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The effect of *Staphylococcus aureus* extracellular products (SECP) on performance, selective innate immune indexes, and humoral immune response against avian influenza and Newcastle disease vaccines in broiler chickens

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Abstract: Improving the immune response is important for many reasons such as vaccine failure, immunosuppressive diseases, and antibiotics misuse. Research on materials likely to have immune-enhancing effects has been on the rise. Factors, substances, or compounds that can regulate different functions of the immune system can be used to enhance specific and even nonspecific responses to vaccines. Different amounts (0, 25, 50, and 100 µg) of *Staphylococcus aureus* extracellular products (SECP) were injected subcutaneously to different groups of chickens during the first vaccination against avian influenza (AI) and Newcastle disease (ND) viruses to investigate the effect of SECP on innate and humoral immunity of broiler chicks. This study showed that the SECP could increase the immune response against AI and ND viruses at 50 µg ($P < 0.05$). Also, innate immune response, including serum complement activity, was boosted at 100 µg, as well as the performance at 50 µg ($P < 0.05$). Hence, SECP is suggested to be used for enhancing chicken immune response and to improve vaccine performance.

Key words: Extracellular products (ECP), *Staphylococcus aureus*, innate immunity, humoral immunity, avian influenza, Newcastle disease vaccine, feed conversion ratio (FCR)

1. Introduction

Research on materials likely to have immune-enhancing effects has been increasing. Factors, substances, or compounds that have the ability to regulate different functions of the immune system can be used to enhance specific and even nonspecific responses to vaccines. In the poultry industry, improving the immune system is important for reasons such as vaccine failure, immunosuppressive diseases, and antibiotics misuse. Research on materials that are likely to have boosting effects on the immune system has been progressively increasing. In birds, what could have been achieved by combining actions such as vaccination and the use of commonly used chemical drugs has almost reached its fullest potential. To study the effects of augmentation or even suppression of the immune system that may exist in products, limited methods are generally used, which may be considered as a standard way to measure the effect of various factors on the functioning of the immune system. Besides, studying the impact of humoral immunity is more common than cell-mediated immunity. The effect of a substance on the function of humoral immunity is typically assessed by measuring its effect on the response of the antibody produced in the body following vaccination, so this method can be considered as a standard method for this purpose. Evaluation of humoral immune response is simpler and less costly than measuring cell-mediated immune function, such as the proliferation of lymphocytes. Therefore, a disease that humoral immunity plays a determining role in protecting the body against should be chosen.

Staphylococcus aureus is a facultative anaerobic gram-positive cocci bacterium. Multiple extracellular materials are produced by *S. aureus*, including common and specific toxins. General toxins such as hemolysin may interfere with the clinical manifestation of general or local infections, and specific toxins such as enterotoxins and epidermolytic toxins are responsible for specific and nonpurulent clinical manifestations of this agent. Enterotoxins are trypsin-resistant proteins that resist boiling heat for half

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an hour. Enterotoxins are considered to be superantigens (superconductors), nonspecific stimulants of lymphocyte clones. These types of toxins have been identified in *S. aureus* and *S. epidermidis*. About 30%–50% of all isolates of *S. aureus* of human origin produce enterotoxin. This type of toxin is produced as a result of the growth of the bacteria in foodstuff and following a meal causes food poisoning with signs of diarrhea and vomiting. Different antigenic types (A, B, C1, C2, C3, D, E, H) have been identified for enterotoxins, which are produced by different strains of *S. aureus* [1–5]. These toxins are powerful stimulants for the production and secretion of cytokines, including IL-1, IL-2, IFN-α, and tumor necrosis factor [6,7].

Avian influenza (AI) is a contagious disease of poultry caused by the type A influenza virus, genus *Orthomyxoviridae*. At present, sixteen hemagglutinin subtypes (H1–H16) and nine neuraminidase subtypes (N1–N9) of influenza A viruses have been recognized. In the present outbreak of AI in Iran, the isolates (H9N2) were characterized as a low pathogenic form of the virus [8]. Avian influenza vaccines do not produce high antibody titer against the AI virus, so we tried to increase the immune response (antibody titer) by immune-stimulating factors.

