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Determining the protective effect of a boron adjuvanted vaccine with an experimental infection model in mice

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Abstract: Boron is a required trace element for plants, humans, and animals and it has significant influence over biological functions and the immune system. Vaccines have aided humans in the control of many contagious diseases around the world. The success of global immunization campaigns may be possible with the development of effective and low-cost vaccines and adjuvants. For this reason, the present study aimed to determine the adjuvant activity of easily accessible boron compounds. In this study, six different vaccines were prepared, including inactive *Staphylococcus aureus* bacteria, boric acid, aluminum hydroxide, montanide ISA 50, and ISA 206, ISA 50 + boric acid, and ISA 206 + boric acid combinations. The rate of survival following the challenge, the bacterial load on internal organs, and histopathological findings at the vaccine injection site and in the internal organs were evaluated in vaccinated mice. In addition, the levels of interferon - γ and tumor necrosis factor - α were measured in the vaccinated seropotency groups. The rate of postchallenge mortality was 50% in the control group, and no mortality was encountered in the boric acid group. The boric acid adjuvanted vaccine decreased the bacterial load and postchallenge abscess in the internal organs and also local inflammatory reactions due to montanide adjuvants in combinations. No difference was found in the interferon - γ and tumor necrosis factor - α levels between the control and vaccine groups. It was concluded that boric acid can be used as an adjuvant in inactivated vaccines.

Key words: Adjuvant, aluminum hydroxide, boron, challenge, vaccine

1. Introduction

Boron is a nonmetal element found widely in nature. It is generally found in water and soil as boric acid (BA) or borate [1], of which BA is the most widely found form of boron in nature. Boron compounds have been widely used in health, agriculture, industry, and cosmetic applications [2].

Boron is a required trace element for plants, humans, and animals, playing significant roles in cell proliferation and development, brain function, hormone and mineral metabolisms, bone development, plasma lipid profile, the prevention of cancerous cell proliferation, cancer therapy, and the immune system [2–5]. Antibacterial, antifungal, and antibiofilm effects of boron and boron based compounds have also been determined [6, 7]. Some important roles of boron have been determined on microbiota health in humans and animals [8].

Vaccines have been continued to be one of the most powerful weapons in the fight against infectious diseases. Thanks to the development of efficacious and safe preventive measures, the most terrifying enemies of humanity have been controlled from many locations around the world, and the importance of vaccines has come to be better understood during the COVID-19 pandemic [9].

The efficacy of any vaccine is not attributable only to the antigen compounds, but also to the adjuvants it contains. Adjuvants are used to improve the power of immunity produced by the antigens, to decrease the cost of the vaccine by decreasing the amount of antigen required, to increase vaccine efficacy in newborns, the elderly, and the immunosuppressed, to increase the rate and duration of the immune reaction, to stimulate mucosal immunity, and to decrease the antigen competition in combined vaccines [10]. The various mechanisms of action of the adjuvants are the depot effect, carrier effect, and immune stimulant mechanisms [9].

The variety of adjuvants used in human vaccines is limited. Currently, the majority of vaccines licensed by

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the European Medicines Agency and the U.S. Food and Drug Administration for human use contain aluminum salts as adjuvants [9]. The adjuvant activity of aluminum hydroxide (AlOH) was first defined in 1920, making it one of the oldest adjuvants used in vaccine formulations. Aluminum salts produce antigen depots that provide long term stimulation to the immune system through the slow release of the antigen at the injection site. Another mechanism of effect is macrophage and complement activation [11]. A vast majority of the injected aluminum is stored in the bones, liver, lungs, and nervous system. Aluminum cannot be eliminated in individuals with chronic renal disease, and it accumulates in the bones and nervous system over time [9]. In addition to the known side effects, other adverse effects, such as the ASIA syndrome (an autoimmune/inflammatory syndrome induced by adjuvants) have been defined [12]. Aluminum penetration into the brain was detected in mice injected with aluminum - 26 [13].

The variety of adjuvants used in veterinary practice is greater than in human medicine. The commonly used adjuvants are aluminum salts, emulsions, oils, particle adjuvants, immunomodulators, and nanotechnological adjuvants [11]. Montanide ISA 50 (ISA 50) and Montanide ISA 206 (ISA 206) are oil-based adjuvants that have a similar mechanism of action as AlOH, being the production of an antigen depot at the injection site [14, 15].

It is important to develop efficient and safe adjuvants since the variety of adjuvants used in human and animal vaccines is limited and the most commonly used aluminum salts and oil based adjuvants cause local reactions at the injection site along with systemic side effects. The aim of the present study, therefore, is to determine the adjuvant activity of boron compounds, which have many positive effects on biological functions and the immune system.

