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Extraction and biological evaluation of external membrane vesicles of Brucella abortus as a candidate for brucellosis vaccine

Bruselloz aşısı için aday olarak Brucella abortus'un dış zar veziküllerinin ekstraksiyonu ve biyolojik değerlendirmesi

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Abstract

Aim: Brucellosis is an endemic zoonotic disease affecting animal and human health. In the last several decades, much research has been performed to develop safer Brucella vaccines to control the disease. Outer membrane vesicles (OMVs) have been considered as immunogenic structures for a subunit vaccine to prevent human brucellosis. The aim of this study was to evaluate the external membrane vesicles of Brucella abortus as a candidate for brucellosis vaccine.

Methods: In this study *Brucella abortus*, S99 strain was used. Extraction of OMV after mass cultivation of Brucella abortus was performed by Ultracentrifugation and Sodium Dosoxycotinate. The SDS-PAGE method was used to observe the protein pattern and electron microscopy, to evaluate the physicochemical properties. The amount of LOS in OMV was also measured by LAL test. After determination of the protein concentration, mice were injected at a specific concentration and blood samples were obtained to evaluate the antibody. Finally, after serum isolation, the antibody production was measured by the ELISA method.

Results: The amount of protein present in OMV was 0.1 mg/ml. The size of OMV was 55 to 150 nm, and in the SDS-PAGE assessment, the protein range was >25 kDa. The LOS value in the LAL test was reported within the authorized range. Significant increase in IgG levels was observed after the first injection (P=0.001) compared to the control group, and in subsequent boosters as well. The highest response rate was observed in the second booster.

Conclusion: OMV maintains its spatial shape during extraction and has the potential to induce the production of a high degree of specific antibodies against Brucella abortus. For this reason, it can be considered as a candidate vaccine after examining clinical phases.

Keywords: External mucous vesicles, Brucella abortus S99, Brucellosis vaccine

Öz

Amaç: Bruselloz, hayvan ve insan sağlığını etkileyen endemik bir zoonotik hastalıktır. Son birkaç on yılda, hastalığı kontrol etmek için daha güvenli Brucella aşıları geliştirmek için çok araştırma yapılmıştır. Dış zar vezikülleri (OMV'ler), insan brusellozunu önlemek için bir alt birim aşı için immünojenik yapılar olarak düşünülmüştür. Bu çalışmanın amacı Brusella abortus'un dış zar veziküllerini bruselloz aşısı için aday olarak değerlendirmektir.

Yöntemler: Bu çalışmada Brucella abortus, S99 suşu kullanıldı. Brucella abortusun kitle kültivasyonundan sonra OMV ekstraksiyonu, Ultracentrifugation ve Sodium Dosoxycotinate tarafından gerçekleştirildi. SDS-PAGE yöntemi, fizikokimyasal özellikleri değerlendirmek için protein paternini ve elektron mikroskopisini gözlemlemek için kullanıldı. OMV'deki LOS miktarı da LAL testi ile ölçüldü. Protein konsantrasyonunun belirlenmesinden sonra farelere belirli bir konsantrasyonda enjekte edildi ve antikoru değerlendirmek için kan örnekleri alındı. Son olarak serum izolasyonundan sonra antikor üretimi ELISA yöntemi ile ölçüldü.

Bulgular: OMV'de mevcut protein miktarı 0,1 mg/ml idi. OMV'nin boyutu 55 ila 150 nm idi ve SDS-PAGE değerlendirmesinde protein aralığı >25 kDa idi. LAL testindeki LOS değeri yetkili aralıkta rapor edildi. İlk enjeksiyondan sonra IgG düzeylerinde (P=0,001) kontrol grubuna kıyasla ve daha sonraki güçlendiricilerde anlamlı artış gözlendi. En yüksek tepki oranı ikinci güçlendiricide gözlenmiştir.

Sonuç: OMV ekstraksiyon sırasında uzamsal şeklini korumaktadır ve Brucella düşüklerine karşı yüksek derecede spesifik antikorların üretimini indükleme potansiyeline sahiptir. Bu nedenle klinik fazları inceledikten sonra aday bir aşı olarak düşünülebilir.

