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The immunomodulatory effect of Neem (*Azadirachtaindica*) seed aqueous, ethanolic extracts and *Candida albicans* cell wall mannoproteins on immune response in mice vaccinated with Brucella Rev-1

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Summary

In the present study, the immunomodulatory effect of Neem (Azadirachtaindica) seed aqueous, ethanolic extracts and Candida albicans cell wall mannoproteins on the immune response of mice vaccinated with Brucella Rev-1 vaccine was investigated. The study was conducted on two main groups(160 mice for each group), Each group was divided into eight subgroups 20 mice for each (I: treated with distilled water, II: treated with the Brucella Rev-1 vaccine, III: treated with mannoproteins, IV: treated with neem aqueous extract, V: treated with neemethanolic extract, VI, VII and VIII: treated with mannoproteins, neem aqueous extract and neemethanolic extract, respectively, then they were vaccinated with Brucella Rev-1). All these treatments were carried out on day 1 and then vaccinated with brucella Rev-1 vaccine on day 4 .Then the mice were tested as follows , on day 8 after vaccination(serum IFN-γ level), day 21 for (anti-Brucella antibody titer). The doses of both plant extracts and mannoproteins represented 10% of the calculated LD50 (neem extracts: 3.8096 g/Kg mannoproteins: 5.7144 mg/Kg), which were given subcutaneously. Mice of the second main group were injected with the immune suppressive drug prednisolone (5mg/Kg) 5 days prior to the treatments, which carried out on mice of the first main group. The results demonstrated clear immunomodulatory effects (improvement of non-specific, humoral immunity) of the tested immunomodulators in mice vaccinated with Brucella Rev-1 as compared with mice that were not treated with Neem extracts or mannoproteins. In this regard, The interferon-γ showed a significant increase ($P \leq 0.01$) serum level in immunomodulator-treated and -vaccinated mice in comparison with negative and positive groups, and again group VII showed the highest increase. The anti-Brucella antibodies assessed by indirect Immunoflourescent test also showed a significant increase titer in immunomodulator-treated and -vaccinated mice in comparison with negative and positive groups .

Conclusion: The aqueous and ethanolicNeem seed extract reported the highest enhancement in all immunological parameters employed in comparison with mannoproteins of Candida albicans cell wall.

Key words: Neem,Candida albicans, Brucella Rev-1,immunomodulatory.

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تأثيربذور نبات النيم و مانوبروتينز الجدار الخلوي للمبيضات البيضاء Candida albicansكمحفز مناعي للفئران الملقحة بلقاح البروسيلا-Brucella Rev-1

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الخلاصة

درس تأثير بعض المحورات المناعية المتمثلة بالمستخلصين المائي والكحولي لبذور نبات النيم (Azadirachtaindica) والمانوبروتينز (Mannoproteins) المستخلصة من جدار خلايا المبيضات (Candida albicans) على الاستجابة المناعية للفئران الملقحة بلقاح البروسيلا العترة Brucella Rev-1.ضمت الدراسة مجموعتين رئيسيتين(160فأرفي كل مجموعة) وأحتوت كل منها على ثمان مجاميع فرعية(20فأر في كل مجموعة) (I: معاملة بالماء المقطر، II: معاملة باللقاح Brucella Rev-1، III: معاملة بالمانوبروتينز، IV: معاملة بالمستخلص المائي للنيم، V: معاملة بالمستخلص الكحولي للنيم، VIو VII و VIII: معاملة بالمانوبروتينز والمستخلصين الما ئي والكحولي لنبات النيم، على التوالي ومن ثم باللقاح). أجريت جميع هذه المعاملات في اليوم الأول وأعطيت لقاح البروسيلا في اليوم الرابع وضحي بالحيوانات في اليوم 8 (المستوى المصلي للأنترفيرون جاما)، في اليوم 21 (عيارية أضداد البروسيلا). أعطيت المحورات المناعية تحت الجلد بجرعة معادلة لـ 10% من نصف الجرعة المميتة (المستخلصين الما ئي والكحولي لبذور نبات النيم:3.9086 ملغم/كلغم؛ مانوبروتينز5.7144ميكروغرام). وقد حقنت فئران المجموعة الثانية بمادة البريسلون (Prednisolone)بجرعة (5ملغم/كلغم) كمثبط مناعي قبل أجراء المعاملات الأنفة الذكر والتي أجريت في حيوانات المجموعة الرئيسية الأولى. أظهرت النتائج تأثيرات واضحة للمحورات المناعية المستخدمة في الدراسة وفي المجاميع الممنعة بلقاح البروسيلا ومن خلال تحسن الاستجابة المناعية غير النوعية والمناعة الخلطية مقارنة مع المجاميع غير المعاملة بالمحورات المناعية، وأشارت النتائج إلى ارتفاع واضح في المستوى المصلي لأنترفيرون جاما في المجاميع الممنعة والمعاملة بالمحورات المناعية بالمقارنة بمجاميع السيطرة السالبة والموجبة وسجلت أيضا المجموعة VII أعلى مستوى. وعند قياس عيارية أضداد البروسيلا بواسطة الوميض الاشعاعي غير المباشر، أظهرت المجاميع الممنعة والمعاملة بالمحورات المناعية ارتفاع مستوى هذه الأضداد مقارنة بمجاميع السيطرة السالبة والموجبة وبفوارق إحصائية معنوية.

