Using T cell lymphokines of hyperimmunized chickens with Salmonella pullorum to enhance immune response of layer hens against avian influenza

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**Abstract**

The current study used immune lymphokines from chickens that had been hyperimmunized with Salmonella pullorum to improve the resistance of layer hens at the production stage to AIV-type (H5N1) infected hens (SP). Two groups (each consisting of 25 pullets) were treated; the first group received three doses of SP vaccination at 12, 14, and 16 weeks; the second group received no vaccination and was used as a control group. At 18 weeks, the T cells of the first group released immune lymphokines (S-ILK), whereas the T cells of the second group produced non-immune lymphokines (N-ILK). The following procedures were then used on a total of 100-layer hens (ISSA brown), divided into four groups of 25 each. G1: S-ILK treatment and H5N1 challenge. G2: N-ILK treatment and H5N1 challenge. G3: Untreated and challenged to (H5N1). G4: untreated or unchallenged. Blood samples were obtained at 31, 32, and 33 weeks of age to assess the (IgG, IFN-γ). Additionally, lung and tracheal tissues were obtained to assess the viral load of influenza RNA copies at (7 and 14) days following the challenge. The experiment's findings indicated that the first group produced the highest mean (IgG and IFN-γ) titers and had the lowest mortality of the other groups. The findings of the viral load test showed that G2 and G3 had the highest number of influenza RNA copies and had significantly lower egg production than G1, which had the lowest viral load and kept egg production at a normal level. The conclusion of the current study shows that using SILK gives layer hens a higher level of homogeneous protection against AIV without any dangers during production.

**Keywords**: H5N1, ISSA brown, Viral load, IgG, IFN-γ

Introduction

Avian influenza (AI) is a significant zoonotic disease that represents a major hazard to both the general public's health and the poultry industry. In 1996, China initially reported an outbreak of the highly pathogenic (HP) H5N1, which later spread to other regions of the world (1). All ages of broiler and layer chickens are susceptible to infection, and the variable mortality rate varies from 30 to 100% depending on the virulence of the virus (2). AIV is one of the viruses with a high potential for devastation in commercial chicken production, which results in massive economic losses, especially in breeder and layer flocks (3). Depending on the strains, hosts, and stages of infection, cytokines can have varying beneficial effects on the pathogenesis of the influenza virus (HPAI) (4,5). Studies on influenza-infected mice showed an increased inflammatory response and dysregulated cytokines, which led to a high mortality rate (6). In order to decrease viral infection and death, the modulation of cytokine production from phagocytic cells promoted the high and rapid proliferation of T cells and natural killer cells. Additionally, other research on mice...
infected with influenza H5HPAI discovered significant mortality as a result of mouse interleukin (IL-6) and TNF deficiency (7,8). Due to the host's depressed immune system and the lack of host-virus interaction, the infection manifests very quickly, the rapid spread of the virus is caused by H5N1 HPAI infection in birds with low immunological response, as confirmed by Kuribayashi et al. (9). Because the killed vaccinations were slow to produce total protection and the influenza virus occasionally underwent genetic mutations, vaccination with a killed vaccine at a young age to prevent HPAI infections did not function unless there was an improvement in the primary response prior to infection (10). By boosting a number of immune cytokines, which are regulatory proteins produced by some immune cells in order to defend the body against infections, the use of lymphokines from Salmonella Typhimurium- immune lymphokines SILK improved the immune response against the ND challenge without the need for a vaccine (11). Mushtaq et al. (12) confirm that the use of SILK in the early stages of the disease activated the immune response to protect the chicks from infectious bronchitis disease in response to the challenge of local isolation of the disease. Mushtaq, (13) also demonstrated the function of SILK in enhancing maternal immunity against avian influenza infection at one day of age.

The goal of this study was to demonstrate the beneficial effects of SILK on layer hens' cellular and humoral immunological tolerance to HPAI (H5N1) during the production stage.