Isolates of NDV are members of the genus *Avulavirus* in the family Paramyxoviridae. At present, a total of 15 avian paramyxovirus serotypes (APMV-1 to APMV-15) have been identified in different species of wild and domesticated birds with the ND virus (NDV) to be APMV–1 [9]. NDV has also been categorized into five pathotypes based on clinical signs in infected chickens, including a) viscerotropic velogenic, b) neurotropic velogenic, c) mesogenic, d) lentogenic or respiratory, and e) asymptomatic [9]. Velogenic strains are the most dangerous and are responsible for the most deaths and severe infections, while deaths resulting from lentogenic strains are negligible. Many species of birds are susceptible and mortality and morbidity rates depend on the strain of the virus. Humans can also become infected. As the effects of *Staphylococcus aureus* extracellular products (SECP) in poultry are unknown, this study was conducted to investigate the effect of this product on innate immune response, feed consumption rate (FCR), and ND virus and AI virus antibody titer in broiler chicks.

2. Materials and methods

One hundred and eighty, 1-day-old broiler chicks (Ross 308) were randomly divided into 6 groups (A through F), each with 3 replicates of 10 chicks. The chicks in group A through D were inoculated twice with Newcastle B_1 strain (commercial vaccines Avishield® ND B1 was provided by Genera Inc., Croatia) via eye-drops. At first vaccination, they were also injected subcutaneously with killed Newcastle + influenza (H_9N_2) commercial vaccine (Gallimune 208 ND + Flu H9 ME was provided by Merial Inc., France) supplemented with 25 µg (group A), 50 µg (group B), 100 µg (group C), and 0 µg (group D) of ECP. The chicks in group E received 100 µg of ECP with no vaccine. Group F chicks constituted the "control group" and received sterile phosphate-buffer saline (PBS) subcutaneously (Table 1).

Ethical permission was granted by the Shahid Chamran University of Ahvaz Ethical Commission for Animal Experiments under verification number ee /97.24.3.70425/ scu.ac.ir.

2.1. ECP extraction

Local isolates of *S. aureus* were cultured on a sac according to the method of Donnelly et al. [10]. For this purpose, a 20-cm segment of activated dialysis tube (Sigma, USA) was filled with approximately 30 mL of Muller-Hinton broth (Merck, Germany); a ligated dialysis tube was placed in an Erlenmeyer flask containing 50 mL of PBS and sterilized by autoclaving at 121 °C for 30 min. The local *S. aureus* isolate was cultured in broth in the abovementioned sterile flask. The cultures were incubated for 48 h at 37 °C, then the dialysis tube was removed, and the remaining broth was centrifuged at 3500 rpm for 10 min. The supernatant was sterilized through filters of 0.22 µm and used as an extracellular product of *S. aureus* after protein assessment and deactivation with 0.1% formalin [10,11].

2.2. DNA extraction

The DNA of a local isolate of *S. aureus* was extracted by a commercial DNA extraction kit (iNtRON, South Korea) according to the manufacturer's instructions with some modifications. Briefly, a colony of *S. aureus* was suspended in 200 µL of lysis buffer and 20 µL of lysozyme (20 mg/mL) were added to the suspension. After 20 min of incubation at room temperature, 20 µL of proteinase K (20 mg/mL) and 5 µL of RNase A (20 mg/mL) were added to the suspension and incubated for 30 min at 56 °C. The suspension was then heated at 80 °C for 15 min and 200 µL of binding buffer were added to it. The suspension was centrifuged at

Table 1. Design of the the experiment groups.