2. Materials and methods

2.1. Vaccine production

Two *Staphylococcus aureus* (*S. aureus*) strains isolated from bovine mastitis and typed according to their phenotypic and genotypic characteristics, which were in the culture collection of the Microbiology Department of the Faculty of Veterinary Medicine of Selcuk University, were used in the study. No. 142 was selected as the vaccine seed strain, and no. 145 as the challenge strain. *S. aureus* strain no. 142 was cultivated in BHI broth at 37 °C for 24–48 h, at aerobic condition, collected by centrifugation, and washed three times using physiological saline. The antigen concentration was adjusted to 5×10^9 CFU (Colony Forming Unit)/mL and inactivated with formalin (0.5%) [16]. The vaccine antigen was adjuvanted with AlOH (% 4, Vetal, Türkiye), ISA 50 (at rate of 1 : 1, Seppic, France), ISA 206 (at rate of 1 : 1, Seppic, France) and BA (11.4 mg/mL $B(OH)_3$, Boron Institute, Türkiye) and mixed for at least 30 min for homogenization [17]. Also, ISA 50 + BA and ISA 206 + BA combinations were made, and six different vaccines were prepared. No combination of boron and aluminum was made since boron has an antagonistic effect on aluminum. Purity and sterility control were performed at each phase of the product for quality assurance, and the harmlessness of the vaccines was tested by injecting subcutaneously each vaccine into five mice at a dose of 0.2 mL [18, 19].

2.2. Research groups and vaccination of mice

Two main groups were formed in the study, namely challenge and seropotency. In both main groups, the placebo, control, ALOH vaccine, ISA 50 vaccine, ISA 206 vaccine, ISA 50 + BA vaccine, and ISA 206 + BA vaccine groups took place. Each of the vaccine, control, and placebo group contained eight 6–8 week old male BALB/c mice (n = 64) that weighed an average of 20 g. The mice were vaccinated twice, 21 days apart, by subcutaneously injecting 0.2 mL of vaccine from the dorsal region. The same amount of physiological saline was injected into the control and placebo groups.

2.3. Determination of challenge strain lethal dose 50 (LD50) in mice

The LD_{50} value of the challenge strain was determined according to the Behrens and Karber method [20]. For this purpose, *S. aureus* no. 145 was cultured in BHI broth (Oxoid) for 24–48 h, at aerobic condition and the bacterial count was determined using the colony counting method [21]. The LD_{50} value was determined in male BALB / c mice aged 8–10 weeks with an average weight of 20–25 g. Nine different doses betweeen 1×10^7 and 9×10^7, and also five different doses betweeen 1×10^8 and 5×10^8 live bacteria were injected intraperitoneally into eight mice in each group. The infected mice were observed for 2 weeks, and deaths were recorded. The deaths were confirmed to be due to *S. aureus* via the cultivation of samples from the internal organs (lung, liver, heart, spleen and kidney) of the dead mice in 5% blood agar (Oxoid) and Baird Parker agar (Oxoid).

2.4. Challenge of the mice

The vaccine and control groups were infected intraperitoneally with the LD50 dose of live *S. aureus* (no. 145) 15 days following the second vaccination. They were observed for 2 weeks, and the mortality rate was recorded. No procedure was performed on the placebo group. The necropsies of the mice that died and were sacrificed 2 weeks after the challenge were performed, and their internal organs (lungs, liver, heart, spleen, and kidneys) were retrieved. The bacterial load was determined using the colony counting method and quantitative Real Time Polymerase Chain Reaction (qRT - PCR), and the level of postvaccination clearance of the internal organs from

the agent was determined. Parts of the internal organs and samples from the vaccine injection sites were fixated in a 10% formaldehyde solution for histopathological analysis.

2.5. Determination of bacterial load on internal organs

All the organs retrieved from the mice were mashed in a sterile mortar. One mg of the tissue sample was mixed with 9 mL of physiological saline and crushed for 10 min. The samples were diluted up to 10^{-9} using physiological saline, and 0.1 mL of the dilutions were inoculated into 3 pieces of plate count agar medium (Oxoid) and cultured for 16–24 hours at 37 °C, at aerobic condition. The number of viable *S. aureus* in a 1 mg tissue sample was determined by colony counting method. DNA isolation was performed from 1 mg of the tissue sample using the method reported by Hein et al. [22]. Briefly, 1 mg of a *S. aureus* negative tissue sample from healthy mice with 1 mL of *S. aureus* no. 145 (1×10^9 CFU/mL). Ten fold dilutions up to 10^{-9} were prepared from the obtained DNA sample. *S. aureus,* primers selected for the *fem*A gene region, and a texas-red marked sequence specific probe (TaqMan) were used for the qRT-PCR analysis (Table 1). Amplification was performed in a Roche LightCycler® 96 Real - Time PCR device by preliminary denaturation at 95 °C for 3 min, denaturation over 30 cycles at 95 °C for 30 s, and annealing at 55 °C for 45 s [23]. *S. aureus* qRT-PCR analyses of the DNA samples were made, standard amplification curves were generated, and the R^2 value was obtained. The obtained data were used as standards, and the total number of bacteria in the samples was determined [22].

2.6. Histopathology

To evaluate histopathological changes in the internal organs and vaccine injection sites in the challenge and placebo groups, samples from dead or killed animals were fixed in 10% formaldehyde for 24 h. Samples washed overnight in running tap water were passed through a series of alcohol (70°, 80°, 90°, 96° and absolute alcohol) and xylol (two times) and embedded in a paraffin wax block. Five µm thick sections were taken from the obtained paraffin blocks, stained with Hematoxylin and Eosin (H & E) stains and evaluated under the light microscope [24].

