Scientific Report

Cytokine Immune Response Following Vaccination against Fowl Pox Disease in Specific Pathogen Free Chickens

Mohammad Reza Azizi[1*], Ismail Asli[2]

1. Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
2. Brucellosis Vaccine Production Department, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Exxtension Organization, Karaj, Iran.

How to cite this article: **Mohammad Reza Azizi, Ismail Asli. Cytokine Immune Response Following Vaccination against Fowl Pox Disease in Specific Pathogen Free Chickens. Archives of Razi Institute. 2025;80(2):525-533.** DOI: 10.32592/ARI.2025.80.2.525

Article Info:
Received: 18 November 2024
Accepted: 1 December 2024
Published: 30 April 2025

Corresponding Author's E-Mail:
mohammadrezaaziz@gmail.com

ABSTRACT

Fowlpox is an infectious disease with a relatively slow spread in all ages of poultry. It is characterized by skin lesions in dry form or diphtheria-like lesions in the mouth in wet form. The disease has been observed to result in diminished growth rates and a reduction in egg production. This disease is among the most ancient diseases known to affect poultry. The virulence of the bacterium was initially demonstrated in 1902 by Marks & Sticher. The disease is widespread on a global scale, affecting birds of all ages, races, and genders. The virus is highly infectious and causes significant mortality in birds. The present study was conducted to investigate the presence of Th1 and Th2 cells, as well as the cytokines IFN-γ and IL-4, in the culture medium of peripheral blood mononuclear cells (PBMCs) stimulated with the mitogen concanavalin A (ConA) in vaccinated and control chickens. The present study comprised three groups of 40 21-day-old specific pathogen-free chicks. One group was inoculated with phosphate-buffered saline (PBS) and served as a negative control, while the other two groups were vaccinated with the Razi Institute fowlpox vaccine and a commercial fowlpox vaccine, respectively. The injection of the vaccine was administered in the wing. The chicks were maintained for a period of five weeks following the inoculation procedure. Blood samples were collected from each group on a weekly basis, continuing until the fifth week following the vaccination. Peripheral blood mononuclear cells (PBMCs) were isolated from each blood sample using a centrifuge Ficoll-Hypaque protocol. The concentration of inflammatory cytokines (IFN-γ) and anti-inflammatory (IL-4) in peripheral blood mononuclear cells was measured using the enzyme-linked immunosorbent assay (ELISA) method. A statistical analysis and a quantitative evaluation ($P<0.05$) were employed to ascertain the significant differences between the groups. The results of this study demonstrated that 7 days after vaccination, 90 to 100% of the vaccinated birds exhibited swelling at the injection site. The ratio of the concentration of IFN-γ to IL-4 in the culture medium of vaccinated chicks was higher than that of the control group. The study posits that the induction of enhanced immune responses subsequent to vaccination against fowlpox is predominantly attributable to the Th1 lymphocytes response.

