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Research article
Ερευνητικό άρθρο

Immunomodulatory potential effects of pomegranate peel in the immunization of chickens against Newcastle disease virus

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ABSTRACT: Newcastle disease (ND) is an acute, transmissible, infectious respiratory disease associated with high mortality and morbidity in chickens. Biosecurity, mass vaccination, and improved immunization are suggested as controlling strategies. Enhancing poultry's innate immunity using natural feed additives eventually activates adaptive immune responses. This research was conducted to determine the effect of a diet supplemented with pomegranate peel (Pop) on the immune status of chickens whether vaccinated against NDV or not. Pop antioxidant capacity was determined by calculating radical scavenging activity. Specific pathogen-free (SPF) chickens' basal diet was supplemented with 1.0% Pop. Chickens were divided into four groups (A-D; n=20): (A) NDV vaccination + basal diet; (B) NDV vaccination + Pop feeding; (C) Pop feeding; and (D) control. Day-old chicks were vaccinated against NDV with a booster dose at 14 days of age. The experimental trial lasted 42 days. Kinetics of IFN-γ and chTLR4 immune response gene expressions and nitric oxide production levels were assessed in primary chicken monocyte-derived dendritic cells isolated from the chicken groups. Humoral and cell-mediated immune responses were also evaluated using hemagglutination inhibition and lymphocyte proliferation assay. Upregulating IFN-γ and chTLR4 expression in the Pop-feeding groups suggested a significant ($P < 0.05$) increase in the innate immunity of chickens. The chickens also showed a marked decrease in nitric oxide production levels, which could be attributable to Pop's high total antioxidant capacity. Slight increases in humoral and lymphoproliferative responses against NDV confirmed the additive effect of Pop. These findings suggest that Pop potentiated the immunomodulatory impact on chicken's innate immunity and served as an additive factor for adaptive immunity against NDV.

Keyword: Newcastle disease; pomegranate; immune responses; IFN-γ; chTLR4; nitric oxide

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INTRODUCTION

World demand for poultry products has significantly increased over the past decades. Poultry health is a critical issue in meat and egg production in the husbandry industries because expanded productivity is associated with the increased susceptibility risk of infectious and metabolic diseases. Most challenges relate to viral diseases like Newcastle disease, infectious bronchitis, highly pathogenic avian influenza, and infectious bursal disease that cause irreparable economic losses in the poultry industry. Newcastle disease virus (NDV) is a negative-sense, single-stranded RNA virus in the family avian paramyxovirus serotype 1 (APMV-1). The NDVs cause substantial economic losses to the poultry industry with high levels of morbidity and mortality. Outbreaks of velogenic or highly virulent strains can result in 80–90% mortality in poultry production farms in developed and developing countries (Alexander, 2001; Ashraf & Shah, 2014). The disease globally represents a threat to food security due to its devastating impact on animal and human health. Therefore, proper vaccination policies and biosecurity measures constitute the most important preventive tools against ND (Dimitrov et al., 2017; Hu et al., 2022)

Administration of live and inactivated vaccines induces efficient immune responses against NDV. In addition to the adaptive immune response of chickens to NDV, the host innate immune response has a major impact on the variability of response to the infection. As the first line of host defense, this system operates in a quick and non-specific manner to protect against pathogens (Kapczynski et al., 2013). Macrophages and dendritic cells (DCs) as key regulatory cells of the immune system recognize the surface ligands of pathogens via pattern recognition receptors including the toll-like receptor (TLR) family (Nawab et al., 2019). Upon ligand recognition, TLR stimulates specific intracellular downstream signaling cascades and activates NF-κB and AP-1 transcription factors and MAPK to initiate host defense reactions (Kogut et al., 2005). Further, TLR signaling activation is pivotal for promoting the maturation of DCs, regulating major histocompatibility complex molecules, boosting lymphoid organs to stimulate T cells, and activating adaptive immune cells (Brownlie & Allan, 2011; Kogut et al., 2005). At least 10 diverse TLRs have been recognized in chickens that classified into six major phylogenetic groups. However, the primary action of chicken TLRs (ChTLRs) during innate immune functions is similar to mammals (Keestra et al., 2013; Kogut et al., 2005). ChTLR4 is highly expressed in heterophils and macrophages and stimulates IFN-γ and pro-inflammatory cytokines production through monocytes. As an effective innate immune response member, chTLR4 links innate and adaptive immunity by regulating the activation of antigen-presenting cells, priming the production of multiple pro-inflammatory cytokines and nitric oxide (NO) (He et al., 2006; Iqbal et al., 2005; Kannaki et al., 2010). Inducible NO synthase (iNOS) enzyme is mainly involved in the innate arm of the immune system such that TLR signaling stimulates iNOS to produce large quantities of NO for prolonged periods led to facilitating innate responses (He et al., 2006).