مفاتيح الكلمات: النيم ,للمبيضاتالبيضاء , بلقاحالبروسيلا ,كمحفزمناعي

Introduction

Understanding the immune system may help in improving immunization protocols inhuman, as well as, animals to develop vaccines, which play an important role in the preventive medicine and provide a rational basis for devising new therapeutic strategies for immune mediated diseases (1). In this regard, immunomodulators are plants and plant products, or biological materials that mediate the effectors mechanisms of the immune system through immune stimulation to a given antigen or potentiate the effectiveness of a vaccine (2). Scientists have began to adopt vaccine strategies that are based on the maximization of antigen presentation for major histocompatibility complex (MHC) class I or class II molecules due to the importance of these molecules in immune response, especially those materials that act as immunomodulators (1).Materials of fungal and/or plant origins have been the interest of different investigators around the globe with their aims to establish the immunomodulator potentials of these materials. Some risks associated with attenuated or killed whole-organism vaccines can be avoided with vaccines that consist of specific purified macromolecules derived from pathogens or in combination with plant materials (2,3and4).The plant extracts, derivatives or their products, have also been the interest of investigators as immunomodulators to overcome the disadvantage of biological and chemical immunomodulators. One of these plants is Azadirachtaindica, which is more popular with the name neem, and has the advantage to

be a medicinal plant with a wide range of applications in folkloric medicine (5). Furthermore, recent investigations demonstrated several biological and pharmaceutical potentials; for instance, anti-viral, anti-bacterial, anti-parasitic, anti-cancer and immune stimulant properties of the Neem (4, 5, 6, 7, 8, and 9). Brucellosis is one of the most wide spread infectious disease in the world that causes fetal death as a single agent in human and animals being. The disease is widely distributed in different countries of the world among humans and animals, and there is a positive correlation of infection between animal and human populations (3). Until now there is no effective available vaccine for protection against brucellosis, although there have been many trials to use combinations of immunomodulators and vaccines to immunpotentiate the immune mechanism in recipient animals (8, 10, 11, 12,13,14,15 and 16). In agreement with such scope, the present study came to add some understanding about the role of biological (Candida albicans cell wall mannoproteins) and a medicinal plant (neem seed aqueous and ethanolic extracts) materials in potentiating the immune response (immunomodulators) against brucellosis in mice vaccinated with Brucella Rev-1 vaccine .