Keywords: Fowl Pox Vaccine; T Lymphocytes; SPF Chickens; PBMC

1. Introduction

Fowl pox is a prevalent disease among unvaccinated backyard chickens. The disease is generally self-limiting. The initial lesions of this disease manifest as a white blister on the shoulder and other areas of the skin. In rare cases, lesions may also be observed on the body, legs, and softer parts of the beak. These blisters subsequently evolve into a dark scab, with a healing period of approximately three weeks. The presence of chickenpox lesions in the buccal and pharyngeal regions of infected birds can result in respiratory distress and even fatality. To mitigate this disease, it is recommended that avian owners vaccinate their birds. Additionally, the management of mosquito populations can contribute to the prevention of chickenpox transmission (1). The virus is classified as belonging to the genus Avipoxvirus, which is a member of the family Poxviridae. The viral genome of the fowlpox virus is classified into three temporal classes: immediate, early, and late. Its substantial linear double-stranded DNA genome, measuring approximately 300 kilobases, features a hairpin loop at each terminus and encodes over 200 genes, including those for DNA polymerase, NTPaseI, uracil glycosylase, and thymidine kinase. A substantial body of research has demonstrated notable discrepancies between the field and vaccine strains of the fowlpox virus through molecular analysis.The virus utilizes glycosaminoglycan receptors present on the surface of the host cell to gain entry. Avian poxviruses have the capacity to replicate within the cytoplasm, a property that is analogous to that of other poxviruses. In contrast to numerous other viruses, avian poxviruses possess the capacity to generate extracellular enveloped viruses through a process known as budding, whereby mature virions emerge directly through the plasma membrane (2). The pathogenic mechanisms of fowlpox virus are characterized by its ability to withstand the typical environmental conditions. This enables the virus to persist in dried scabs for extended periods. The presence of photolyase and A-type inclusion body protein genes in the genome of fowlpox virus appears to offer protection against environmental challenges. The incubation period of fowlpox in chickens and turkeys is typically between four and ten days (3). There have been reports of the virus infecting other bird species, including ducks, geese, pheasants, quail, canaries, and hawks. In contrast, mammals are not susceptible to natural infection by the fowlpox virus or any other avipoxviruses. The virus is typically transmitted through contact with the skin abrasions of infected birds. Shed skin lesions (scabs) from recovering birds in poultry houses can serve as a source of aerosol exposure for susceptible birds. Furthermore, mosquitoes and other biting insects may act as mechanical vectors for transmission. In multiple-age poultry complexes, the virus exhibits a propensity to persist for extended periods, a phenomenon attributable to its languid diffusion and the presence of susceptible birds (4, 5). The clinical manifestations of varicella-zoster virus (VZV), more commonly known as chickenpox, exhibit a wide spectrum of presentations. In chickens and turkeys, the disease is characterized by the presence of proliferative lesions on the skin, which eventually develop into thick scabs. This form of the disease is known as the cutaneous form. Furthermore, the presence of lesions in the upper gastrointestinal and respiratory tracts has been observed, a phenomenon referred to as the diphtheritic form. In more severe cases, virulent strains of the virus can cause lesions in the internal organs, resulting in the systemic form. The localization of lesions in the vicinity of the nostrils can result in nasal discharge, while cutaneous lesions on the eyelids may lead to the complete closure of one or both eyes. Gaseous patches that exhibit a strong adherence to the mucosa of the mouth and larynx, or proliferative masses, may form in the diphtheritic form. Mouth lesions have been observed to disrupt feeding patterns, while tracheal lesions have been associated with respiratory distress. Distinguishing between laryngeal and tracheal lesions in chickens and those caused by infectious laryngotracheitis is imperative. It is noteworthy that a single bird may exhibit multiple forms of the disease, including cutaneous, diphtheritic, or systemic manifestations. The disease often progresses slowly within a flock, with an incubation period ranging from two to eight weeks. Severe infection in a layer flock can result in a decline in egg production (6).The diagnosis of cutaneous fowlpox infections is typically made through microscopic examination of affected tissues, which are then stained with hematoxylin and eosin (H&E). Furthermore, the application of fluorescent antibody and immunohistochemical methods is a standard practice in this field. The isolation of the fowlpox virus can be achieved through the inoculation of the chorioallantoic membrane of developing chicken embryos, susceptible birds, or cell cultures of avian origin. Among the available options, chicken embryos from a specific pathogen-free (SPF) flock, aged between 9 and 12 days, are the preferred and most convenient host for virus isolation (7). In light of the fact that the majority of fowlpox outbreaks in previously vaccinated chickens are attributable to strains harboring a genome containing full-length REV, the utilization of REV envelope-specific primers to ascertain the presence of full-length REV is advantageous in such instances. It has been demonstrated that both naturally infected and vaccinated birds elicit humoral and cell-mediated immune responses. The humoral immune response can be assessed through ELISA, agar gel precipitation (AGP), or virus neutralization tests. While AGP is a straightforward and convenient test, its sensitivity is comparatively lower than that of ELISA (8). There is currently no specific effective treatment for birds infected with fowlpox virus; therefore, prevention is key. The most effective method of disease control is through the implementation of vaccination protocols. In regions where fowlpox is endemic, it is strongly recommended that poultry such as chickens and turkeys be vaccinated with a virus vaccine that is propagated using a live-embryo or cell-culture method. The most commonly utilized vaccines are live, attenuated fowlpox virus and