Anahtar kelimeler: Dış mukoza vezikülleri, Brucella abortus S99, Bruselloz aşısı

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Introduction

Brucellosis is a zoonosis affecting approximately 500,000 people annually around the world. The disease remains endemic in many regions of the world including Latin America, Middle East, Africa, Asia, and the Mediterranean basin [1]. Research focused on the development of an ideal Brucella vaccine to prevent brucellosis in animals and humans has been performed since the beginning of twentieth century [2]. Currently, no licensed human or canine anti-brucellosis vaccines are available. In bovines, the most successful vaccine (S19) is only used in calves. Over the years, a wide variety of killed vaccines have been developed for protection against brucellosis. They have had limited acceptance and success. None have approached the protection levels afforded by live attenuated vaccines.[3]. Several antigenic fractions extracted from *Brucella* have been tested as a vaccine candidate, in association with a variety of adjuvants. Some of them included cell envelopes [4] and outer membrane proteins [5]. Vaccination is probably the most economic measure for control of brucellosis in endemic areas. Many countries have developed control measures for the eradication of the disease in livestock animal. These programs minimized the economic losses due to the abortion, infertility, and weak offspring and decreased milk production [6]. Presently, the vaccination programs are based on control of brucellosis mainly due to *B. melitensis* and *B. abortus* [7]. The use of the live-attenuated vaccines against brucellosis represents a risk due to its potential ability to revert to virulence, cause abortion in pregnant animals, and the fact that it is shed in milk. Live strains could infect people coming into contact with the vaccine for example, farmers, abattoir workers, and veterinarians. Smooth live attenuated vaccines suffer shortcomings such as residual virulence and serodiagnostic interference. Inactivated vaccines, in general, confer relatively low levels of protection. Recent developments to improve brucellosis vaccines include generation of knockout mutants by targeting genes involved in metabolism, virulence, and the lipopolysaccharide synthesis pathway, as well as generation of DNA vaccines, mucosal vaccines, and live vectored vaccines, which have all produced varying degrees of success. An ideal vaccine for use either in humans or in animals should meet the following criteria: It should be effective, avirulent and induce long-lasting protection [8]. Subunit vaccines, like recombinant proteins, are promising vaccine candidates because they are less biohazardous, well defined, avirulent, noninfectious, and nonviable [9]. The aim of this study was to evaluate the immunological properties of external membrane vesicles extracted from Brucella abortus in the animal model BALB/C.

Materials and methods

Bacterial strain

Brucella abortus strain 19 (S19) was obtained from the bacterial collection center. The culture was revived by inoculating on potato infusion agar (PIA) slant and incubating at 37°C for 72 hours, and subsequently plating on PIA plates to obtain a single colony.

Bulk massage

In order to achieve the proper cell mass for purification of OMV, a high volume of culture of bacteria should be prepared. At first, the bacterium was cultured in Brucella Agar, then the colonies were washed with buffer phosphate, transferred to Brucella broth medium and placed in a shaker incubator for 2 hours. Then the bacteria were transferred to fresh culture medium and kept at 37 °C for 24 hours. The microbial suspension was weighed in Brucella agar containing booths, thus a dephasic environment was provided for optimal growth of microorganisms, and again placed at 37 °C for one day and finally, the cell biomass was collected.