2.7. Vaccination and sampling of the seropotency group

The "Balb–C" mice aged 6–8 weeks in the seropotency group were vaccinated twice with 0.2 mL of the prepared vaccines, 21 days apart. The same amount of physiological saline was injected into the mice in the control group. Blood samples were taken into anticoagulated (lithium heparin) and without anticoagulant test tubes for plasma and serum from the hearts of the mice in the seropotency-control group under ketamine/xylazine anesthesia on day 1 of the study. Also, blood samples were taken from the mice in the seropotency vaccination group 10, 21 (during the second vaccination), 41, and 60 days after the initial vaccination, from six mice each time (6×6 groups = 36). Serum samples were used to measure humoral immunity in our previous study [17]

2.8. Interferon - γ (IFN - γ) and tumor necrosis factor - α (TNF - α) measurements in the seropotency group

For cytokine measurements, mononuclear cells were isolated from anticoagulated blood samples and cultured together with *S. aureus* sonicated antigen. Strain no. 145 was used to produce the antigen. The antigen was prepared according to the method described by Bhaduri and Demchick [25]. An antigen containing 10 µg/mL protein was used in the study. Mononuclear cells were isolated by Ficoll - Gradient (Histopaque 1077, Sigma Aldrich) centrifugation (400 × g, 30 min). Live cells were diluted in a RPMI - 1640 medium (Sigma Aldrich), including 5% fetal calf serum (Sigma), up to a final cell count of 2×10^6 cells/mL. A hundredµL of the cell solution was taken into sterile microplate wells and cultured for 24 h together with the *S. aureus* sonicated antigen in an incubator including 10% CO_2. The cultured supernatant obtained by centrifugation (500 × g, 10 min) at the end of the incubation was stored at –80 °C for cytokine measurement [26, 27]. IFN - γ and TNF - α levels were measured using an Abcam (UK) Mouse Interferon Gamma ELISA Kit (IFNG) (ab100689) and a Mouse TNF Alpha ELISA Kit (ab100747), respectively, according to the manufacturer's instructions.

2.9. Statistical analysis

All statistical analyses were performed using the "one - way ANOVA" test.

2.10. Ethics statement

This research was approved by the Ethics Committee of the Experimental Medicine Research and Application Center of Selcuk University in Konya, Türkiye (protocol number: 2015/44).

Table 1. Primers and probe used in the quantitative Real Time Polymerase Chain Reaction (qRT - PCR) analysis [23].

Oligonucleotide	**Sequence**
*fem*A – F	ACTGTGACG ATGAATGCGACAA
*fem*A – R	ATGTTGTGGTGTTCTTATACCAAATCC
*fem*A – Probe	**Texas red** – 5' - CGACAACTGGCACATTGGCTAT CGCTT T - 3' - BHQ-2

3. Results

3.1. Challenge results

For male BALB/c mice 8–10 weeks old, weighing 20–25 g on average, the *S. aureus* strain no. 145 LD_{50} value was determined as 2.5×10^8 CFU. While death was observed in 4 of 8 (50%) mice in the control group and 1 of 8 (12.5%) mice in the ISA 206 group after the challenge, no death was observed in the other vaccine groups. Following the bacterial colony count on the internal organs, the bacterial load in all vaccine groups was found to be statistically significantly lower than in the control groups ($p < 0.05$) (Table 2). The lowest and highest numbers of bacteria were seen in the ISA 50 (42.500 CFU/ mg) group ($p < 0.05$) and the ISA 206 (5.334,000 CFU/mg) and ISA 206 + BA (11,097,830 CFU/mg) groups, respectively. The number of bacteria isolated was lower in the BA (218.500 CFU/mg) group than in the AlOH (237.500 CFU/mg) group, though no significant difference was detected between the two groups ($p < 0.05$). The number of bacteria was found to be significantly higher in combinations with the adjuvants ISA 50, ISA 206, and BA ($p < 0.05$). The R^2 value was determined to be 0.98 based on the amplification of the DNA samples of the bacteria (1×10^3 - 1×10^9 in number). DNA samples of bacteria equal to or lower than 1×10^2 were found to be impossible to amplify by qRT - PCR. According to the qRT - PCR results, the bacterial load on the internal organs in the vaccine groups was statistically significantly lower compared to that in the control groups ($p < 0.05$), while the bacterial load on the internal organs determined by qRT - PCR was found to be significantly lower than that detected using the culture method ($p < 0.05$) (Table 2).

3.2. Histopathological findings

Subcutaneous abscesses the size of a chickpea and with white-colored content were noted at the vaccine injection site in all mice in the ISA 50 group, while the abscesses were smaller and more limited in the ISA 50 + BA group (Figures 1a, 1b). Subcutaneous abscesses had developed in four mice in the ISA 206 group but in only one mouse in the ISA 206 + BA group. Two mice in the AIOH group developed small abscesses, whereas no abscesses were found in the BA or control groups. A macroscopic evaluation of the internal organs revealed gray-white foci in the kidney, liver, and heart in four mice and in the cecal serosa in one mouse in the control group (Figure 2), in the heart in one mouse in the ISA 50 group, in the kidney in

Table 2. Bacterial loads in internal organs by colony counting and qRT-PCR in challenge groups.