pigeon pox virus isolates, which possess high immunogenicity and low pathogenicity. In regions characterized by a high risk of infection, it is recommended to administer a live, attenuated virus vaccine of cell-culture origin within the initial weeks following hatching. Subsequent revaccination is advised at 12–16 weeks of age. The timing of vaccinations should be based on the health of the birds, the level of exposure, and the type of operation. Given the gradual progression of the infection, vaccination can play a pivotal role in curtailing its spread within affected flocks, particularly when administered prior to the manifestation of lesions in more than 20% of the birds (9). It is imperative to note that passive immunity has the potential to impede the replication of the vaccine virus. Consequently, it is advisable to exercise caution when vaccinating birds from recently vaccinated or infected flocks, and only proceed with vaccination once passive immunity has subsided. Subsequent to the administration of the vaccine, it is imperative to observe avian subjects for any signs of swelling or scab formation at the site of injection for a period of one week. The absence of a vaccine take can be indicative of a number of factors, including the potency of the vaccine, the presence of passive or acquired immunity, or the technique employed during the vaccination. In such cases, revaccination with a different vaccine lot number may be necessary (9). IFN-γ, also known as type II interferon, is a cytokine that plays a vital role in the immune response to viral, bacterial, and protozoan infections. It activates macrophages (a type of immune cell) and helps increase the expression of class II molecules that are essential for immune recognition. Abnormal levels of IFN-γ have been associated with various autoimmune and inflammatory diseases. Its importance in the immune system is attributable to its capacity to directly inhibit viral replication and enhance immune responses. Interferon gamma (IFNG) is primarily produced by natural killer (NK) cells and natural killer T (NKT) cells during the initial immune response. Subsequently, IFNG is produced by CD4 Th1 and CD8 cytotoxic T lymphocyte (CTL) cells as the immune response becomes more specific to the antigen. Levels of type II IFN increase in response to the production of interleukin cytokines, such as IL-12, IL-15, and IL-18, in conjunction with type I interferons (IFN-α and IFN-β). Conversely, IL-4, IL-10, transforming growth factor-beta (TGF-β), and glucocorticoids have been identified as factors that can reduce type II IFN levels. Interleukin 4 (IL4, IL-4) is a cytokine that facilitates the conversion of naive helper T cells (Th0 cells) into Th2 cells. Upon activation by IL-4, Th2 cells proceed to synthesize additional IL-4, thereby initiating a positive feedback cycle. IL-4 is predominantly derived from mast cells, Th2 cells, eosinophils, and basophils. Interleukin 4 (IL-4) plays a number of pivotal roles in the human body. It has been demonstrated to facilitate the proliferation of both activated B cells and T cells, while concomitantly promoting the transformation of B cells into plasma cells. It plays a pivotal role in both the humoral and adaptive immune systems. IL-4 has been observed to promote the transition of B cells towards the production of IgE, while concurrently augmenting the synthesis of MHC class II. Conversely, IL-4 has been observed to impede the proliferation of Th1 cells, macrophages, IFNγ, and dendritic cells IL-12. In vitro experimentation has demonstrated the remarkable efficacy of IL-4 and IL-10 in their capacity to function as potent inhibitors of Th1 effector responses (10). The present study aims to provide a comprehensive definition of the chicken cytokine response to fowl pox vaccination. To this end, two vaccines were utilized in this experiment: Razi and another imported fowl pox vaccine. The level of cytokine expressions was then assessed by ELISA in the PBMC cell culture medium following stimulation with concavalin A.

2. Materials and Methods

In the third week of age, 120 SPF chicks were randomly divided into three groups, with each group comprising 40 individuals. The initial group constituted the target population for the vaccine developed by Razi. The second group received a vaccine that had been imported for use. The third group was inoculated with an equivalent volume of phosphate-buffered saline, thereby establishing a non-vaccinated control group for the study. All chickens that had been vaccinated, as well as those in the control group, were observed for a period of five weeks following the vaccination. At the fifth day after the vaccination, a sign that the vaccine had been taken was noted at the injection site, indicating the accuracy of the vaccination. Refer to Figure 1.

2.1. Blood Samples

Blood samples were obtained from five chicks per group on a weekly basis for a period of five weeks following vaccination. A total of 1 cc of blood was extracted from the heart of each chick under sterile conditions and mixed with EDTA. Subsequently, all samples from each group were pooled.