Extraction of OMV

Extraction of external membrane vesicles was performed on the basis of ultrasound diffusion and using solutions containing dezocyticulate and sucrose gradient. The Brucella passive cell body was centrifuged for about an hour at 6000 rpm at 4 °C. After washing twice with phosphate buffered saline (PBS pH=7.2), a solid mass of the cell was suspended in chloride buffer and homogenized for 30 minutes, after which the wet weight was determined. The suspension was centrifuged again at 6500 rpm for an hour at 4 °C. Cell suspension at 7.5 times of wet weight was suspended with a 0.1-m^3 buffer of triazole and 10 ml of ethylene diaminete tracycloacetic acid (EDTA). Then suspension was homogenized with a volume of 20/1 ratio, a 0.1 M Tris buffer solution containing 10mM EDTA and sodium deoxycholylate 100g/L and shaken vigorously. After 10 minutes, the cell mass was treated with sodium deoxycholate and separated by ultrasound diffusion for 1 hour at 16500 rpm at 4 °C. Then, cell pellet was centrifuged for 2 hours at 42,000 rpm at 4 °C and the suspension was obtained from a precipitate in a 0.1 molar buffer containing 10 mm EDTA and 5 g /L sodium, and shaken vigorously. After 10 minutes, the cell mass was treated with sodium deoxycholate and separated by ultrasound diffusion for 1 hour at 16500 rpm at 4 °C. Then, cell pellet was centrifuged for 2 hours at 42,000 rpm at 4 °C and the suspension was obtained from a precipitate in a 0.1 molar buffer containing 10 mm EDTA and 5 g/l sodium deoxycholate. For 2 hours, the clock was at 16500rpm and 4 °C. Then OMV was deposited in 15 ml of distilled water containing 3% soluble sucrose, passed through 0.2 micron filters to collect in sterile glass vials.

Electron microscopy

Membrane vesicles were ultrasonically treated to disperse the vesicles followed by attaching to Formvar/carbon-coated nickel grids. Grids were washed with a 0.01 M PBS supplement, 0.1% gelatin (PBG), and 0.5% BSA. The vesicles on the grids were fixed with 1% glutaraldehyde in PBS at 4°C for 60 minutes and negatively stained with 1% potassium phosphotungstate pH 7.5. The grids were examined using a Zeiss EM10C transmission electron microscopy operated at 80 KV.

Nanodrop

The standard concentration of the proteins of the extracted vesicles was measured using a nanodrape. The basis of this device is spectrophotometry.

Endotoxin determination

The biological activity of the endotoxin was measured using the QCL-1000® chromogenic Limulus amebocyte lysate (LAL) endpoint assay (Lonza, Walkersville, MD, USA). To this

end, 0.1 mL of LAL with 0.1 mL of the sample was incubated for 88 minutes at 38°C. Then, by adding 0.5 mL of the substrate, the mixture was incubated again for 3 minutes. Finally, after adding 0.1 mL glacial acetic acid solution, the reaction stopped. Optical density (OD) of the reaction mixture was determined by a spectrophotometer at 405 nm and the amount of Endotoxin in the sample was calculated using the standard curve.

LAL test

For the measuring the LPS in the sample, a Thermo Scientific Pierce LAL Chromogenic Endotoxin Quantitation Kit was used according to the manufacturer's protocol.

Pyrogenic test

The extracted external vesicle was tested on four healthy albino New Zealand rabbits. At the beginning of the study, the animal weight was limited between 1.8 and 3.8 kg. Three rabbits were used for testing and one for control. The OMV was injected into rabbit peripheral ear veins at a dose proportional to the rabbit weight. The animals' rectal temperature was measured using a digital thermometer. If the rabbit did not show a temperature rise of 0.6ºC or greater or the sum of the three single temperature increases did not exceed 1.4◦C, the test material was considered to have no toxicity.

Mice immunization

5 to 6-week-old female rats were used and randomly divided into two groups of ten each. One group received OMV at a concentration of 50 µg and the other group (control group) received PBS.

Evaluation of the level of total (IgG) antibody against OMV in immunized mice by ELISA

After blood sampling and serum collection, the level of antibody against the protein was evaluated by ELISA. Protein was prepared in PBS buffer at a concentration of 5 µg/100 µl. The mouse serum used was prepared in dilutions of 1: 250-1: 500-1: 1000 and 1: 2000. The mouse IgG concentration of 1.6000 was used and the samples were then read at 405nm wavelength.

Statistical analysis

Data were analyzed using Graphpad software, with which graph was depicted (San Diego, California USA). In addition, Analysis of variance and Tukey post-hoc test were used for comparing the means of groups and two by two comparisons were performed with SPSS version 20 (SPSS Inc. Chicago, IL, USA).