Group	Live+killed ND vaccine + Influenza vaccine	*Staphylococcus aureus* ECP (µg)
A	+	25
B	+	50
C	+	100
D	+	-
E	-	100
F	-	-

13,000 rpm for 5 min, 350 µL of supernatant from this step were transferred to a new microtube, 200 µL of absolute ethanol were added and the mixture was mixed well. The mixture was applied to the spin column and centrifuged at 13,000 rpm for 2 min; the column was washed with 700 µL of washing solutions A and B, respectively. Finally, 50 µL of elution buffer was added to the column, it was placed into a new collecting tube, and after 1 min incubation at room temperature, the column was centrifuged at 13,000 rpm for 1 min. The eluted solution was used as a template DNA in the PCR test.

2.3. PCR reaction for polymerization of *aroA* gene

The *aroA* gene is one of the important genes for the production of aromatic amino acids and is essential for bacterial viability. It has been shown that the *aroA* gene sequence is different in bacteria. In this study, we used the specific primer of *aroA* gene in *Staphylococcus aureus* (GenBank database accession no. L05004) and the PCR method was used to identify this bacterium [12]. The FA1 (5′-AAGGGCGAAATAGAAGTGCCGGGC-3′), and RA2 (5′-CACAAGCAACTGCAAGCAT-3′) primers (Bioneer, South Korea) were used to amplify a 1153 bp segment of *aroA* gene with a full length of 1283 bp.

The PCR was carried out according to the method of Marcos et al. [12] with some modifications. The *aroA* gene was amplified using 12.5 µL of 2X mastermix (Ampliqon, Denmark), 4.5 µL (10 pg) of template DNA, 1.5 µL of 10 µM of forward and reverse primers, and 5 µL of PCR grade water in a final volume of 25 µL. Microtubes were transferred to a thermal cycler (Eppendorf, Germany) and the following thermal conditions were carried out: One cycle at 95 °C for 5 min, 40 cycles at 95 °C for 60 s, 58 °C for 60 s, 72 °C for 90 s, and 1 cycle at 72 °C for 5 min. Finally, 7 µL of PCR product was evaluated by electrophoresis in 1.5% agarose gels containing safe stain (Sinagen, Iran). The gels were read under UV light (Uvitec, England) and samples with a PCR product of about 1153 bp band were considered positive (Figure).

2.4. Blood collecting and serological evaluation

Ten chicks of each group were bled randomly and blood samples were collected prior to vaccination and on days 14 and 21 postvaccination. Antibody titers against ND and AI viruses were determined. Blood samples obtained from the brachial vein and sera were separated and frozen at –20 °C until the serological tests were performed. Serum samples were analyzed by hemagglutination inhibition (HI) test to identify antibodies against Newcastle and Avian influenza disease vaccines [13].

2.5. Complement activity

In order to measure the activity of the complements, the recommended method by Budiño et al. [14] was used with slight modifications. In a sterile 0.5 microtube, 25 µL of each serum sample were added to 375 µL of complement buffer (Veronal buffer, pH = 7.5, containing 0.5 mM magnesium chloride and 1.5 mM calcium chloride) and 100 µL of 10% rabbit RBC. The mixture was gently mixed and incubated at 18 °C for 1 h. Microtubes were then centrifuged for 4 min at 400 rpm, 200 µL of the supernatant of each sample were transferred to a well of a flat-bottomed plate, and optical density (OD) was measured with a plate reader at 430 nm. An inactive serum (a serum heated at 56 °C for 30 min) was used as a negative control. Serum hemolytic activity was calculated by subtraction of OD_{430} of each sample from negative control and was considered as serum complementary activity.