Materials and methods

Ethical approve

This work approved by College of Veterinary Medicine, University of Baghdad under the code 164 dated 1/12/2021.

Prepared lymphokines

A Salmonella pullorum (OM988162.1) isolate was obtained from the University of Baghdad's Department of Diseases and Poultry Diseases-College of Veterinary Medicine. The isolate was cultivated in nutritional broth and peptone water, and it was then incubated at 37 ºC for 24 hours until turbines and a small amount of white sediment were visible. Then, a sediment sample was cultured on Salmonella-selective media such as SS agar and Macconkey agar. The LD₃₀ of the isolate was determined to be exactly 5x10⁵ bacteria per bird at a dilution of 10⁻², which causes 50% mortality. Then, at the fields of Al-Rashidiya, two groups, each with 25 pullets, were grown. At 12, 14, and 16 weeks, the first group received three doses 1x10⁸ from the same isolate (OM988162.1); the second group received no shots and served as the control group. In contrast to the T cells in the second group, which produced non-immune lymphokines, the first group's T cells released immune lymphokines (SILK) at the 18-week mark Non immune lymphokines (NILK). After the infected spleens were crushed, T-cells were extracted from the infected bird spleens by centrifugation at 1500 rpm. Then, Concavalin-A was added to T-cell cultures on RBM media to promote the release of lymphokines. Centrifugation of the filtrate at 3000 rpm was used to separate the lymphokines (14).

Prepared the viral inoculum

The department of pathology and poultry diseases-college of veterinary medicine-University of Baghdad had an unpublished isolate of the H5N1 influenza virus (allantoic fluid). The ELD₅₀ was determined to be 10⁹⁵ by a hemagglutination test in embryonated chicken eggs, which was then utilized to challenge in 100ELD₅₀ 10⁵ using Reed and Muench's approach (15). The sample was kept until usage in a deep freezer at -80ºC.

Experimental Approach

100-layer hens (ISSA brown) 30 weeks old, divided into 4 groups (25 each group) G1: intraperitoneal injection with SILK, then intranasal challenge with H5N1 after 5hr from injection with SILK. G2: intraperitoneal injection with NILK, then intranasal challenge with H5N1 after 5hr from injection with NILK. G3: Untreated but H5N1 challenge. G4: untreated and unchallenged. Following the challenge all the clinical symptoms, egg production, pathological changes, and mortality rates were recorded.

Sampling

At 31, 32, and 33 weeks, each group had five blood samples taken from the right jugular vein. The serum was separated from the blood samples using glass tubes free of anticoagulant in a centrifuge for 15 minutes at 1000 rpm. At 2 and 4 days after the challenge, each group received five samples of lung and tracheal tissues, which were kept in a deep freezer. The HPAI RNA copies were calculated using RT-PCR (H5N1).

Mortalities and morbidities

Clinical symptoms for the birds that survived the peracute infection were recorded throughout the experiment. Coughing, sneezing, whistling, and rales were all noted as respiratory symptoms. Other symptoms included torticollis, opisthotonos, paralysis, lack of coordination, and drooping wings. They measured the proportion of eggs produced before and after the challenge. During the experimental period, pathogenic lesions were noted, including skin lesions such as facial swelling, cyanosis in the comb and wattles, edema, and red discoloration of the legs and feet due to subcutaneous ecchymotic bleeding; visceral lesions such as petechial hemorrhage in internal organs and muscles; nose and mouth colored with blood; and acute infections such as green diarrhea.
Real time (RT-PCR)
According to Sun et al., the number of RNA copies of the influenza virus HPAI (H5N1) in lung tissues was determined using this assay, and the findings were examined (16). Utilizing the TRIzol reagent, RNA was isolated from the infected birds’ lung tissue (Invitrogen, Carlsbad, CA, USA). For the detection of influenza viruses, specialized primers forward: 5’-AAG CCG AGA TCG CAC AGA AAC TTG AAG ATG TCT TT GC-3’. Reverse: 5’-GCA AAG ACA TCT TCA AGT TTC TGT GCG ATC GCT GCTT-3’. and TaqMan (17) probes were used to detect AIV RNA. A real-time PCR test was performed using a single-step Prime Script RT-PCR kit and the LightCycler® 480 real-time PCR system from Roche Diagnostic Deutschland GMBH, Mannheim, Germany (16).

