; G2, G3 and G4 recorded low bacterial count with a significant difference ($P<0.01$) as compared to the control. In conclusion, this study indicated that immunized mice with salt-Extractable *Brucella abortus* S19 antigens and *β*-glucan were effective vaccine, the activated innate immunity was an important key to activate the protective Th1 responses through a significant decrease in the bacterial splenic count after challenge.

Keywords: Salt-extractable antigens, *Brucella abortus* S19, *β*-glucan, Immune response.

Introduction

Brucellosis is a major zoonotic disease that causes severe economic losses for livestock farms which are transmitted between animals, both vertically and horizontally (1). *Brucella abortus* is the primary cause of brucellosis or Bang's disease in cattle, bison, and elk all over the world (2).There is a general agreement that the most successful methods for prevention and control of brucellosis in animals is through vaccination (3). Both killed and live, attenuated vaccines have been examined for their potential role in the control and eradication of brucellosis in cattle, goats, and swine (4). Currently, *B. abortus* S19, *B. abortus* RB51 is used to immunize cattle, whereas, *B. melitensis* Rev 1 is used to immunize goats and sheep. The immunity derived from their use tends to be cell-mediated and long lasting and the organism is allowed to replicate within the host, thus making them less expensive (5**).** The *B. abortus* strain S19, a spontaneously attenuated strain, has been used as a vaccine strain in vaccination of cattle against brucellosis (6). However, this vaccine has several disadvantages, including abortion in pregnant animals, and induces an antibody response to long O-side chain (7 and 8). This strong antibody response is the reason for this vaccine's effectiveness, but it also causes a problem as the antibodies produced will create a false positive in a serological test for *Brucella* infection (9). For these reasons, different strategies are being sought for the production of safe, non-replicating vaccines that are easy to reproduce with consistent quality. Several outer membranes, periplasmic and cytoplasmic proteins were tested as a candidate for subunit and recombinant vaccine in various species of animals as they had a possible role in the immune response against brucellosis (10 and 11). Salt extractable antigen excluding protoplasmic proteins are those present in the matrix of the outer membrane and in the periplasmic space of gram-negative bacteria, salt-extractable protein antigens from *B. abortus* may be of potential

value as a vaccine and as a diagnostic reagent for the prevention and diagnosis of bovine brucellosis (12 and 13).

B. abortus activates innate and acquired immunity over the course of infection. Macrophages are key elements in innate immune responses and recognition of *Brucella* components. The important bactericidal mechanisms of macrophages are nitric oxide production (14). Th1 polarization of the adaptive immune system is crucial in the host controlling infection. Vaccination can enhance the protection afforded by the host's immune system due to priming of the adaptive immune system. (15). Natural products containing *β*-glucans have been used for thousands of years for the benefits of human health; recently the active components of *β*-glucans were identified. Since then, they have been studied extensively for immune stimulatory effects and developed for the treatment of several diseases, including cancer, infectious diseases (16-18). *β*-glucan is a safe and very potent biological response modifier that nutritionally activates the immune response through the Macrophage immune cells to yield various therapeutic effects (19). Current vaccines (*B. abortus* and *B. melitensis*) are effective in preventing abortion and transmission of brucellosis, but poor at preventing infection or seroconversion. In addition, they can induce abortions in pregnant animals and are infectious to humans (20).

The Aim of this study was to evaluate cellular immunoprotective responses of salt-extractable antigen of *Brucella abortus* S19 with soluble *β*-glucan as an adjuvant in Balb/c mice that later challenged with virulent *B. abortus*.

Materials and Methods

The Aborvac-S Cow™ *Brucella abortus* S19 vaccine was kindly supplied by the State Company for Veterinary Services from Company VETAL, HAYVAN SAGLIGI URUNLERi A.S. / TURKEY. The field isolate was kindly supplied by the State Company for Veterinary Services. It was isolated from diagnostic *Brucella* infected cow and identified biochemically as well as serologically.

Preparation of salt extractable *B. abortus* Antigens (SEBA): The salt extractable antigen was prepared from *B. abortus* S19 as described by (21). The total protein content in the SEBA was 2.4 g /dl; while the total carbohydrates were 1.5 mg/ml. The total protein content of the whole cell sonicate WCS was 3.3 g/dl and the carbohydrates were 2.3mg/ml. The *β*-glucans was kindly supplied from Prof. Dr. Nidhal Raoof Mahdi. The aqueous extract of soluble *β*-glucans was extracted from baker's yeast (*Saccharomyces cerevisiae*) according to Williams, (22) with the concentration of 1mg/ml.