Group/Method	Mean number of bacteria in 1 mg of tissue	
	Colony count	qRT – PCR
Control	218,266.66 ± 13.04	14,400 ± 31.62
BA	218,500 ± 246.98	135,000 ± 296,64
AlOH	237,500 ± 316.22	164,300 ± 228.03
ISA 50	42,500 ± 252.98	33,100 ± 303.31
ISA 50 + BA	308,800 ± 404.96	165,000 ± 316.22
ISA 206	5334 ± 17.20	3982.50 ± 14.14
ISA 206 + BA	11,097.83 ± 38.37	7243.40 ± 22.28

Abbreviations; BA: Boric acid, ALOH: Aluminum hydroxide

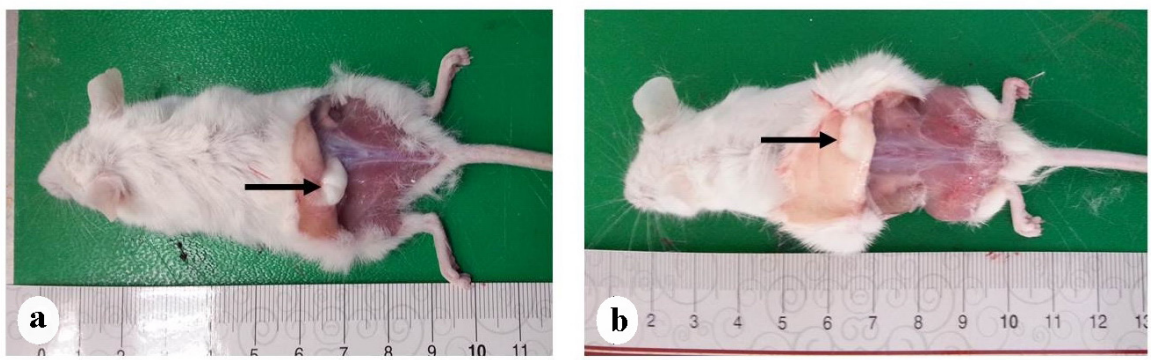

Figure 1. a. Subcutaneous abscess (arrow) with white colored content in the size diameter of 1 × 1 × 1 cm, ISA 50 group. **b.** Smaller, well-defined, and thickly encapsulated abscess (arrow), ISA 50 + BA.

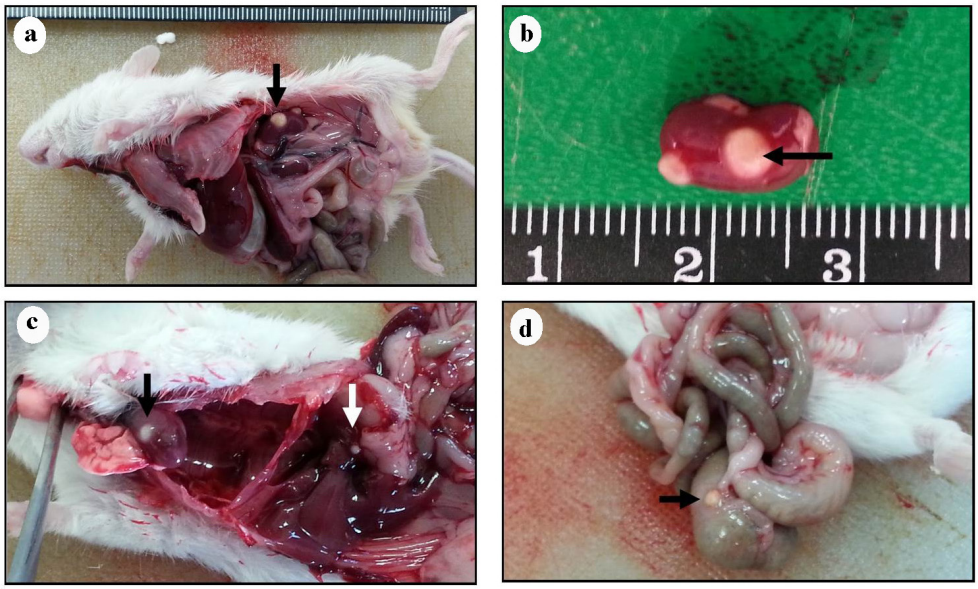

Figure 2. Control group, abscesses in the internal organs, **a, b.** in the kidney (arrow). **c.** in the heart (black arrow) and liver (white arrow); **d.** in the cecum (arrow).