ELISA test
The ELISA test was performed to identify IgG and chicken IFN-γ against H5N1 in the serum of infected birds; the ELISA kit used was produced by the company ProFlock AIV and (SunLong Biotech).

Analytical statistics
The dataset was examined using the Statistical Analysis System (SAS). By using the least significant difference (LSD) test, the means were separated. The P<0.05 was used to determine statistical significance.

Results

Humoral and cellular immunity against AIV
Ten hens were randomly selected and divided into groups at the age of 30 weeks in order to evaluate the humoral immunity IgG and IFN-γ in the serum. 2764.3±365.2 and 21.4±2.1 were the outcomes, respectively. The study illustrated how SILK enhances the immune response to the H5N1 challenge. IgG and IFN-γ titers against AIV differ considerably at the level at all times, as demonstrated in (Tables 1 and 2) G1 had the highest mean for IgG and IFN-γ titer without mortality, whereas G2 and G3 had the highest IgG and IFN-γ titer with significant mortality at 33 weeks. The IgG and IFN-γ against AIV significantly decreased in the control group compared to G4, however.

Table 1: Statistics are used to describe the IgG titer against AIV

<table>
<thead>
<tr>
<th>Groups</th>
<th>31 weeks</th>
<th>32 weeks</th>
<th>33 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>5632.4±334.2 A</td>
<td>8764.2±522 A</td>
<td>10455.7±897 B</td>
</tr>
<tr>
<td>G2</td>
<td>1022±121.7 C</td>
<td>675±32.3 C</td>
<td>19437.2±999.2 A</td>
</tr>
<tr>
<td>G3</td>
<td>1134.2±122 C</td>
<td>876±30.4 C</td>
<td>20768±1243 A</td>
</tr>
<tr>
<td>G4</td>
<td>2234.5±322 B</td>
<td>1922±476.1 B</td>
<td>1640.6±154 C</td>
</tr>
<tr>
<td>LSD</td>
<td>488.14</td>
<td>372.8</td>
<td>876.6</td>
</tr>
</tbody>
</table>

Five samples were collected. Capital letters denote a significant difference at the level at P≤0.05.

Table 2: Statistics are used to describe the IFN-γ titer against AIV

<table>
<thead>
<tr>
<th>Groups</th>
<th>31 weeks</th>
<th>32 weeks</th>
<th>33 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>38.4±11.2 A</td>
<td>53.5±10.2 A</td>
<td>78.4±21.3 B</td>
</tr>
<tr>
<td>G2</td>
<td>8.8±2 C</td>
<td>12.4±3.1 C</td>
<td>85.2±23.6 A</td>
</tr>
<tr>
<td>G3</td>
<td>7.6±1.8 C</td>
<td>16.2±3.1 C</td>
<td>87.1±28.6 A</td>
</tr>
<tr>
<td>G4</td>
<td>20.2±4.7 B</td>
<td>18.7±5.3 B</td>
<td>14.5±2.7 C</td>
</tr>
<tr>
<td>LSD</td>
<td>7.3</td>
<td>22.4</td>
<td>19.5</td>
</tr>
</tbody>
</table>

Five samples were collected. Capital letters denote a significant difference at the level at P≤0.05.

Viral load results
Table 3 lists the findings of the RT-PCR used to measure viral load following the challenge with H5N1 G3 and G2 exhibited a marginally significant increase in the number of AIV RNA copies in the trachea and lung tissues at 7 days PI in comparison with G1, which recorded the lowest number. However, by 14 days, G3 and G2 showed a more noticeable increase in the number of AIV RNA copies, whereas G1 showed the lowest number.