Preparation of whole cell sonicate (WCS) *B. abortus* S19 antigens: Ten ml of Viable *B. abortus* (S19) organisms were seeded on tryptic soya agar and incubated at 37°C for 48 h. Bacteria were harvested by saline and the bacterial suspension was examined with gram stain. The bacterial suspensions were washed three times by centrifugation the by cool centrifuged 4°C, 5000 rpm for 15 min, the sediments were diluted in 1:4 ratio (w/v) with PBS pH 7.2 and sonicated on ice with a probe sonicator. The sonication process was performed by using the sonicat0r (at Al-Candi Company) with ultrasound waves for 15 min of actual working with 30 Volt. The cell debris of *B. abortus* S19 in the suspension were removed by centrifugation 16000 rpm for 30 min at 4°C, the supernatant was filtered through 0.22 µm pore size. WCS was streaked onto tryptic soya agar to confirm purity from bacterial contamination prior to use. Then the total protein of the supernatant was measured by using biuret method and it contained 3.3 g/dl and the LPS was 2.3mg/ml

Animals and Experimental Design: One hundred and twenty four female white Swiss BALB/C mice, aged 6-8 weeks and weight (20-25g), were used in this study. They were housed and maintained in a conventional animal facility, with controlled conditions of temperature (20 ±5°C) and 10-14 hours of light and dark respectively. The animals were fed on special formula feed pellets and water was provided on an *ad libitum* basis. Throughout the experiments, mice were housed in a plastic cage containing hardwood chip as bedding, the bedding was changed weekly to ensure a clean environment.

The first experiment was carried out to determine the best concentration of *B. abortus* salt-Extractable *Brucella abortus* S19 antigens (SEBA); three different concentrations of prepared antigen were used. In this experiment 40 mice were divided equally into 4groups: G1: The 10 mice were i.p. injected with 0.2ml of Nacl normal saline solution, G2: The10 mice were i.p. injected with 0.2ml SEBA (0.24 mg/ml concentration), G3: The10 mice were i.p. injected with 0.2ml SEBA (0.72mg/ml concentration) and G4: The 10 mice were i.p injected with 0.2ml SEBA (1.2mg/ml concentration).

The second experiment was designed according to the results in the first experiment; the best concentration of salt extractable antigen was 0.72 mg/ml of SEBA. One hundred female mice were divided into four groups each group involved 25 mice that were treated as follows: G1: The 25 mice were i.p. injected with 0.2ml Nacl normal saline solution, G2: The 25 mice were subcutaneously (S.C.) injected with 0.1ml (10^8 CFU/mouse) of *B. abortus* S19 (23), G3: The 25 mice were i.p injected with 0.2ml SEBA, and G4: The 25 mice were i.p injected with 0.2ml SEBA with soluble *β*-glucan in concentration of 1mg/ml (17). The phagocytic activity of peritoneal macrophages was assayed by determination of the Nitric oxide concentration that was measured by Phagocytic assay as described by (24).

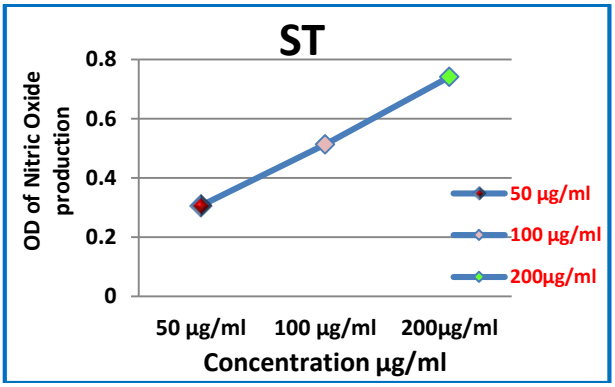
Delayed type hypersensitivity (DTH): Mice were injected intradermal in one footpad with 0.05 ml of WCS and in the contralateral footpad with an equal volume of saline, as negative control. The footpad thickness was measured 24, 48, and 72 hrs. later by using a digital caliper with a precision of 0.01 mm, and the mean increase in footpad thickness (expressed in mm) was calculated according to the following formula: Footpad thickness injected with sonicated antigen-footpad thickness injected with saline (25**).**

Four weeks after immunization, all groups of mice were challenged with virulent strain of *B. abortus*. Each mouse was given approximately 4×10^4 CFU in 0.2ml of saline solution. The challenge dose inoculum of approximately10^4 of *B. abortus* per 0.2ml of saline was used according to the previous study indicated that this dose consistently caused moderate signs of brucellosis in non-vaccinated Balb/C mice within 1 month of exposure (26). The bacterial burden in spleen of the immunized and nonimmunized groups were enumerated (27).