2.2. Ficoll–Hypaque Density Gradient Centrifugation

A total of 5 cubic centimeters of blood spiked with ethylenediaminetetraacetic acid (EDTA) was meticulously transferred into a Falcon tube. This was followed by the subsequent addition of 5 cubic centimeters of phosphate-buffered saline (PBS). In a separate sterilized falcon tube, 3 cc of ficoll with a density of 1.077 g/ml was poured. The 10-centimeter cubic centimeter of blood mixed with Phosphate Buffered Saline (PBS) was poured onto the Ficoll solution, ensuring that the Ficoll settled at the bottom of the tube and the blood remained on the top. Subsequently, the tubes were subjected to a centrifugal process at a low temperature of 8°C and at a speed of 400g for a duration of 20 minutes. Following the process of centrifugation, four discernible layers were identified, which are designated as follows: plasma, PBMC, ficoll, and blood cells, in sequential order, from the uppermost layer to the lowermost layer. The PBMC layer was extracted with meticulous care. Subsequent to the extraction of the PBMC,

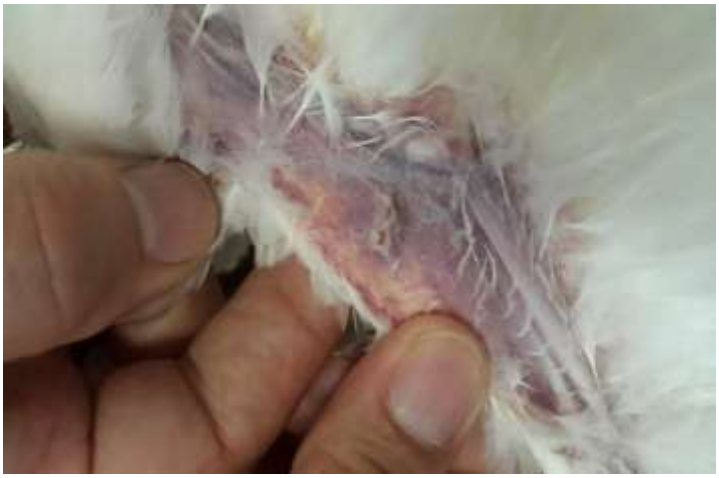

Figure 1. Take is visible after the injection of the vaccine.

the sample was transferred to flask tubes, and the volume was adjusted to 10 cc by the addition of PBS (Figure 2). Subsequently, the samples were subjected to a centrifugal process at 100 g for a duration of 10 minutes, followed by a vortex mixer. This step was repeated, and the resulting fluid containing PBS was discarded. In conclusion, the 2 cc precipitate was subsequently amalgamated with a 3% solution of PBS/BSA.

2.3. Culture of PBMC Cells and Stimulation of Cells with ConA Mitogen

The collected PBMC cells were then subjected to two washes with RPMI 1640 culture medium, devoid of any antibiotics. The assessment of cell viability was conducted by staining the cells with Trypan Blue. In a 12-well microplate, 2 cc of a cell suspension containing 107 line PBMC cells in RPMI 1640 culture medium, inclusive of 10% fetal bovine serum (FBS), was cultured in each well. The plates were then subjected to an incubation period of 24 hours at a temperature of 37°C in an environment containing 5% CO2. A volume of 200 microliters of ConA, with a concentration of 10 micrograms per milliliter, was added to each well. The cells were then incubated for an additional three days. Following a 72-hour period, the cell culture media from each well was extracted by means of a centrifugal process at an angular velocity of 300 g for a duration of 5 minutes. This liquid containing secreted cytokine was stored in a freezer set at -20°C until the time of cytokine measurement.

2.4. Measurement of Cytokines

The level of cytokines produced in the culture medium of peripheral blood mononuclear cells (PBMCs) following stimulation with concanavalin A (ConA) mitogen was measured using an enzyme-linked immunosorbent assay (ELISA) test kit from Biosource. In summary, samples and standards (in different dilutions) were added in separate wells of the plate in duplicate at a volume equal to 100 µl. Following a two-hour incubation period at laboratory temperature, the monoclonal antibody directed against a particular cytokine (interferon gamma or interleukin 4) was introduced into each well, with a volume of 100 µl.

Following the incubation of the plates for a period of one hour, a solution consisting of 100 µL of the conjugate per well was added. Following a 60-minute incubation at laboratory temperature and a five-time wash cycle, 90 µL of the chromogen substrate was added to each well. After 30 minutes, the reaction was halted with 50 µL of a stop solution, with the plates being kept in the dark. Then, the optical density (OD) of the plate was measured in an ELISA reader at a wavelength of 450 nm. The quantity of cytokines present in each specimen was determined by calculating the corresponding values according to the standard curve.