one mouse in the ISA 50 + BA group, in the heart in two mice in the ISA 206 + BA group, and in one mouse in the AlOH group. No macroscopic abscesses were found in the internal organs of the BA, ISA 50 + BA, and ISA 206 + BA groups. In the histopathological examinations, localized abscess formations with diffuse purulent inflammation in the dermis were evident at the vaccine injection site in the ISA 50 and ISA 206 groups (Figures 3a–3c). The inflammation was noted to continue in the ISA 50 + BA group, but was found to be converted into a subacute and chronic form with lowered neutrophil infiltration, and more limited and with fewer masses of pus than in both the ISA 206 and ISA 50 groups (Figures 3b–3d). Diffuse purulent inflammation was seen in the subcutaneous connective tissue in the AlOH group (Figures 4a, 4b), while a substantially large abscess was noted in one case in the AlOH group. In the BA group, generally, mild chronic inflammation was present in the dermis, while chronic reactive inflammation including isolated neutrophils was encountered in one mouse (Figure 4c). In the internal organs, focal mononuclear cell infiltrations, identical to each other, were seen in the kidneys of the vaccine groups (Figure 5a). A marked abscess in the cortex was seen in only one mouse in the ISA 50 + BA group (Figure 5 b). Significant purulent inflammation was observed in the cortex of three mice in the control group (Figures 5c, 5d). While no changes were observed in the vaccine sites and internal organs in the placebo and BA groups, four mice in the challenge control group had purulent inflammation in the kidneys, liver, heart, and cecal serosa (Figures 6a–6c). Nonpurulent myocarditis (Figure 6 d) is characterized by focal (in the ISA 50 group) and multifocal (in the ISA 206 + BA group) mononuclear cell infiltrations, and focal nonpurulent pericarditis in one case in the AlOH group (Figure 6 e).

3.3. IFN - γ and TNF - α measurements results

For INF - γ and TNF - α, the measurement ranges for the ELISA kits used in cytokine measurement were 31.25–1000 pg/mL and 0–6000 pg/mL, respectively. The levels of INF and TNF in culture supernatants obtained from cultured mononuclear cells (2×10^6 cells/mL) by *S. aureus* sonicated antigen (10 mg/mL) in vaccinated mice were found to be comparable to those in the control group. No significant or regular change was observed in the cytokine levels on the different sampling days postvaccination (Table 3).

4. Discussion

The importance of vaccines has been better understood during the recent COVID-19 pandemic, and also the importance of accessing effective vaccines to prevent potential threats of a new pandemic. The current adjuvants may contribute to savings in doses, although in pandemic situations, vaccines should be low-priced and formulated to provide immunity with as minimal doses as possible to support extensive global vaccination campaigns and subsequent immunization [28]. The effectiveness of vaccines has increased with the introduction of modern technologies such as nanotechnology and molecular biology into the production processes of both antigen and adjuvant components [9].

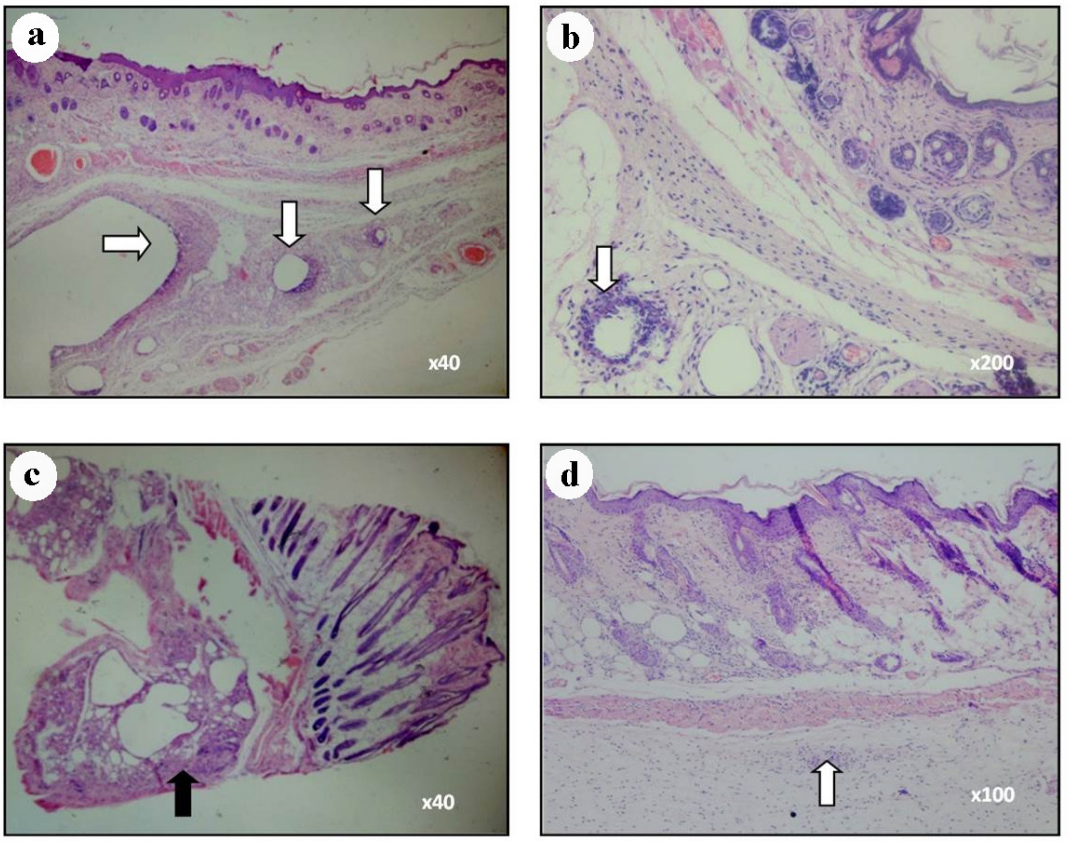

Figure 3. a, c. Diffuse purulent inflammation together with patchy abscess formations and pus accumulation in the subcutaneous tissue (**a.** ISA 50, **c.** ISA 206). **b, d.** Mild inflammatory granulation tissue and patchy masses of pus in the subcutaneous tissue (arrow) (**b.** ISA 50 + BA) and focal neutrophil infiltrations (arrow) (**d.** ISA 206 + BA) in the subcutaneous tissue. H & E (Hematoxylin and Eosin).