Statistical analysis was performed using the computer software by David S. Walonick, Ph.D. (Copyright © 1996-2010, StatPac Inc.) Student *t* test was used to compare mean values between groups. ANOVA test was used to find out the significant differences of mean of more than two groups of continuous variables. The statistical significance was accepted as *P* value < 0.05.

Results and Discussion

In the first experiment determination of phagocytic activity, the Nitric oxide production was determined for assessing the phagocytic activity of the peritoneal macrophage at day 7 in G2, G3 and G4 that were inoculated with different concentrations of SEBA (0.24, 0.72, 1.2) mg/ml respectively; also the G1. The standard curve of Nitric oxide obtained from different concentrations of sodium nitrate; accordingly, the concentration of Nitric oxide produced by different concentration of SEBA demonstrated that the peritoneal macrophages of mice in G3 that were injected 3% SEBA had high value (163.3 µg/ml) as compared to G2 (110 µg/ml), G4 (83.33 µg/ml) while in G1the OD value was less than the OD value of standard curve (Fig. 1).



Figure, 1: The standard curve of sodium nitrite used to determine the Nitric oxide production.

It has been reported that IFN-γ is the only cytokine that may be capable of inducing Nitric oxide production by itself. Nitric oxide

is crucial mediator in host defense and is one of the major killing mechanisms within macrophages (28). The resistance to intracellular bacterial pathogens such as *Brucella* is strongly dependent on the ability of the organism to develop a cellular type of immune response Thl, which is mediated by cytokines such as IFN-γ, IL-12, TNF-α. These cytokines activate macrophages, lymphocytes, and other effectors of the innate or adaptive immune system to optimize their capacity of pathogen clearance (29).

The results of DTH of SEBA were summarized in (Table, 1) which shows measurement of the foot pad thickness (mm) after day 21 of immunization in groups (G1, G2, G3 and G4).

Table, 1: The Mean ± SD mm thickness of footpad in groups (G1, G2, G3 and G4) at day 21 after inoculation with different concentration of SEBA.

Time/hour / Groups	Thickness of footpad (mm) Means ±SE 24 hours	Means ±SE After 48 hours	Means ±SE After 72 hours
G1	0.30±0.02 Ca	0.32±0.11 Ca	0.30 ±0.11 Ca
G2	0.38±0.19 Bb	1.21±0.38 Ba	0.28±0.08 b
G3	1.67±0.38 Ab	2.62±0.13 Aa	0.29±0.06 A
G4	1.4±0. 8 Aa	0.78±0.11 Cb	0.07±0.02 c

The different capital letters in column refer to significant difference (P≤0.05) between groups. The different capital letters in row refer to significant difference (P≤0.05) among periods.

According to the results, the DTH test for cellular immune response in G3 SEBA (0.72 mg/ml concentration) was increased after 24hrs. (167±0.38) as compared with G1, G2 and G4; their means thickness of footpad was 0.30±0.02, 0.38±0.19, 1.4±0.8, respectively. At 48hrs. the results showed that G3 also differ significantly (2.62±0.13) from G1, G2 and G4; their means thickness of footpad was 0.32±0.11, 1.21±0.38, 0.78±0.11, respectively. The results are in agreement with (30) who concluded that DTH test depend on ability and activity of Th cells to recognize antigen and secrete IL-1 which enhanced proliferation and differentiation of other T-cell into Th-cells which secrete IL-2 as a chemoatractive factor to attract macrophage around the area of activated T-cell which also secrete INF- γ that enhance the cytolytic activity of accumulated macrophages leading into skin thickness.