Mortalities and morbidities
G3 and G2 had the highest prevalence of clinical symptoms in all infected birds and the highest rates of morbidity 84 and 80%, respectively, whereas G1 had the lowest incidence of clinical signs and the lowest rates of death 76 and 68% in birds exposed to H5N1 (Table 4).

Table 3: RT-PCR analysis of H5N1 RNA copies in tracheal and lung tissues

<table>
<thead>
<tr>
<th>Groups</th>
<th>7 days PI</th>
<th>14 days PI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trachea</td>
<td>Lung</td>
</tr>
<tr>
<td>G1</td>
<td>342±9.2 B</td>
<td>232.3±90.2 B</td>
</tr>
<tr>
<td>G2</td>
<td>1765±245 A</td>
<td>1123.5±123.1 A</td>
</tr>
<tr>
<td>G3</td>
<td>1567.2±380 A</td>
<td>1342±312 A</td>
</tr>
<tr>
<td>G4</td>
<td>0±0 C</td>
<td>0±0 C</td>
</tr>
<tr>
<td>LSD</td>
<td>723</td>
<td>451.7</td>
</tr>
</tbody>
</table>

Five samples were collected. Capital letters denote a significant difference at the level at P≤0.05.
That would suggest S. Pullorum stimulates the Th1 immune system between 7 and 35 days after being ingested. The findings are in accordance with He et al. (27), who report that activated lymphocytes induce the production of IL-18, which influenced by adherent macrophage cells, and attach to lymphocytes on day 7 after infection and release along with them into the cell medium. According to Asif et al. (28), a number of lymphokines with antiviral capabilities against a variety of viral diseases as well as an immunomodulatory impact have been discovered in recent investigations into avian lymphokines the first type of interferon IFN-α, is produced by cells after viral infections and inhibits the localization of the virus by preventing its reproduction. Layton et al. (29) also noted the significance of IFN-γ in preventing the HPAI H5N1 infection, which is produced by CD4+ T cell and natural killer cells, stimulates and multiplies CD8+ T cells and cytotoxic T lymphocytes (CTL) to direct interaction with the antigen-presenting cell is necessary for the effector T lymphocyte to activate its cytolytic activity (21). The S-ILK-treated and H5N1 HPAI-challenged G1 groups had the least amount of virus these findings support Mustaq (13) findings that the G1 group of broilers treated with SILK, had the lowest viral proliferation at 7, 14, 21 and 28 days after H5N8 exposure. Gonzales and Elbers (30) noted that the losses from lower egg production have been encountered as a result of AIV infections, our not their findings that there has been a drop in egg production with significant mortality in G2 and G3 are consistent with their findings. An AI epidemic that affected three nearby laying farms led to a large drop in egg production (from 13 to 80%) and a total death rate of 69%, but had no impact on G1 egg production. These findings of mortality and production are consistent with previous research (31,32). Current study confirmed that SILK’s significant contribution to lowering the systemic viral load of the H5N1 virus in infected tracheal and lung tissues.