Results

Concentration study using Nanodrop

Nanodrop was used to estimate the concentration and quality of membrane vesicles extracted. Physicochemical analysis of vesicles extracted from Brucella abortus S99 revealed that the total protein content of membrane vesicles by nanodrop device was 1.42 mg/ml, which is acceptable.

Electrophoresis of membrane vesicles by SDS-PAGE

Electrophoretic Motion Analysis of Proteins in the Extracted Vesicles of Brucella abortus are shown in figure 1. According to the markers, the protein bands are in the 25 kDa region.

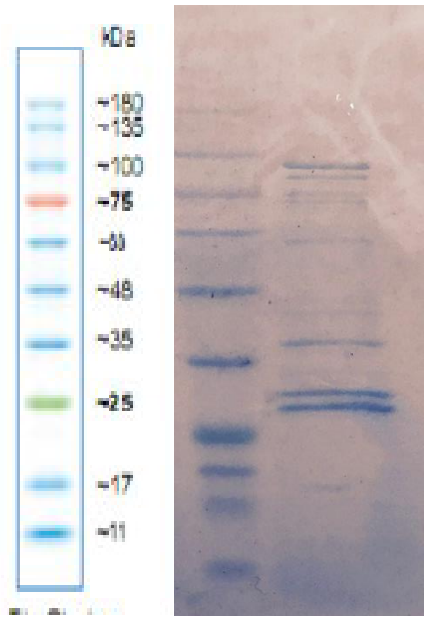

Figure 1: Protein pattern of outer membrane vesicles by SDS_PAGE method

Electron microscopy

The stability of the natural form of membrane vesicles at different stages of the purification process was investigated by electron microscopy. The spatial properties of the extracted membrane vesicles in negative contrast staining with the EM 900 electron microscope are shown below. As can be seen in the Figure 2, the extracted vesicle is about 50-50 nm and retains its spatial properties at various stages of extraction and purification.

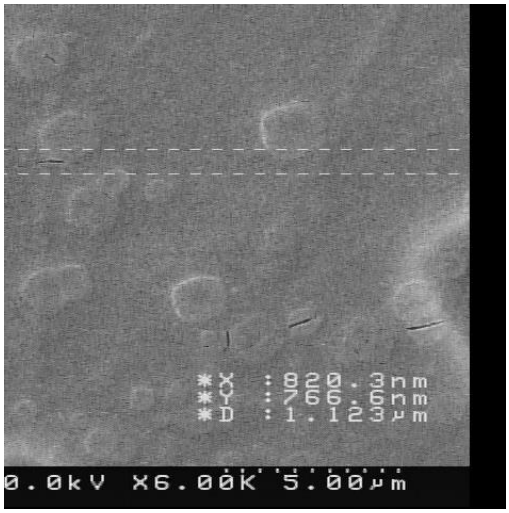

Figure 2: Electron micrograph of outer membrane vesicles

Determination of endotoxin levels in membrane vesicles by chromogenic Limulus amebocyte Lysate (LAL)

Results in this semi-quantitative test showed that the extracted vesicles were allowed to be used in the animal model within the safe limit (less than IU300).

Pyrogenic test

After the test, no significant increase in temperature was observed in rabbits, indicating a lack of pyrogenicity.

Evaluation of total IgG responses against OMV by ELISA

Results showed a significant increase in IgG antibody titers at all three doses compared to the control group (Figure 3). The highest response was seen on day 42 (the second injection booster) which showed a significant increase compared to the first injection and booster injection (P=0.001).