In the second experiment DTH assay: The increased footpad thickness (mm) at 24 hrs., 48 hrs. and 72 hrs. were determined after 27 days of immunization for G1, G2, G3 and G4. As shown in (Table, 2) the footpad thickness was significantly increased (P<0.05) in vaccinated groups as compared to non-vaccinated group at 24 hrs., 48 hrs. and 72 hrs. However, there was a significant increase (P<0.05) between G4 (2.96 ±0.4, 2.98 ±0.04, and 2.38 ± 0.3, respectively) as compared to G3 (2.03 ± 0.4, 2.63±0.5 and 1.66 ± 0.5, respectively) and G2 (1.42 ± 0.07, 1.7±0.09 and 1.12 ± 0.09, respectively) at 24 hrs. 48 hrs. and 72 hrs. In addition, there was a significant increase (P<0.05) in periods at 24 hrs., 48 hrs. and 72 hrs. for G2, G3, and G4 as compared to G1. At 48 hrs. There was a significant increase in G4 as compared to G2 and G1. At day 72 hrs. there was a significant difference (P<0.05) between G4 as compared to G3, G2 and G1.

Table, 2: The mean of footpad thickness in vaccinated groups of mice (G2, G3, G4) and non-vaccinated group (G1) after 27 days of immunization.

Time/hr. / Groups	Thickness of footpad (mm) Means ±SE 0 hrs.	Means ±SE After 24 hrs.	Means ±SE After 48 hrs.	Means ±SE After 72 hrs.
G1	0.59 ± 0.3	0.55 ± 0.3 C	0.22 ± 0.05 C	0.15 ± 0.02 C
G2	0.4 ± 0.06 b	1.42 ± 0.07 Ba	1.7±0.09 Ba	1.12 ± 0.09 Ba
G3	0.97 ± 0.13 c	2.03 ± 0.4 Bb	2.63±0.5 Aa	1.66 ± 0.5 Bb
G4	0.5 ± 0.3 b	2.96 ± 0.4 Aa	2.98 ±0.04 Aa	2.38 ± 0.3 Aa

The different capital letters in column refer to significant differences (P<0.05) among Group. The different small letters in row refer to significant differences (P<0.05) between period.

The mean increased in footpad thickness increase in beta glucan treated group (G4) was inconsistent with (31) who found that the intraperitoneal application of lentinan

improved the capacity of peritoneal macrophages to produce IL-12, which directed the immune response towards Th1 and stimulated the T lymphocytes to produce IFN-γ. The results of this study were in agreement with other studies where the β-glucan had the ability to activate the phagocytic activity of macrophages and PMNCs (32) and proliferation of monocytes and macrophages (33). The effect of *β*- glucan can be attributed to inhibition of the synthesis of cytokines, such as IL-10 (34), promotion of cytokine receptor secretion (35), and release of macrophage arachidonic acid metabolism in response to soluble *β*-glucan (36). The administration of *β*-glucan improved the capacity of peritoneal macrophage to produce IL-12 which directed the immune responses toward Th1 and stimulated the T- lymphocyte to produce IFN-γ due to binding to dectin-1 (37). The DTH results of this study were in agreement with (38) who demonstrated that the outer membrane and inner membrane protein of *Brucella bovis* induced high DTH response in mice by production IL-2 and INF-γ.

To establish the protective efficacy in the vaccinated groups, bacterial burden in spleen of the immunized and non-immunized mice were enumerated after 7 and 14 days of virulent challenge with *B. abortus* field strain. As shown in (Table, 3), the mean $\log^{10}$ CFU/spleen counted of G4 demonstrated a level of protection at day 14 post challenge ($8.5\pm1.3\times10^3$). There was highly significant difference ($P<0.01$) between the vaccinated groups (G2, $1.2\pm0.1\times10^4$; G3, $1.4\pm0.4\times10^4$ and G4 $1.3\pm0.2\times10^4$) at day 7 as compared to the control G1, $5.4\pm0.9\times10^4$. While at day 14 post challenge there was high significant difference was noticed ($P<0.001$) between the treated groups G2, $7.8\pm1.5\times10^3$; G3, $9.4\pm0.7\times10^3$ and G4, $8.5\pm1.3\times10^3$ and the control group G1, $6.2\pm2.9\times10^4$.

The results of G4 demonstrated that the *β*-glucans are potent immunostimulant which initially trigger the immunobiological function of macrophage. The activation of macrophage consists of several inter-connected process, including increased chemokines, chemotaxis, migration of macrophage to particles to be phagocytosed and degranulation leading increased expression of adhesive molecules on macrophage surface, in addition, β-glucan binding also triggers intracellular processes characterized by respiratory burst after phagocytosis of invading cells by formation of reactive oxygen species, increasing of content and activity of hydrolytic and metabolic enzyme and signaling processes leading to activation of other phagocytes and secretion of cytokines (39). Other studies (17) had demonstrated, the mice treated with *β*-glucan showed a high protection by lower the CFU\spleen, as compared to infected group with *Salmonella Typhimurium* not treated with the soluble *β*-glucan.