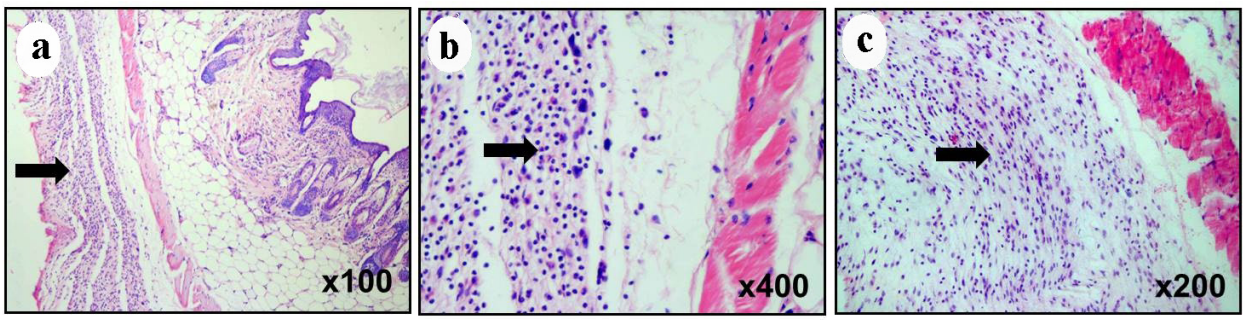

Figure 4. a, b. Diffuse purulent inflammation (arrow) in the subcutaneous connective tissue in the AlOH group. **c.** BA group, patchy neutrophil infiltrations in chronic inflammatory granulation tissue in the dermis (arrow). H & E.

The main hypothesis of this study is that boron compounds, whose immunomodulatory effect has been demonstrated by many studies [29–31], can be used as adjuvants in inactivated vaccines. The effect of boron on the immune system has been suggested to be associated with the production of certain cytokines [32]. Boron has been shown to have an immunomodulatory effect by raising the number of NK cells in the blood when it is added to the diet of mice with arthritis [29]. Performance, bone development, and immune functions may be augmented in layer hens when B is added to their rations in appropriate concentrations [33]. Jin et al. [34] reported that the addition of B to the drinking water of broiler pullets (<100 mg/L) had a positive effect on the development of

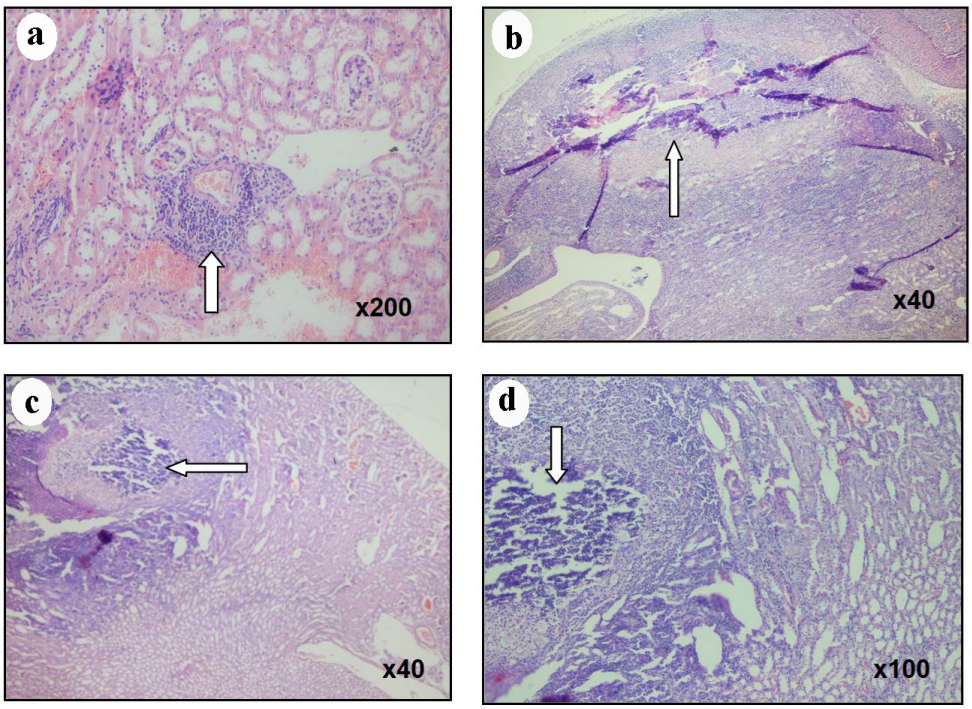

Figure 5. Kidney, H & E. **a.** Focal mononuclear cell infiltration in the corticomedullary border in the ISA 50 group (arrow). **b.** A quite large purulent inflammation in the cortex in the ISA 50 + BA group (arrow). **c, d.** Purulent inflammation in the cortex in the control group (arrow).

immune organs after 4 weeks. Furthermore, the addition of B to the diet has been determined to increase neutrophil, NK, and CD8+/CD4 cell concentrations [30] and WBC concentrations in humans [35].