Supplementation of chick feed with bioactive compounds including probiotics, antioxidants, and antibiotics at sub-therapeutic doses improves poultry performance and supports the sufficient innate immune system function (Dalia et al., 2018; Dhama et al., 2011). Emergence of antibiotic-resistant bacteria due to the misuse or positive selection, and particularly potential risk of transmission into human through the food chain has become an inevitable challenge for poultry industry. The function of probiotics in growth efficiency and improvement of gut microbiological homeostasis as well as immune modulation is well documented (Brisbin et al., 2011; Mahfuz et al., 2017; Palamidi et al., 2016). The use of antioxidants is another management and nutritional strategy in the poultry industry to maintain productive and reproductive performance, improve immune function, and protect against infectious disease. Herbal antioxidant ingredients have gained interest for their potential benefits in poultry nutrition (Giannenas et al., 2018). The pomegranate *(Punica granatum L.)* peel (Pop) contains a large number of polyphenolic metabolites including flavonoids (anthocyanins, catechins, and other complexed flavonoids) and hydrolyzable tannins (punicalin, punicalagin, gallic acid, and ellagic acid). The compounds have a highly antioxidant capacity (Ismail et al., 2012; Saad et al., 2012) that can boost innate immunity and restore poultry health parameters. The effect of dietary Pop on the primary and secondary immune responses to sheep red blood cell antigen, as well as T cell proliferation in the cellular immune system, was evaluated. The findings indicate that Pop can enhance the overall immune function of broiler chickens (Saleh 2015). The effectiveness of Pop in poultry nutrition is well documented; however, its influence on the immune status of chicks following

vaccination against avian pathogens has not been reported. In this study, the effect of Pop on immune responses against NDV vaccination of chickens was evaluated using indicators of immune cell gene expression, nitric oxide production, and the induction of humoral and cell-mediated immune responses.

MATERIALS AND METHODS

Pomegranate peel

The Pop powder was purchased from GiyahKala, Iran. HPLC analysis shows that at least two components of ellagitannin (ponicalin and ponicalagin) dominate over other polyphenols in the Pop powder. Prior to chicken immunization trial, 1 mg Pop powder was diluted in 1 L nutrient broth and cultured for sterility in fluid thioglycollate medium incubated at 30-35 °C and soya-bean casein digest medium incubated at 20-25 °C for 14 days. The antioxidant activity of Pop was determined by measuring its ability to scavenge radicals using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Solutions of Pop and DPPH were prepared in methanol. A 100 µL Pop solution (1 mg/mL) was mixed with 2.0 mL of DPPH working solution (0.1 mM) and incubated in the dark for 20 min. Absorbance was measured at 517 nm against methanol. The radical scavenging activity expressed as the inhibition percentage was calculated using the formula $(A_C\text{-}A_S)/A_C \times 100$ where A_C is the absorbance of the control and A_S is the absorbance of the sample.