Table, 3: The protective efficacy against B. abortus virulent strain that conferred by immunization with SEBA with β-glucan or non β-glucan compared to *B.abortus* S19 or non-vaccinated group at 7 and 14 days postchallenge.

Postchallenge	Mean $\log_{10}$ of *Brucella* ± SD in spleen	
Group	7 days	14 days
G1	$5.4 \pm 0.9\times10^4$	$6.2 \pm 2.9\times10^4$
G2	$1.2 \pm 0.1\times10^4$	$7.8 \pm 1.5\times10^3$
G3	$1.4 \pm 0.4\times10^4$	$9.4 \pm 0.7\times10^3$
G4	$1.3 \pm 0.2\times10^4$	$8.5 \pm 1.3\times10^3$

Accordingly, this study was designed to prepare the salt extractable *Brucella abortus* S19 antigens that contain proteins and trace of carbohydrate with the usage of soluble *β*-glucan as immunoadjuvent as a safe vaccine for controlling the incidence of brucellosis in pregnant cows.

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الحماية المناعية الخلوية لفئران BALB/C الملقحة بالمستضدات المستخلصة بالاملاح للبروسلا المجهضة S19 مع المساعد المناعي بيتا كلوكان للتحدي بالعترة الضارية للبروسيلا المجهضة

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الخلاصة

هدفت الدراسة تقييم الإستجابة المناعية الخلوية لمستضدات البروسيلا المجهضة S19 المستخلصة بالاملاح مع المساعد المناعي بيتا كلوكان القابل للذوبان في الفئران BALB/C تحدّى لاحقا مع عترة ضارية للبروسيلا المجهضة. استعمل 0.72 ملغم/مل من مستضدات البروسيلا المجهضة S19 المستخلصة بالاملاح وفقاً لنتائج حُصل عليها من التجربة لتحديد أكسيد النيتريك المنتج من الخلايا البلعمية وتفاعل اختبار فرط الحساسية المتأخر. مائة فأره قسّمت بالتساوي إلى أربعة مجاميع. حُقنت المجموعة الأولى (G1) بالتجويف البرتوني 0.2 مل من الملح الفسيولجي، والمجموعة الثانية (G2) لقّحت تحت الجلد 0.1 مل (10^8 CFU/فأر) من *B. abortus* S19، والمجموعة الثالثة (G3) لقّحت بالتجويف البرتوني 0.2 مل من مستضدات البروسيلا المجهضة S19 المستخلصة بالاملاح، أما المجموعة الرابعة (G4) فقد لقّحت بالتجويف البرتوني 0.2 مل من مستضدات البروسيلا المجهضة S19 المستخلصة بالاملاح و0.2 مليلتربيتا كلوكان. في اليوم 27 بعد التلقيح، أجرى فحص فرط الحساسية المتأخر بوسادة القدم والذي أظهر فرق معنوي ($P<0.05$) في زيادة السُمك للمجموعة الرابعة مقارنة مع المجموعة الثانية والثالثة والأولى. في يوم 30 من التلقيح جميع الفئران الباقية حقنت بالتجويف البرتوني بـ 0.2 مل من$10^4 \times 1$ CFU / مل جرعة التحدي للعترة الضارية للبروسيلا المجهضة. يوم 7 و 14 بعد التحدي، المجموعة الثانية والثالثة والرابعة سجلت انخفاض في العد البكتيري للطحال مع وجود فرق معنوي ($P<0.01$) مقارنة مع مجموعة السيطرة. نستنتج من هذه الدراسة أنّ الفئران المحصّنة بـمستخلص البروسلا الملحي مع البيتا كلوكان كان لقاح فعّال، حيث أن تنشيط المناعة الفطرية هي مفتاح مهم لتنشيط Th1 الوقائي، حيث لوحظ انخفاض ملحوظ في العد البكتيري للطحال بعد التحدي.

الكلمات المفتاحية: المستضدات المستخلصة بالأملاح، البروسيلا المجهضة S19، **بيتا كلوكان، الاستجابة المناعية.**