3. Results

The objective of this study was to measure the induction capacity of IFN-γ and IL-4 cytokines in the culture medium of peripheral blood mononuclear cells (PBMCs). The PBMCs were stimulated with ConA mitogen, and the samples were collected from vaccinated and control chickens. The results demonstrated that the IFN-γ concentration of chicken PBMC cell culture media was elevated in comparison to the control group (Figure 3). The investigation revealed that the level of Th1 cytokines, such as IFN-γ, exhibited an increase in the vaccinated group. This finding indicates that Th1 cell activity undergoes an enhancement following vaccination (Figure 2). Furthermore, the concentration of IL-4, predominantly secreted by Th2 cells, demonstrated higher levels in the cell culture medium of the vaccinated group during the fourth and fifth weeks in comparison to the control group. As demonstrated in Figure 3, a statistically significant discrepancy was observed ($P>0.05$). However, no substantial disparities were identified between the groups that received the Razi S vaccine and the groups that received the imported vaccine. As demonstrated in Tables 1 and 2, the induction of cytokines IFN-γ and IL-4 in PMBC cells of vaccinated chickens was observed to be significantly higher than that of the control group following stimulation with mitogen Con A.

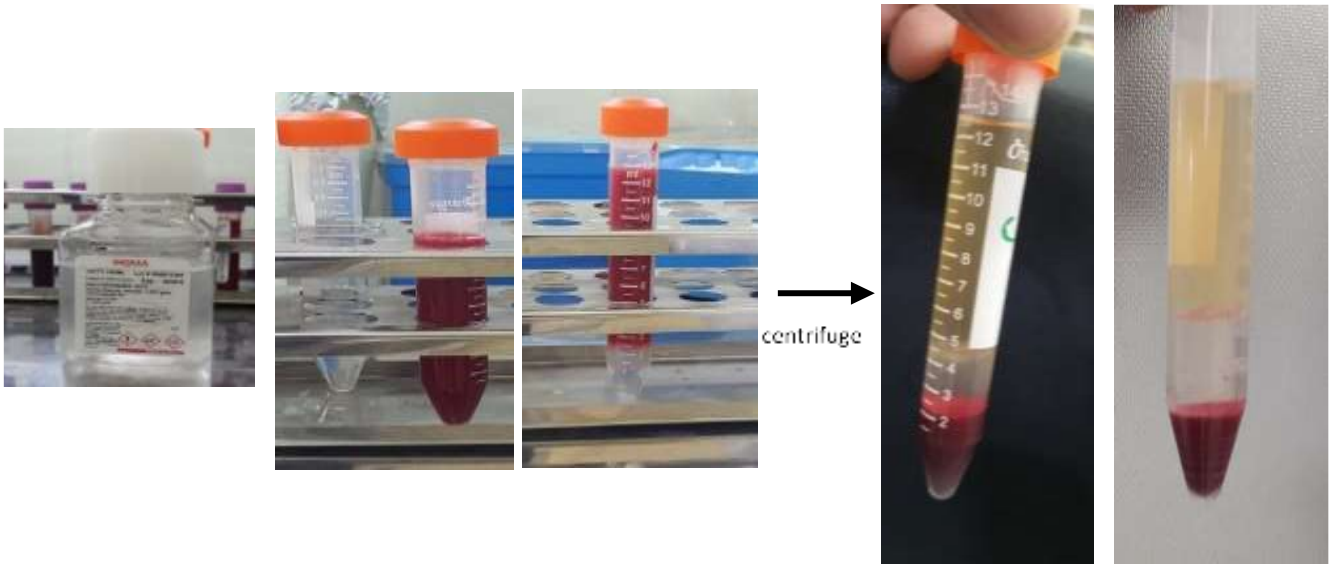

Figure 2. Preparing a PBMC with a Ficoll. By creating a 4-layer centrifuge, the image is created in accordance with the image. The first layer (from above) contains the plasma. The second layer contains PBMC cells. The third layer is the ficoll, and the fourth layer (bottom and red) contains red blood cells and multi-nucleated cells.