Materials and Methods

All experiments was done on 320 male and female albino mice (Balb-c).Their age range at the start of experiments 6-8 weeks. They were housed in bio-clean hoods at 20-25ºC with light: dark periods of 14:10 hours. They had free access (*ad libitum*) to food (standard pellets) and water, and their average weight was 22 ± 3 grams at the start of experiments. Before carrying out the experiments, the mice were left in separate cages for one week to experience the acclimatization period. The study was conducted on two main groups(160 mice for each group), Each group was divided into eight subgroups 20 mice for each (I: treated with distilled water, II: treated with the *Brucella* Rev-1 vaccine, III: treated with mannoproteins, IV: treated with neem aqueous extract, V: treated with neemethanolic extract, VI, VII and VIII: treated with mannoproteins, neem aqueous extract and neemethanolic extract, respectively, then they were vaccinated with *Brucell a* Rev-1). All these treatments were carried out on day 1 and then vaccinated with brucella Rev-1 vaccine on day 4 .Then the mice were tested as follows , on day 8 after vaccination(serum IFN-γ level), day 21 for (anti-*Brucella* antibody titer). The doses of both plant extracts and mannoproteins represented 10% of the calculated LD_{50} (neem extracts: 3.8096 g/Kg mannoproteins: 5.7144 mg/Kg), which were given subcutaneously. Mice of the second main group were injected with the immune suppressive drug prednisolone (5mg/Kg) 5 days prior to the treatments, which carried out on mice of the first main group. The following kits were used in the experiments of the study: Mouse IFN-γ ELISA quantitative determination (Bender Med Systems, Austria) / Rat anti-mouse IgG conjugated with fluorescin (Bethyl, USA) Two extracts of Plant Neem Seeds (aqueous and ethanolic) were prepared(10). The doses of both extract were prepared after the determination of the LD_{50} (17) .The Mannoproteins were prepared from the cell wall of a *Candida albicans* isolate (18). The isolate, which was obtained from the vaginal swab of a healthy woman, was supplied by the Central Health Laboratory (Iraq/Baghdad). The *C. albicans* sample was maintained on yeast extract peptone glucose agar supplemented with amino acids (18) .The dried lyophilized seed of *Brucella*Melitensis Rev-1 strain was supplied by the Central Veterinarian Laboratory Iraq/Baghdad, and this laboratory received the strain from the Food and Agriculture Organization. Indirect Fluorescent Antibody Test (IFAT) .The IFAT was used to assess anti-*Brucella* antibody titer in the sera of mice that were immunized with *Brucella* Rev-1 vaccine in different treatment regimens (3). The procedure of WHO was adopted to determine such titer. Quantitative Determination of Interferon-γ Serum Level was carried out using a mouse IFN-γ ELISA kit (Bender Med Systems, Austria), which is an enzyme-

linked Immunosorbent assay for quantitative detection of murine interferon-γ (IFN-γ) in murine serum. The values of the investigated parameters were given in terms of means ± standard errors (S.E.), and differences between means were assessed by analysis of variance (ANOVA), least significant difference (LSD) and Duncan test, using the computer programmer SPSS (Statistical Package of Social Sciences) version 7.5. The difference was considered significant when the probability value was equal or less than 0.05. A further estimation was also given; it was treated efficiently (19), which were calculated according to the following equation:

Treatment efficiency (%) = $\left(\frac{A - B}{B}\right) \times 100$ A=Treated groups, B=Negative control group.

Results and Discussion

After the tabulated procedures and calculations presented in this study, it was found that the LD_{50} of *C. albicans* cell wall mannoproteins was (5714.4 µg /kg), while the corresponding LD_{50} for neem seed aqueous or ethanolic extract was (3809.6 mg/kg). Based on these findings, 10% of the $LD_{50}$of each material (mannoproteins: 5.7144mg/kg/bodyweight; aqueous or ethanolicneem extract: 3.8096mg/kg/body weight) was considered as the immunomodulator dose in the study (17). The sera of animals in groups I, III, IV and V of both treatments (with or without prednisolone treatment) showed no anti-*Brucella* antibodies at the start titer 1:16, while the other groups showed some variations. In mice of group II of both treatments, three mice out of four (75%) showed a positive Immunoflourescent reaction at the titer 1:32, while in mice without prednisolone treatment of groups VI, VII and VIII, a positive reaction was observed at the titer 1:64 (50, 75 and 100%, respectively).The latter groups of mice with prednisolone treatment showed 100% positive reaction at the titer 1:32 (Table-1).

Table -1: Anti-*Brucella* antibody titer by indirect Immunoflourescentin sera of treated mice.