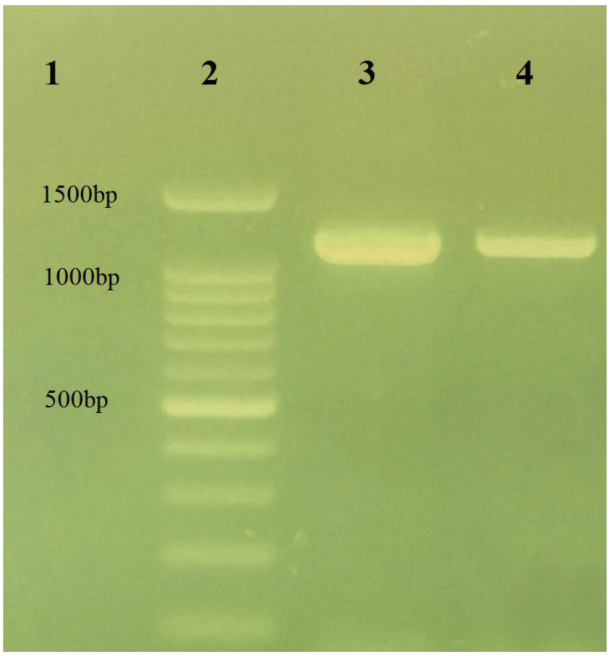

Figure. PCR product of *aroA* gene of *S. aureus*. Molecular marker of lane 1: negative control; lane 2: 100 bp ladder; lane 3: positive control; lane 4: local *S. aureus* isolate.

2.6. Serum lysozyme activity

The activity of lysozyme was measured using the method recommended by Budiño et al. [14]. In the wells of a flat-bottomed microplate, 25 µL of each serum sample were mixed with 75 µL of lysozyme buffer (0.05 M mono- and disodium phosphate, pH = 6.2) and 100 µL of *Micrococcus lysodeikticus* (0.2 mg mL^{-1}; Sigma). The OD_{450} of wells was read after 1 and 30 min. Due to the effect of lysozyme that results in bacterial degradation and reduction of optical absorption, the rate of OD450 reduction was considered as a standard of serum lysozyme activity.

2.7. Bactericidal activity of serum

To measure the bactericidal effect of serum, the method recommended by Budiño et al. [14] was used with some

modifications. A local isolate of *Pasteurella multocida* was cultured at 37 °C in tryptic soya broth (Merck, Germany) for 6 h. The medium was centrifuged and washed twice with PBS, a suspension of 2×10^7 cfu mL^{-1} of *P. multocida* was prepared in TSB medium (using McFarland standard), and 133 µL of the suspension was added into a 96-well plate in two replicates. Also, 133 µL of PBS was considered as the negative control. Moving forward, 33 µL of serum was added into three representative wells and the plate was incubated at 37 °C for 6 h. At the end of the incubation time, 86 µL of 2 mg mL^{-1} of MTT solution was added to all wells and incubated for 15 min at room temperature. Finally, OD_{630} of wells was measured. The percentage of bactericidal activity was calculated according to the following formula:

Bactericidal activity (%) = 1 – (mean OD_{630} of the serum – OD_{630} negative control) × 100

2.8. Serum total protein

To measure the total protein of the serum, a Biuret-based commercial kit (Pars Azmun, Iran) was used. Twenty microliters of the serum sample was mixed with 1000 µL of reagent 1 and 2, and then incubated at 37 °C for 5 min. The OD_{546} of the mixture was read and the total protein of the serum was calculated according to the standard.

2.9. Serum total globulin

The concentration of serum globulin was measured by the method described by McEvan et al. [15] using zinc sulfate turbidity (ZST). To do this, 25 µL of the serum sample or standard was added to 1400 µL of 0.7 M zinc sulfate solution with pH = 5.8. The resulting mixture was shaken well and after 2 h of incubation at room temperature, OD_{590} of the mixture was read and total globulin of the serum was calculated according to the standard.

2.10. Hemagglutination inhibition (HI) test

A beta procedure of microplate HI test was carried out in U-bottomed 96-well microtiter plates to find out the titer of antibodies in sera of different groups. One percent chicken erythrocytes were used in this test. The test used a constant 4 HA unit of NDV and 4 HA unit of AI viruses [13].