Boron has been reported in some studies to be toxic to humans and animals [36], although the toxic effect has been determined to be dose-dependent [37]. The dose of boron administered in the diet should be above 100 mg/kg of live weight in order to exert its toxic effects, according to the results of animal experiments [2]. The boron dose used in this study (11.4 mg/mL) caused no hypersensitivity reactions or intoxication symptoms in vaccinated mice.

Boron has been determined to have an antiinflammatory effect in experimental animal studies [26, 29, 31, 33]. Yıldiz et al. [38] reported that the application of intraperitoneal boric acid at a dose of 50 mg/kg for 7 days in mice inflicted with experimental septic arthritis by *E. coli* caused a significant decrease in inflammation, both histopathologically and biochemically. Different opinions have been reported on the mechanism behind the antiinflammatory effect of boron. Armstrong and Spears [26], Armstrong et al. [31], Naghi et al. [39] suggest that boron exerts its effect by decreasing the production of inflammatory cytokines. Hazman et al. [40] reported that the inhibitory effect of boron on inflammation in a cisplatin induced nephrotoxicity model may be related to mRNA expression and the monocyte chemoattractant protein - 1 (MCP - 1) gene. Some studies have shown that BA has an antiinflammatory effect by reducing oxidative stress [41, 42]. In this study, the macroscopic appearance of vaccine injection sites was observed similarly to our previous seropotency study [17]. Based on this finding, the inflammatory reactions seen at the injection sites of ISA 50 and ISA 206 adjuvanted vaccines were decreased when the vaccine was combined with boron. The inflammation was observed to continue in the vaccines with the boron combination group, although it was seen to be more limited and with decreased neutrophil infiltration due to the conversion into subacute and chronic forms.

Benderdour et al. [43] discovered that pigs fed boron containing diets in various concentrations had higher serum IFN - γ and TNF - α levels after LPS injection than controls fed a boron free diet. The increase in cytokine levels was found to be dose dependent. Fry et al. [27] discovered that following a BHV - 1 challenge in calves fed diets containing 5 mg/kg and 15 mg/kg B, serum IFN - γ and TNF - α levels increased in the 5 mg/kg group but decreased in the 15 mg/kg group compared to controls. They observed that the cytokine levels did not change in blood monocytes isolated from these cattle and stimulated