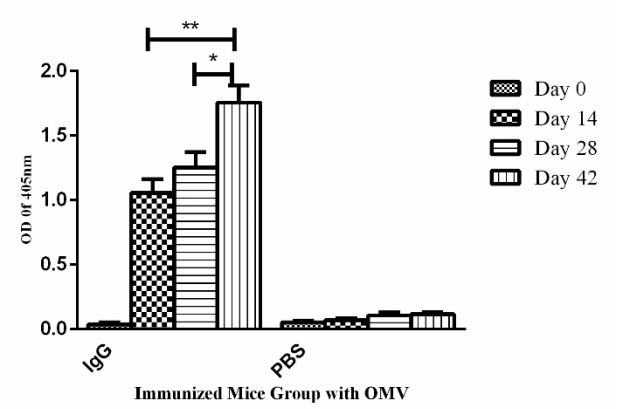

Figure 3: IgG antibody titer level against OMV Brucella on days 14, 28 and 42 after injection

Discussion

Control of zoonotic diseases in human populations has relied heavily on the control of animal disease. Over the last century, human brucellosis has been controlled by vaccination and culling within cattle, goat, and sheep herds. Despite past and current efforts to eradicate brucellosis, many human cases are reported annually worldwide [10].

Brucella can enter the host cell, especially macrophages, during its pathogenic process and grow and proliferate within these cells. In vaccination against Brucella infections in the animal model, attenuated live strains are usually injected [11]. The vaccine also has some disadvantages, such as abortion in pregnant animals and the possibility of bacterial secretion in vaccinated animal milk. The animal brucellosis vaccine is not an effective and safe vaccine against Brucella virulent strains. Therefore, extensive studies are currently underway to understand the protective mechanism against Brucella and the development of effective human Brucella vaccines [12]. Today, the control of brucellosis is based on the three principles of killing infected livestock, pasteurizing dairy products and vaccination. Human brucellosis vaccines, which are based on complete inactivation of the bacterium or live strains, are associated with two basic problems: First, they sometimes lead to disease, and second, to hypersensitivity reactions.

In recent years, immunogenicity with various antigens of Brucella species has been investigated in monovalent, conjugated, or recombinant forms. The key to designing a vaccine against this bacterium is to pay attention to the way of bacterial life [13].

The Brucella survival cycle in the body is intracellular, so only cellular immune responses are able to remove this bacterium from the body. In other words, the ability of macrophages to kill intracellular bacteria is mediated by the induction of this ability with the release of cytokines such as INF-γ. Therefore, it is important to select the antigen and the type of antigen-presenting cell unique to the body to stimulate the desired responses to eliminate this bacterium.

Protective immunity against brucellosis has been studied extensively in mouse models. From a clinical point of view, the most important strategy used to enhance the immunogenicity of compounds is the binding of a polysaccharide to a suitable protein vector, resulting in non-T lymphocyte-dependent antigen [14].

LPS has been selected as a potent protective antigen in all Brucella strains to induce non-T lymphocyte-dependent, T-dependent, and lymphocyte-induced responses by conjugating with a protein carrier and forming a hapten-carrier complex. The type of T lymphocyte response is very important. Stimulation of Th1 responses results in the secretion of cytokines such as INF-γ and stimulation of Th2 responses associated with antibody production. Although the presence of these antibodies may play a role in bacterial restriction, the importance of cellular response and cytokine production, including interferon-gamma, has priority in killing bacteria and clearing the body [15].

The outer membrane vesicles can be considered as a new vaccine candidate, due to the presence of various compounds including outer membrane proteins, lipopolysaccharide, peptidoglycan, and the recently discovered DNA and RNA in the OMV structure. It carries several bacterial native antigenic compounds and has therefore been considered for vaccine development. The remarkable properties of membrane antigens and their exposure to OMV have led to the physicochemical stability of its structure and therefore the protective and genomic properties of OMV have been confirmed by many bacteria [16]. In 2014, Acevedo et al., in a review of bacterial outer membrane vesicles and vaccine applications, demonstrated that OMVs were more applicable than previously understood and remain an important technology for the development of laboratory vaccines [17]. The LAL test showed the LPS level within the limit; although this macromolecule is a toxic compound, its small amount acts as a strong adjuvant. A previous study showed that discrepancy in the protein's bands could be used as protein effective biomarker for tuberculosis diagnosis. [18].

Limitations

Further research of the Molecular pathobiology and immunological properties would lead to the development of better and safer vaccines.

Conclusions

The results indicate that these structures may be suitable candidates for vaccination and be considered as a new generation of vaccines against Brucella infections, although further studies in this field are needed to evaluate antibody subclasses and responses.

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