Chicken immunization procedure

The trial was ethically approved by the Animal Care and Use Committee of Razi Vaccine and Serum Research Institute. Chickens were hatched from specific pathogen-free (SPF) White Leghorn chicken eggs (Venky's Company, India). Eighty SPF chickens were randomly divided into four equal groups (A-D) in separate rooms. All chickens were maintained at a constant temperature of 37.5°C with a relative humidity of 50% to 70%, and received the standard feed and water *ad libitum* throughout the experiment. Chickens in group A fed the basal diet supplemented with 1.0% Pop for 3 weeks, group B received the same regimen and vaccinated with the NDV Clone12IR vaccine (RVSRI, Iran) which contains $\geq 10^6$ EID_{50}/dose via eye drops and boosted after 2 weeks of prime. Chickens in group C fed the basal diet and vaccinated against NDV and group D received only PBS (not vaccinated and not fed Pop). Blood samples were collected in vacutainer tubes containing EDTA at 7, 14, and 28, and 42 days of age. Sera samples were also collected and stored at −20°C until used.

Immune cells gene expression

The immunomodulation effect of Pop was evaluated on days 7, 21, and 42 post-immunization (PI) by expression levels of IFN-γ and chTLR4, and NO production assay. Primary chicken monocyte-derived dendritic cells (chMoDCs) were isolated from the experimental chicken groups. Briefly, heparinized blood samples taken from SPF chickens in each group were diluted 1:1 with sterile PBS, mixed with lymphocyte separation medium, layered onto a Ficoll (Histopaque®-1077, Sigma-Aldrich), and centrifuged for 30 min at 1500 rpm. The upper layer containing the peripheral blood mononuclear cells (PBMCs) was harvested and washed twice in PBS through centrifugation at 3000 rpm for 5 min to remove thrombocytes and non-adherent lymphocytes. Adherent cells or primary chicken monocytes at 2×10^6 cells/mL concentration were cultured in 24-well plates for 6 days in RPMI-1640 (Gibco™) complete medium containing chicken serum, penicillin and streptomycin at 41°C and 5% CO_2. Recombinant chicken granulocyte–macrophage colony stimulating factor (GM-CSF; KingfisherBiotech, USA) at 25 ng/mL and IL-4 (KingfisherBiotech, USA) at 12.5 ng/mL were added to generate mature macrophage differentiation. The culture medium was replaced by fresh supplemented medium to remove the unattached cells and cell debris. The morphology, cell aggregation, and growth pattern of cells were monitored up to day 7 of the culture. On day 7, expression of CD11c as maturation marker was examined in chMoDCs using flow cytometry.

All four sets of chMoDCs (A-D groups) at 1×10^6 cells/mL concentration were seeded in a 96-well microplate and incubated overnight at 37 °C in 5% CO_2. The expression levels of IFN-γ and chTLR4 mRNA were assessed by quantitative real-time PCR using β-actin as the housekeeping gene (Table 1). The reaction consisted of 10 µL of 2X SYBR Green Master Mix (Roche Diagnostics), 1 µL of each forward and reverse primers (5 µM), 3 µL PCR-grade water and 5 µL of cDNA. An initial denaturation at 95°C, followed by amplification for 40 cycles consisting of 95°C for 10 sec, annealing at 60°C for 20 sec, and extension at 72°C for 10 sec were applied. The fold change in gene expression mRNA was estimated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Another set of cultured chMoDC prepared on

Table 1. Gene-specific primer pairs used in real-time quantitative RT-PCR

Gene	Primer sequence (5′-3′)	Production size	Accession number
IFN-γ	F: CGCACATCAAACACATATCTG R: GATTCTCAAGTCGTTCATCGG	118	NM205149
chTLR4	F: GAGAACCTCAATGCGATGC R: ATAGGAACCTCTGACAACG	272	AY064697
β-actin	F: TGCCTCTAGCTCTTCCCTGGA R: CCAATGGTGATGACCTGACCA	63	L08165

days 42 PI was used to evaluate the NO production by the Griess assay. First, a 3.13–200 μM $NaNO_2$ nitrite standard dilution series was set up according to the manufacturer's instructions to get a standard curve. Then, chMoDC supernatants were harvested from triplicate wells and transferred to another microplate. An equal volume of Griess reagent was added to the PBMC supernatant and standard wells. The optical density (OD) of the final colorimetric product was measured at 540 nm to qualify the nitrite concentration of each sample per the standard curve and values were expressed in μM.