Groups	Number of Mice with a Positive Anti-*Brucella* Antibody Titer / 4 Animals									
	Without Prednisolone Treatment					With Prednisolone Treatment				
	1:16	1:32	1:64	1:128	1:256	1:16	1:32	1:64	1:128	1:256
I	0	0	0	0	0	0	0	0	0	0
II	4	3	0	0	0	4	3	0	0	0
III	0	0	0	0	0	0	0	0	0	0
IV	0	0	0	0	0	0	0	0	0	0
V	0	0	0	0	0	0	0	0	0	0
VI	4	444 4	2	0	0	4	4	0	0	0
VII	4	4	3	0	0	4	4	0	0	0
VIII	4	4	4	0	0	4	4	0	0	0

Anti-brucella antibodies showed an increased titer in all immunized groups treated with the immunomodulators used in the study, especially group V111 (Brucella vaccine + ethanolicneem extract solution) as compared to the control group that received vaccine only. Such observation suggests that the immunomodulation involved the humoral immune response, although the pathway may be through the modulation of macrophages and T lymphocytes as both types of cells are required to enhance the B lymphocytes to produce immunoglobulin (1). Such findings came to confirm previous results reported by (8, 6, 16, 17 and 18). Also the results agreed indirectly with (6), who demonstrated that mice and rats immunized with breast tumor antigen (BTA) and a neem leaf preparation (NLP) have a higher antibody response. They also suggested that the use of NLP in vaccination was involved in the induction of a T_H1 response, as evidenced by the enhanced secretion of IFN-γ and decreased release of IL-10 from spleen cells. The results of IFN-γ Serum Level were given in (table-2), while the treatment efficiency for each group of treated mice was presented in (figure-1). A significant increased serum level of IFN-γ was observed in groups IV, VI, VII and VIII (114.00 ± 22.90 , 130.00 ± 37.00, 170.00 ± 10.00 and 126.70± 1.76pg/ml, respectively) of mice with prednisolone treatment as compared to group I (43.33± 23.40pg/ml), and such differences were associated with treatment efficiencies of 163, 200, 292 and 192%, respectively. In mice with prednisolone treatment, similar results were obtained, and group VIII recorded the highest significant increased level of IFN-γ, but their values were significantly showed mean value significantly lower (P≤0.01)as a result of breakage down of the immune response by immunosuppressed drug prednisolone.

Table-2: Interferon-γ serum level by ELIZA in treated mice.

Groups	Interferon-γ Serum Level (mean ± S.E.; pg/ml)*		Probability**≤
	Without Prednisolone Treatment	With Prednisolone Treatment	
I	43.33 ± 23.40 [c]	24.67 ± 4.40[c]	0.01
II	85.00 ± 14.00[bc]	74.67 ± 2.85[ab]	0.01
III	44.67 ± 14.70 [c]	29.00 ± 11.53	0.01
IV	114.00 ±	91.33 ± 14.7 [a]	0.01
V	44.67 ± 17.30 [c]	38.33 ± 15.3[bc]	0.01
VI	130.00 ±	88.00 ± 19 [a]	0.01
VI	170.00 ± 10.00	80.67 ± 4.9 [a]	0.01
VI	126.70 ± 1.76[ab]	73.67 ± 11.7[ab]	0.01

***Different letters: Significant difference (P≤0.05) between means of the same Colum **The comparison is between means of the two columns (horizontal comparison).**