2.11. Feed conversion ratio

FCR in each group was calculated using the following formula: % (average feed intake/average weight gain).

The amount of feed that is required for the production of a unit of meat is known as feed conversion ratio (FCR). FCR is a mathematical relationship between the quantities of feed provided (fed) and the weight gained by consuming it. It can be calculated by dividing the total input of the given feed by the total weight gain. Feed conversion ratios are important because they help the farmer to know how much feed will be required in the growth cycle of chickens. This serves as a powerful tool by letting the farmer know what choices he should make in order to maximize the profitability of his business.

2.12. Statistical analysis

All of the measured parameters were introduced to analysis of variance using SPSS version 18.0. One-way ANOVA LSD test was carried out to demonstrate the significant differences in each group. Means were compared at a significance level of 5%.

3. Results

3.1. Feed conversion rate (FCR)

At 1–21 and 21–35 days of age, there was a significant difference between the mean conversion rates of group B and group F chicks; the mean conversion rate in group B (receiving 50 µg of ECP) was less than group F (control). There was no significant difference among the rest of the groups in these two periods (Table 2).

3.2. The effect of ECP on the serum complements activity

The mean ± standard error of the mean (SEM) of complements activity in different groups is shown in Table 3.

Comparing the mean complements activity in different groups after 14 days of vaccination, there was a significant difference between groups A, B, C, and D and group F. There was also a significant difference between groups C and E. It should be noted that group C had the greatest impact on the level of complements activity. Also, there was a significant difference between groups C and E 21 days after vaccination, and also between groups C and F. It should be noted that in this blood sampling period, group C, among all groups, had the highest effect on complements activity ($P < 0.05$) (Table 3).

3.3. The effect of ECP on the serum lysozyme content

The mean ± standard error of the mean (SEM) of lysozyme activity in different groups is shown in Table 4.

A comparison of mean serum lysozyme levels in chicks 14 days after vaccination showed that groups A, B, C, and E had a significant difference compared to groups D and F. Lysozyme levels were higher in these groups compared with groups D and F. It should be noted that group E had the highest amount of lysozyme ($P < 0.05$). The mean serum lysozyme at 21 days after vaccination showed no significant difference among all groups (Table 4).

3.4. The effect of ECP on serum bactericidal activity

The mean ± standard error of the mean (SEM) of the bactericidal activity of the serum in different groups is shown in Table 5.

A comparison of mean serum bactericidal power 14 and 21 days after vaccination showed that there was no significant difference among all groups (Table 5).

3.5. The effect of ECP on serum total protein

The mean ± standard error of the mean (SEM) of total protein in different groups is shown in Table 6.

Table 2. The mean ± standard error of the mean (SEM) of feed conversion ratio.

Group \ Day	1–21	21–35
A	1.46 ± 0.057 [ab]	1.77 ± 0.057 [ab]
B	1.43 ± 0.055 [a]	1.71 ± 0.057 [a]
C	1.48 ± 0.05 [ab]	1.78 ± 0.15 [ab]
D	1.46 ± 0.04 [ab]	1.76 ± 0.11 [ab]
E	1.46 ± 0.057 [ab]	1.77 ± 0.06 [ab]
F	1.53 ± 0.057 [b]	1.85 ± 0.1 [b]

The columns which have no common superscripts are significantly different (P < 0.05).

Table 3. The effect of *Staphylococcus aureus* extracellular products on serum complements in broiler chicks (light density caused by rabbit RBC lysis).

Group	Days after first vaccination		
	0	14	21
A	0.085 ± 0.004	0.179 ± 0.021 [ab]	0.195 ± 0.005 [ab]
B		0.187 ± 0.016 [ab]	0.198 ± 0.012 [ab]
C		0.193 ± 0.039 [a]	0.212 ± 0.014 [a]
D		0.182 ± 0.023 [ab]	0.194 ± 0.034 [ab]
E		0.162 ± 0.024 [bc]	0.180 ± 0.041 [b]
F		0.152 ± 0.031 [c]	0.182 ± 0.037 [b]

The columns which have no common superscripts are significantly different (P < 0.05).