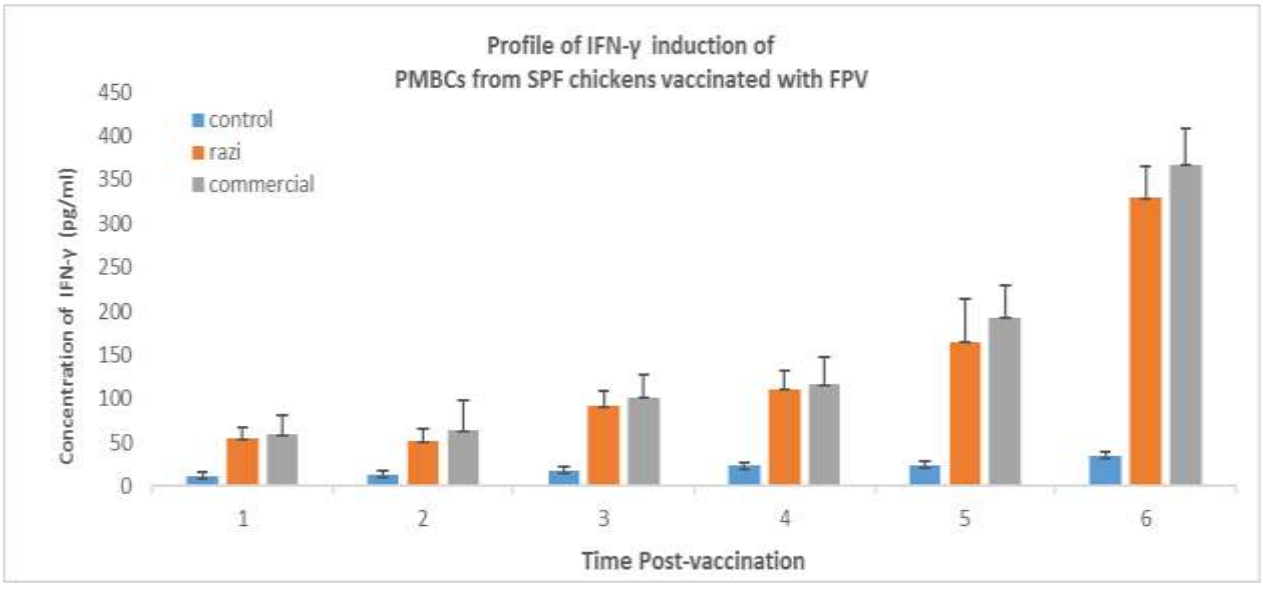

Figure 2. Cytokine induction of IFN-γ in PMBC cells of vaccinated chickens after stimulation with mitogenCon A compared to the control group.

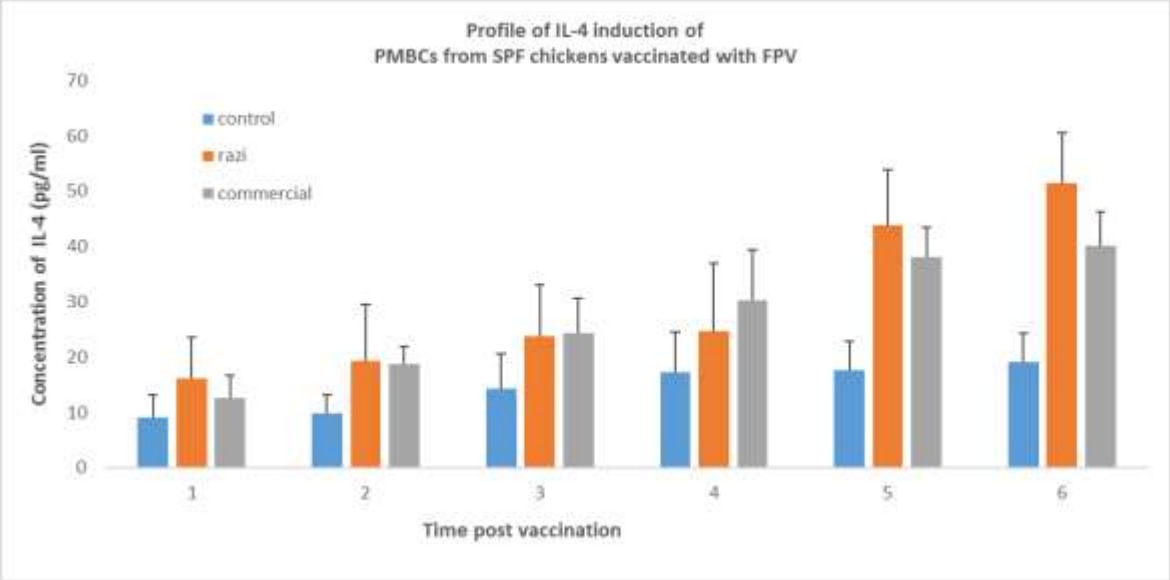

Figure 3. IL-4 cytokine induction in PMBC cells of vaccinated chickens after stimulation with Con A mitogen compared to the control group.