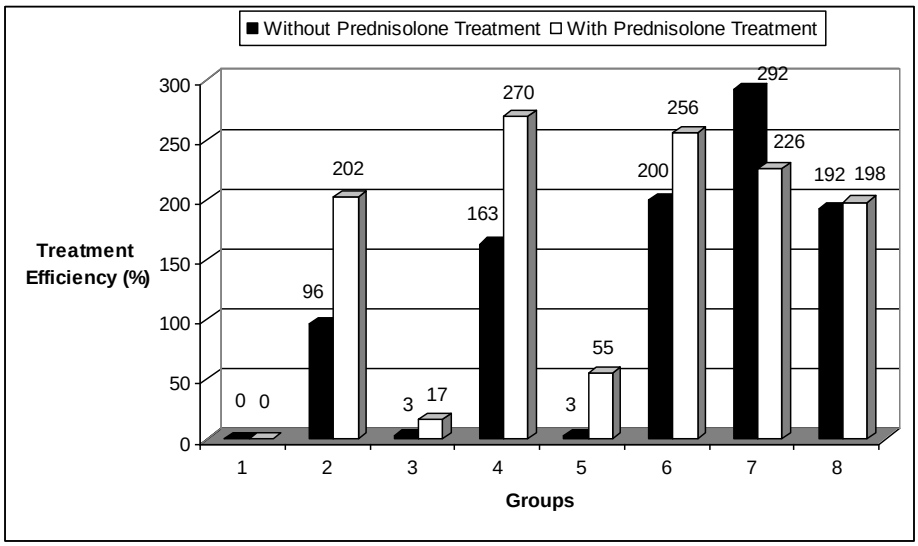

Figure 1: Treatment efficiency of IFN-γ serum level in treated mice

One of the cytokines is IFN-γ, which was evaluated in the present study through different experiments in regard to *Brucella* vaccination and immunomodulators (mannoproteins and neem extracts). The results demonstrated a significant increased serum level of IFN-γ in mice treated with the tested immunomodulators especially groups without prednisolone treatment because of the immunosuppressed effect of prednisolone. Such findings highlight the importance of ethanolic and aqueous neem seed extracts, as well as, mannoproteins of *C. albicans* cell wall as immunomodulators, especially when we consider the immunological importance of IFN-γ in enhancing the cellular immune response, which is important in controlling *Brucella* infection. However, the pathway by which these materials can act as immunomodulators, especially for the neem extracts, is not well understood, but investigators interested in the immunomodulation of neem extracts suggested that such extracts are rich in chemical constituents that may act positively on the immune system and enhance the immune response (5, 7, 8, and 16). Furthermore, these investigators agreed that the action of neem extracts is a cytokine-mediated activation, and the cytokine most often implicated is IFN-γ, which enhances both oxygen-dependent and oxygen-independent killing mechanisms in phagocytes. A further inspection of this implication has been recently addressed, in which neem leaf glycoprotein (NLGP)-mediated immune activation and associated immune polarization was investigated, and NLGP-induced activation was reflected in up regulation of early activation marker CD69 on lymphocytes, Monocytes, and dendritic cells. Activation is also denoted by CD45RO enhancement, with a decrease in CD45RA phenotype and CD62L (L-selectin). A also suggested that NLGP-activated T cells secrete a greater amount of the T_H1 cytokine IFN-γ and a lower amount of the T_H2 cytokine IL-4. The antigen-presenting monocytes and dendritic cells are also involved through the up regulation carried out by IL-12 and tumor necrosis factor-α (1). The aqueous and ethanolicneem seed extract reported the highest enhancement in all immunological parameters employed in comparison with mannoproteins of *C. albicans* cell wall, with the exception of DTH parameter, in which the latter immunomodulators showed the higher level of immunomodulation. In recent years the understanding and importance of antigen-specific immune responses after vaccination has completely changed. In the past the focus for monitoring a vaccine-specific immune reaction was principally based on the humoral branch of the immune system, and the efficacy of vaccines, as assessed by the induction of protective immunity was mainly correlated with antibodies and antibody titers. However, this correlation is often failed and other parts of

the immune system have also to be considered; namely the innate immune system and the cellular branch of the antigen-specific immune system. The innate immune system plays its main role in the effective activation of the antigen-specific immune response, in antigen-uptake and antigen-presentation. Furthermore, in order to achieve an effective vaccination, the activation of all T-cell subpopulations is of advantage, but more important is the generation of antigen-specific memory T and B lymphocytes(1and 2).

Conclusion, that the neem seed extracts (aqueous and ethanol) and the cell wall mannoproteins of *C. albicans* might be a potential immune adjuvant for inducing active immunity against *brucella*, and may act as Immunopotentiators through increasing microsomal proteins. These proteins have a binding activity to antigens, and such binding helps in extending the half-life of the antigen by a gradual release of it over a long period

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