A comparison of total protein in different groups showed that there was a significant difference between groups D, E, and F in 14 days after vaccination and that group D had the lowest amount of total protein among all groups. It should be noted that no significant difference was observed between any of the groups 21 days after vaccination (Table 6).

3.6. The effect of ECP on serum globulin

The mean ± standard error of the mean (SEM) of total protein in different groups is presented in Table 7.

A comparison of mean serum globulin in different groups showed that there was no significant difference in any of the groups 14 days after vaccination. Twenty-one days after vaccination, group E had a significant difference compared to group F. The lowest amount of globulin belonged to group E (Table 7).

Table 4. The effect of *Staphylococcus aureus* extracellular products on serum lysozyme in broiler chicks (reduced light density due to micrococcus lysis).

Group	Days after first vaccination		
	0	14	21
A	0.027 ± 0.005	0.037 ± 0.007 [a]	0.037 ± 0.005
B		0.037 ± 0.008 [a]	0.036 ± 0.02
C		0.038 ± 0.01 [a]	0.034 ± 0.013
D		0.028 ± 0.005 [b]	0.035 ± 0.008
E		0.039 ± 0.005 [a]	0.035 ± 0.007
F		0.027 ± 0.005 [b]	0.04 ± 0.015

The columns which have no common superscripts are significantly different (P < 0.05).

Table 5. The effect of *Staphylococcus aureus* extracellular products on serum bactericidal power in broiler chicks (%).

Group	Days after first vaccination		
	0	14	21
A	50.26 ± 5.7	54.5 ± 1.5	50.93 ± 5.01
B		49.83 ± 1.2	54.2 ± 2.05
C		49.81 ± 4	53.63 ± 1.1
D		54.43 ± 8.3	53.15 ± 10.03
E		53.5 ± 6.1	54.1 ± 2.49
F		49.45 ± 1.6	51.93 ± 3.34

The columns which have no common superscripts are significantly different (P < 0.05).

3.7. The effect of ECP on the ND virus titer

The mean ± standard error of the mean (SEM) of anti-NDV titer in different groups is shown in Table 8.

A comparison of the mean ND antibody titer in different groups of each age suggests that there is a significant difference among all vaccinated groups (A, B, C, and D) and unvaccinated groups (E and F) 14 and 21 days after vaccination (P < 0.05). Also, there was a significant difference between groups B and D 14 and 21 days after vaccination, so the mean antibody titer in group B (receiving 50 μg of ECP) was higher than in group D (vaccine only recipient). It should be noted that there was no significant difference among the groups receiving the different doses of ECP, but the mean antibody titer in group B was higher than in groups A and C (Table 8).

Table 6. The effect of *Staphylococcus aureus* extracellular products on serum total protein in broiler chicks (g/dL).

Group	Days after first vaccination		
	0	14	21
A	4.18 ± 0.26	4.93 ± 0.45 [ab]	5.42 ± 0.7
B		5 ± 0.87 [ab]	5.51 ± 0.54
C		5 ± 0.39 [ab]	5.13 ± 0.96
D		4.5 ± 0.26 [b]	5.25 ± 0.61
E		5.41 ± 0.93 [a]	5.45 ± 0.55
F		5.51 ± 0.74 [a]	5.43 ± 0.89

The columns which have no common superscripts are significantly different (P < 0.05).

Table 7. The effect of *Staphylococcus aureus* extracellular products on serum globulin in broiler chicks (light absorption increments at 590 nm).