Table 1. Cytokine induction of IFN-γ in PMBC cells of vaccinated chickens after stimulation with mitogen Con A compared to the control group.

Week	Control (pg/ml)	Razi FPV vaccine	Commercial FPV vaccine
week1	12.15 ± 6.83	54.33 ± 13.21	59.51 ± 21.34
week2	13.61 ± 4.21	51.25 ± 14.11	64.24 ± 34.18
week3	18.36 ± 3.14	91.62 ± 16.45*	101.42± 26.19*
week4	23.21 ± 10.16	110.98 ± 21.53*	116.71 ± 30.87*
week5	24.48 ± 13.11	164.97 ± 48.75*	192.97 ± 35.62*
week6 (Challenge)	35.23 ± 10.21	329.93 ± 35.68*	367.13 ± 41.23 *

*: (P < 0.05) Indicates a significant difference with the control group using paired t-test.

Table 2. Induction of cytokine IL-4 in PMBC cells of chickens of the vaccinated group after stimulation with mitogen Con A compared to the control group.

Week	Control	Razi FPV vaccine	Commercial FPV vaccine
week1	9.04 ± 4.17	16.23 ± 7.26	12.67 ± 4.11
week2	9.91 ± 3.35	19.3 ± 10.23	18.72 ±3.21
week3	14.31 ± 6.21	23.84 ± 9.28	24.36 ±6.19
week4	17.25 ± 7.25	24.76 ± 12.22	30.19 ± 9.11
week5	17.62 ± 5.26	43.78 ± 10.16*	38.15 ± 5.27*
week6 (Challenge)	19.11 ± 5.13	51.39 ± 9.21*	40.11 ± 6.18*

*: (P < 0.05) The indicator of significant difference with the control group using paired t-test.