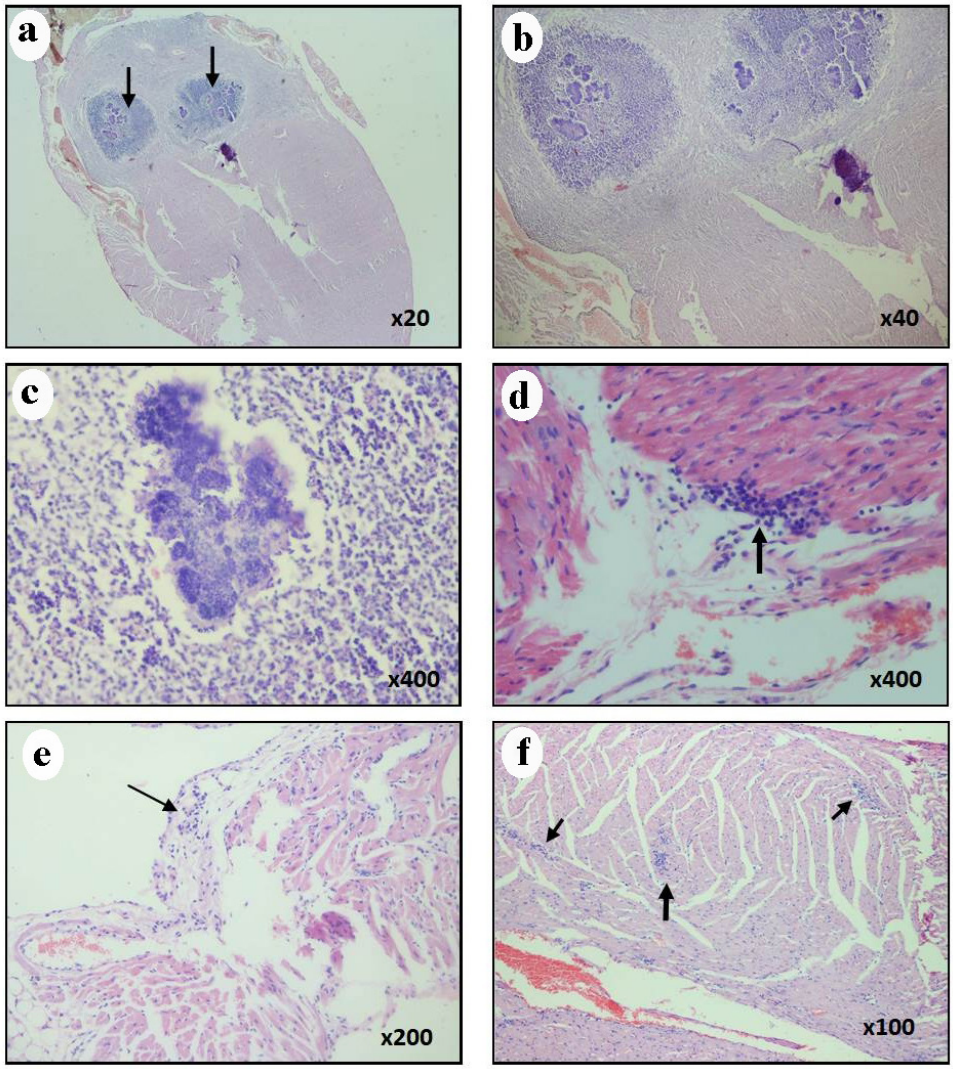

Figure 6. a–c. Abscess formation in the heart in the challenge group (arrows) **d.** Focal nonpurulent myocarditis in the ISA 50 group (arrow). **e.** Focal nonpurulent pericarditis (arrow). **f.** Multifocal nonpurulent myocarditis in the ISA 206 + BA group (arrows). H & E.

Table 3. Interferon - γ (IFN - γ) and tumor necrosis factor - α (TNF - α) levels (pg/mL) in the seropotency groups.

Sampling day	Groups													
	Control		BA		AlOH		ISA 50		ISA 50 + BA		ISA 206		ISA 206 + BA	
	IFN	TNF	IFN	TNF	IFN	TNF	IFN	TNF	IFN	TNF	IFN	TNF	IFN	TNF
10th day	145.2	946.2	155.7	1007.7*	112.7*	755.9*	92.7*	639.2*	103.2*	700.6*	145.2	946.2	134.7	1069.1*
21st day	91.7	633.0*	113.7*	762*	123.2*	817.3*	113.7*	762.0*	145.2*	946.2*	124.2*	823.4*	103.2	700.6*
40th day	130.5	860.3	103.2*	700*	145.2*	946.2*	103.2*	700.6*	134.7	884.8	134.7	884.8	113.7*	516.3*
60th day	113.7	762	134.7*	884.8*	134.7*	884.8*	134.7*	884.8*	113.7	762.4	113.7	762	124.2*	823.4*

* control group vs. treatment groups ($p < 0.05$).

with LPS. In another study suggesting a dose dependent effect on immune function, Zhang et al. [44] evaluated cell proliferation, apoptosis, cytokine secretion, and immune response-related gene levels in *Ostrich chick* splenic lymphocytes cultured in different concentrations of a BA solution and reported that boron in low concentrations (0.01–10 mmol/L) increased cell proliferation, cytokine secretion (IFN - γ and IL - 6), and cell apoptosis. High dose boron (25–100 mmol/L) has been reported to exert a contrary effect, while the positive effects of boron on the immune system have been suggested to be associated with the application dose. Similarly, the INF and TNF levels in the culture supernatants obtained from cultured mononuclear cells using the *S. aureus* sonicated antigen in vaccinated mice were found to be comparable to those in the control group of the current study. Furthermore, no significant or regular changes in cytokine levels on different sampling days were detected following vaccination. The unchanged cytokine levels in the B vaccine group may be attributed to the B dose and the route of application of the vaccine. Boron has generally been used orally in model studies evaluating its effects on the immune system. In this study, Boron was administered as a vaccine adjuvant by subcutaneous injection.

In bacterial reisolation from internal organs, the bacterial load on the internal organs in all vaccine groups was found to be decreased when compared to the control group ($p < 0.05$). The lowest bacterial load was seen in the ISA 50 group, compatible with the anti - *S. aureus* antibody titers noted in our previous study [17]. Bacterial reisolation rates in combinations of ISA 50 and ISA 206 adjuvants with BA were determined to be higher than single use adjuvants. BA adjuvanted vaccine has been shown to reduce bacterial load on internal organs just as much as AlOH adjuvanted vaccine. A qRT - PCR analysis revealed the bacterial counts in the groups to be proportional to the culture results but lower. This condition has been suggested to originate from bacteria or DNA loss during DNA isolation.

In challenge groups, microscopic findings of internal organs after histopathology showed that there was no difference between BA and other vaccine groups.

In conclusion, BA has been observed to exert antiinflammatory effects and to decrease reactions, especially to ISA 50, when the macroscopic and histopathological data at the vaccine injection sites are considered. Their efficacy, however, has been observed to decrease due to the possible breakdown in the oil structure in BA - oil combinations. BA has been proposed as a vaccine adjuvant based on the relative survival rates, the bacterial reisolation rates from the internal organs following the challenge, and the histopathological findings of the internal organs.

Acknowledgment/disclaimers/conflict of interest

As a result of this study, a patent has been obtained from the "Turkish Patent and Trade Mark Office" for "The production method of a vaccine adjuvanted with boron" (patent number: TR 2015 04069 B).

Part of this study was presented in abstract form as a poster presentation at the VET İstanbul Group Congress 2016, Sarajevo, Bosnia and Herzegovina.

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The authors declare that they have no conflict of interest.

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