Group	Days after first vaccination		
	0	14	21
A	0.171 ± 0.032	0.28 ± 0.031	0.278 ± 0.078 [ab]
B		0.284 ± 0.023	0.267 ± 0.051 [ab]
C		0.254 ± 0.044	0.267 ± 0.045 [ab]
D		0.252 ± 0.039	0.264 ± 0.053 [ab]
E		0.274 ± 0.035	0.225± 0.026 [b]
F		0.256 ± 0.019	0.291 ± 0.018 [a]

The columns which have no common superscripts are significantly different (P < 0.05).

3.8. The effect of ECP on the influenza virus titer

The mean ± standard error of the mean (SEM) of anti-AI viruses titer in different groups is presented in Table 9.

A comparison of the mean AI antibody titer suggests that there is a significant difference between all vaccinated groups (A, B, C, and D) and unvaccinated groups (E and F) at 14 and 21 days after vaccination (P < 0.05). Also, there was a significant difference between groups B and D at 14 and 21 days after vaccination, so the mean antibody titer in group B (receiving 50 μg of ECP) was higher than in group D (vaccine only recipient). It should be noted that there was no significant difference among the groups receiving the different doses of ECP, but the mean antibody titer in group B was higher than in groups A and C (Table 9).

4. Discussion

It is important to improve the immune system of poultry for reasons such as vaccination failure, immunosuppressive diseases, and antibiotics misuse. Research on materials that are likely to have reinforcing effects on the immune system has been increasing. In the case of birds, what could have been achieved through a combination of measures such as vaccination and the use of conventional chemical drugs has almost reached its utmost level. In order to study the effect of reinforcing or even attenuating immunity, which may be present in a product, there are generally limited methods that may be considered as the standard method for measuring the influence of various factors on the function of the immune system. At this point, the study of the efficacy of humoral immunity is more common than cell-mediated immunity studies. The effect of a substance on humoral immune function is also commonly assessed by measuring its effect on the antibody response produced

Table 8. The effect of *Staphylococcus aureus* extracellular products on HI antibody titer against Newcastle disease vaccine (log 2).

Group	Days after first vaccination			
	Maternal ab titer	0	14	21
A	5.7 ± 0.94	3.13 ± 0.75	6.73 ± 0.94 [ab]	7.15 ± 0.83 [ab]
B			7.26 ± 0.75 [a]	7.95 ± 0.51 [a]
C			6.83 ± 0.94 [ab]	7.25 ± 0.75 [ab]
D			6.16 ± 0.98 [b]	6.9 ± 0.75 [b]
E			1.4 ± 1.5 [c]	-[c]
F			1.48 ± 0.64 [d]	-[d]

The columns which have no common superscripts are significantly different (P < 0.05).
* Mean of antibody titer according to log 2 ± standard deviation

Table 9. The effect of *Staphylococcus aureus* extracellular products on HI antibody titer against AI vaccine (log 2).

Group	Days after first vaccination			
	Maternal ab titer	0	14	21
A	5.04 ± 0.05	3.06 ± 0.2	3.2 ± 0.4 [ab]	4.6 ± 0.3 [ab]
B			3.9 ± 0.2 [a]	4.97 ± 0.5 [a]
C			3.3 ± 0.5 [ab]	4.7 ± 0.52 [ab]
D			2.9 ± 0.45 [b]	3.92 ± 0.2 [b]
E			1.3 ± 0.63 [c]	-[c]
F			1.1 ± 0.52 [d]	-[d]

The columns which have no common superscripts are significantly different (P < 0.05).
* Mean of antibody titer according to log 2 ± standard deviation

in the body following vaccination, so the mentioned method can be considered as a standard method for this purpose.

Jeon et al. stated that IL-6 gene expression in spleen cells was induced in chickens immunized with the B subunit of the enterotoxin. They reported that per os administration of this adjuvant increases immune responses against poultry typhoid [16].

Nandre et al. also found that when the B subunit of *Escherichia coli* enterotoxin is used as a vaccine adjuvant it can reduce the contamination of eggs with *Salmonella enteritidis* [17].