Discussion

The present work demonstrated the biological function of lymphokines obtained from hyperimmunized chickens with Salmonella pullorum in supplying very powerful and efficient protection against the challenge of HPAI at the production stage. According to reports, creating recombinant live vaccines with attenuated Salmonella not only boosts the immune system’s capacity to fight against Salmonella infection but also considerably improves the immunological impact of the exogenous gene carried by the vaccine (18,19). According to these findings, NDV and AIV vaccinations have a high protective impact against S. pullorum infections (20). These findings are consistent with those of Baumgarth et al. (21), who showed that the efficient response of Th2 cells to cytokines generated by activated phagocytic cells in mice aids in reducing viral growth in lung tissue. Similar to this, Mass et al. (22) revealed the crucial significance of IL-4 to decrease the HPAI in comparison with cellular immunity (IFN-γ), which is shown by the considerable reduction in viral shedding, despite the high pathogenicity and multiplication of the virus. The results were in line with those reported by Ke Ding et al. (20), who showed that lymphocytes play a role in controlling the host immune response and signaling between protective cells through the production of cytokines, which are soluble particles produced by many cells, including T, B, macrophages, and dendritic cells. S. Pullorum causes a higher Th2-like response than the Th1-type response, that means crucial particularly cellular immune response for the clearance of S. Pullorum post infection (23). The cellular immune response was investigated by using a lymphocyte proliferation test. Additionally, cytokines actively control immune cells throughout development and homeostasis, innate or acquired responses, as well as both (24). Furthermore, highly virulent H5N1, could induce high levels of IFN. As showed in present study table 2, as noted by Moulin et al. (25). After being exposed to HPAI H5N1 infection, these cells aggregation inhibits the spread of the virus and influences and shapes the humoral immune response. Activated macrophages release IL-18, an inflammatory cytokine with IFN-inducing properties (Th1 immunological mechanism) S. Pullorum increased IL-10 levels (26).

Table 4: The mortality, morbidity and egg production rates after the H5N1 challenge after 10 days

<table>
<thead>
<tr>
<th>Groups</th>
<th>Morbidity</th>
<th>Mortality</th>
<th>Eggs production</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>32% (8) d</td>
<td>12% (3) d</td>
<td>80% (20) d</td>
</tr>
<tr>
<td>G2</td>
<td>80% (20) d</td>
<td>68% (17) d</td>
<td>16% (4) d</td>
</tr>
<tr>
<td>G3</td>
<td>84% (21) d</td>
<td>76% (19) d</td>
<td>12% (3) d</td>
</tr>
<tr>
<td>G4</td>
<td>0% (0) d</td>
<td>0% (0) d</td>
<td>88% (22) d</td>
</tr>
</tbody>
</table>

Five samples were collected. Capital letters denote a significant difference at the level at P≤0.05.

Acknowledgments

In addition to thanking everyone who helped me in any way throughout the project's completion, I extend my greetings and blessings to the Al-Rashidiya farm for providing all my research needs.
Conflict of interest

With regard to scientific and practical interests, there is no disagreement or conflict; rather, scientific reality is associated with fresh scientific endeavors and concepts.

Reference

7. Salomon R, Hoffmann E, Webster RG. Inhibition of the cytokine response does not protect against lethal H5N1 influenza infection. Proc Natl Acad Sci. 2007;104:12479-12481. DOI: 10.1073/pnas.0702589104
استعمال ليمفوكينات الخلايا التائية للدجاج المعزز مناعيا بالسالمونيلا بللورم لتنشيط الاستجابة المناعية للدجاج البياض ضد إنفلونزا الطيور

مشتاق طالب الزهيري
فرع الأمراض وأمراض الدواجن، كلية الطب البيطري، جامعة بغداد، بغداد، العراق

الخلاصة


الملفوكينات المناعية والليمفوكينات غير المناعية تم تجميعها من خلال إعطاء الجرعات الثلاثة خلال الأسبوع 12 و 14 و 16. ودخنت كل مجموعة، وتم قم بتحليل نتائج الدراسة من خلال قياس مستويات كلوبيلين ج والإنترفيرون كام في الدم. وتم استخدام نسخة الحمض النووي الريبي للإنفلونزا بعد 7 و 14 يوم من الإصابة.

النتائج: أظهرت النتائج أن المجموعة الأولى أنتجت أفضل متوسط معين للكلوبيلين المناعي والإنترفيرون كام. واستنتجت الدراسة أن استخدام الليمفوكينات المناعية يعطي الدجاج البياض مستوى أعلى من الحماية المتجانسة ضد الإنفلونزا بدون أي آثار جانبية أثناء الإنتاج.