4. Discussion

Cytokines are proteins that are secreted from immune cells, thereby stimulating and regulating inflammatory and immune reactions. The specific biological effects of each cytokine have been demonstrated to result in disease and immune system disorder when there is an absence or excess of them. Cytokines have been utilized as a therapeutic agent or as a target for the development of specific antagonists in a variety of immune and inflammatory diseases. A robust immune response that results in the substantial secretion of various cytokines can precipitate inflammatory diseases. It is imperative to quantify the levels of cytokines in different bodily tissues before and after pharmaceutical interventions (11). Conversely, certain cytokines, such as INFγ and TNFα, have been implicated in the onset or progression of inflammatory diseases. In contrast, IL-4, a distinct cytokine, has been shown to attenuate chronic inflammatory diseases (12, 13). The assessment of cytokine secretion is typically conducted through the implementation of various assays, including the enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, and reverse transcription-polymerase chain reaction (RT-PCR). Immunoassays, such as the enzyme-linked immunosorbent assay (ELISA), are preferred due to their simplicity. However, these assays have been observed to encounter challenges in detecting low concentrations of cytokines. Furthermore, the correlation between mRNA levels measured by RT-PCR and protein levels is not always direct, underscoring the intricacies inherent in cytokine measurement (14-16). In a previous study, the author evaluated the cell-mediated immune response using flow cytometry on CD4+ and CD8+ cells. The study demonstrated that the vaccination with fowlpox virus induced cell-mediated immunity (17). The present study demonstrated that the IFN-γ concentration of chicken peripheral blood mononuclear cell (PBMC) culture medium was elevated in comparison with the control group. This finding suggests that following vaccination against fowl pox, the activity of Th1 cells is augmented. Furthermore, the concentration of IL-4, predominantly secreted by Th2 cells, exhibited a marked increase in the cell culture medium of the vaccinated group during the fourth and fifth weeks in comparison to the control group. This observation revealed a significant difference, suggesting that the fowlpox vaccine elicits a cytotoxic T cell immune response, which is classified as cell-mediated immunity. In 2014, Roy et al. conducted a study on the detection of target antigens of B and T cells of domestic chickens. The study revealed a high level of antibodies against the smallpox virus in the tested birds. In this study, a significant increase in cell-dependent responses was observed in inoculated chickens, and the increase in T lymphocytes indicated cell-dependent responses (18). In 1994, a study was conducted by Astrila, Vaino, and Lasila regarding the central role of CD4+ T lymphocytes in birds. The present study demonstrated that T lymphocytes are the primary agents responsible for combating the viral agent (19). In 2006, Jianing Wang and colleagues employed an indirect enzyme-linked immunosorbent assay (ELISA) method to assess the specific antibody response of the smallpox virus. The non-specific humoral response was evaluated by injecting two T-cell-dependent antigens, sheep red cells and bovine serum albumin. A notable finding was the absence of substantial variation in antibody responses to the smallpox virus among chickens infected with disparate isolates and strains of the virus within both groups. In contrast, two weeks after inoculation, the antibody responses were found to be significantly lower. It has been posited that the humoral immune response is not implicated in the immune response of chickens against the fowl pox vaccine or virus. The cellular immune response was measured by in vitro lymphocyte proliferation assays and PHA-P skin tests. It was observed that cellular immunity was significantly enhanced in chickens infected with both pox virus strains and vaccine strains. (20). In 2016, Bareda conducted a study that compared the immune response induced by a commercial fowlpox virus vaccine and the lesion at the inoculation site. In this research, the performance of an enzyme-linked immunosorbent assay (ELISA) on the antigen prepared from the cell culture medium demonstrated a direct relationship between the ELISA values and the presence of lesions after vaccination (21). In 2003, Puehler and his colleagues demonstrated that in cells infected with fowl pox virus, the high activity of chicken interferon-gamma is destroyed by trypsin. In this study, the laser ionization time analysis method of a mass spectrometer was utilized to demonstrate that the viral interferon gamma binding protein is encoded by the FPV 016 gene. In addition, the researchers demonstrated that, in contrast to the prior findings concerning cellular interferon-gamma receptors and smallpox viruses that contain domains similar to fibronectin type 3, the interferon-gamma binding protein of the birdpox virus contains an immunoglobulin domain that does not interact with any cellular protein. Furthermore, no other virus has been observed to possess a homologous structure of this nature. In this study, an increase in immunoglobulins (which ultimately led to an increase in gamma interferon) was observed in the vaccinated groups (22). In 2008, Holecheck et al. conducted a study of a recombinant smallpox virus to express the recombinant fungal interferon gamma gene. The study's objective was to administer the recombinant virus to mice, with the aim of providing concentrated levels of the cytokine to the tissue microenvironment. The study's results showed a significant increase in the level of MHC class 1 expression (23). The decreased mRNA expression of the IFNγ gene at late ages post-vaccination may be attributed to the transient nature of cytokine secretion, which occurs alongside the transcription of their respective genes. A multitude of factors have been identified as influential in the immune response to vaccination. These include maternal antibodies, antigen characteristics, adjuvants, host age, nutritional status, genetics, and stress. It is imperative to comprehend immune responses and the efficacy of vaccines in order to develop and optimize

vaccination strategies (24). The selection of an appropriate method for quantifying cytokines is imperative in the context of immunological studies, as it exerts a significant influence on the precision of evaluating cellular responses to antigens. The judicious selection of assays is predicated on the specific capabilities of the laboratory and the nature of the immunological responses being investigated, as it is not feasible to apply a singular technique universally to all scenarios. The present study demonstrated that the immune system may be associated with an increase in cellular immunity. This increase in cellular immunity resulted in the proliferation of T lymphocyte cells and an increase in the ratio of the concentration of IFN-γ to IL-4 in groups of vaccinated chickens compared to the control group. The present study posits that the induction of enhanced immune responses is predominantly attributable to Th1-type responses. The results of the study demonstrate that the cytokine test for measuring IFN-γ and IL-4 can be considered a suitable method for measuring CMI in poultry. Therefore, ELISA tests can have a high ability to measure the role of CMI in protection against poultry infectious diseases and following vaccination.

Acknowledgment
The authors wish to express their deepest gratitude to our esteemed colleague at the Razi Institute for their invaluable contributions to each phase of this research study.

Authors' Contribution
Study Concept and Design: I.A. and M.R. A.
The initial phase of the research process is the acquisition of data: I.A. and M.R. A.
A. Data Analysis and Interpretation: I.A. and M.R. A.
Preparation of the Manuscript: N.M. and M.R. A. Critical revision of the manuscript for its intellectual content: The abbreviation "N.M." is used to denote the state of New Mexico.

Ethics
As this study did not involve human or animal subjects and the data were collected from previous studies conducted in Iran, ethical committee approval was deemed unnecessary.

Conflict of Interest
The authors affirm that they have no conflicts of interest.

Data Availability
The data that underpin the conclusions of this study are available upon request from the corresponding author.

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