The Department of Immunology and Molecular Biology of the US Army Medical Research Institute in 2013 stated that staphylococcal exotoxins, staphylococcal A-E enterotoxins (SEA-SEE), and toxic shock syndrome toxin 1 (TSST-1) were immune system activators. These toxins are called superantigen due to their ability to activate T lymphocytes and produce cytokines. These toxins cause T cell proliferation [18].

Hu et al. [7] examined the effect of intracutaneous injection of *E. coli* enterotoxin on enhancing humoral and cellular immunity in 8–12-week-old mice and reported that the compound could enhance innate immunity, humoral immunity, and cellular immunity in mice.

In 1985, Platsoucas et al. reported a significant increase in natural killer cells and antibody-dependent cellular cytotoxicity[1] after treatment with *Staphylococcus aureus* protein A. They stated that this increase was associated with an increased IFN-γ level [19]. These results are consistent with the results of the present study in terms of the positive effect of SECP on some of the innate immune indices.

In 2002, Ami et al. showed that Staphylococcus enterotoxin A (SEA) stimulates IFN-γ production in natural killer cells through IL-12. In addition, SEA induces regulatory CD8 +, producing a massive amount of IFN-γ, which has potent antitumor cell cytotoxicity compared to CD4 +. It has also been shown that 3 h after treatment with SEA, the number of natural killer cells increased, but no increment was observed in levels of IFN-γ and IL-2 [20]. These results are in agreement with the results of the present study in terms of the positive effect of SECP on some of the innate immune indices.

Lavelle et al. [21] have shown that the cholera toxin is a potent mucosal adjuvant, which is also considered to be a stimulant and antiinflammatory factor. This toxin improves the activity of Th2 and induces an increase in the IL-10-producing regulatory T cells. Also, laboratory studies with macrophages and dendritic cells showed that the cholera toxin increased IL-10, IL-6, and IL-1β levels in the presence of LPS-restricting doses. These results are consistent with the results of this study in terms of the positive effect of SECP on some of the innate immune indices.

In 2010, Ortega et al. concluded that superantigenic toxins of *Staphylococcus aureus* stimulate immunity in primates and rodents by increasing cytokine levels. To reduce toxicity and enhance the superantigenic activity, some mutated strains of staphylococcal enterotoxins, such as mSEA and mSEC2, have been produced for treatment against tumor and infection [22].

Stich et al. proved that activating T cells is the first step in the inflammatory or toxic processes after exposing rabbits to Staphylococcal superantigens. They reported that the cells activated by superantigens migrate from the circulation to organs such as the liver and spleen. IL-2 gene expression after the challenge with TSST-1 was diagnosed mainly in the spleen. T cells in the spleen were also identified as the primary source of TNF-α in response to SEB in galactosamine-sensitive mice. They also showed that the induction of proinflammatory cytokines, including IFN-γ, IL–1β, TNF-α, and especially IL-6 after placing rabbits exposed to TSST-1, was greater than the results observed after induction by a single toxin. These cytokines are mainly produced by macrophages and dendritic cells. Their results clearly show that the early signal produced by T cells activates IL-6 expression by macrophages and dendritic cells. There has also been a slight increase in TLR-4 expression after TSST-1 administration in rabbits [23].

The present study showed that the simultaneous use of 100 μg of SECP and vaccines 14 and 21 days after vaccination had a synergistic effect on the amount of complement activity and it is recommended that the vaccine plus 100 μg of SECP be administered together to stimulate the complement system. The present study also showed that the extracellular products of *S. aureus* at 50 μg could increase the immune response against AI and ND virus and could improve FCR. Moreover, receiving different doses of SECP 14 days after vaccination, with or without the vaccine, significantly increased lysozyme activity.

Besides, ECP administration had no positive effect on serum bactericidal power, total protein, and serum globulin levels.

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Conflict of interest
No potential conflict of interest was reported by the authors.

[1] ADCC

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