Humoral immune responses

To determine the trend of humoral immune responses, serum samples were collected from the experimental chickens at 7-day intervals up to the 42 days PI. The NDV-specific HI antibody levels were evaluated by the hemagglutination inhibition (HI) test. Briefly, 25 μL of each serum was serially diluted two-fold into PBS (25μL) in a 96-well microplate. Then an equal volume of 4 hemagglutinating units (HAU) of NDV Clone strain was added to each well. After 30 min incubation at room temperature, 25 μL of 1 % (v/v) chicken red blood cells (RBCs) were added to each well and the microplate was incubated again. The results were recorded as $\log_2$ of the reciprocal of the highest serum dilution that completely inhibited hemagglutination of the chicken (WOAH, 2021).

Lymphocyte proliferation assay

Induction of cell-mediated immunity was determined at the 2nd and 4th weeks PI by evaluating lymphocyte proliferation in MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The PBMCs from each group were cultured with RPMI-1640 medium and seeded in each well of a 96-well microplate at $2x10^5$ cells/ml concentration. The cells were stimulated with Phytohemagglutinin (PHA, Gibco™) 5 μg/mL in RPMI-1640 as a positive control, NDV antigen (10^6 EID_{50}/mL in RPMI-1640), and plain growth medium as a negative control taken in triplicate wells. Following incubation and adding MTT solution, the OD was measured at 630 nm. The lymphocyte proliferative response was represented as stimulation index (SI) calculated by the mean OD of stimulated lymphocytes–mean OD of blank divided by the mean OD of unstimulated lymphocytes (Ebrahimi et al., 2023).

Statistical analysis

Statistical analyses were carried out using the ANOVA and Student's *t*-test with SPSS 17.0. Data are represented as mean ± SEM of three independent experiments for each group. Differences were considered statistically significant for $P < 0.05$.

RESULTS

Antioxidant activity of Pop

Microbial contamination of Pop was assayed using microbiological testing and no microbial growth occurred during incubation. The methanol extract from Pop powder was investigated for its antioxidant activity using DPPH. The total antioxidant capacity of Pop was found to be 83.95 ± 0.23 at a concentration of 1 mg/mL.

Characterization of chMoDCs

The chMoDCs were characterized based on their morphologic changes on incubation days and CD11c mRNA expression. On day 4, the mononuclear cells were enlarged, aggregated, and more granulated due to GM-CSF and IL-4 stimulation. Cytoplasmic projections indicating immature DCs characteristic were observed. These dendritic protrusions prolonged gradually up to day 7 (Figure 1a). The maturation of chicken DCs was confirmed by the expression of CD11c, which was found to be significantly up-

regulated in the mature phenotypes compared to the immature ones ($P < 0.05$) (Figure 1b).

Quantification of IFN-γ and chTLR4 genes, and NO production

We examined whether Pop could modulate innate immunity-related gene expression. The expression of the IFN-γ gene was upregulated in the immunized chickens against NDV at all sampling days, which increased on day 7 and peaked on day 21. As shown in Figure 2a, the fold change was significant ($P<0.05$) in the vaccinated and Pop-feeding group compared to the vaccinated-alone group. The expression of chTLR4 transcripts was similar to the pattern of IFN-γ but peaked on day 21 and rapidly declined (Figure 2b). Next, the nitrite release rates were measured in experimental chicken groups using the Griess assay. Supplementation of the basal diet with Pop resulted in approximately a two-fold increase in NO production in groups A and B compared to group C (112-114 μM/mL vs. 48.3 μM/mL) (Figure 2c). The differences in NO production between the Pop-fed and control groups were significant ($P<0.05$).

Humoral immune response

Serum samples from each group were collected and evaluated for NDV-specific antibodies. As expected, antibody titers (log_2) in the vaccinated groups A and B were significantly ($P<0.05$) increased and persisted throughout the entire trial period. Figure 3 presents the impact of Pop feeding on the HI antibody titers in vaccinated chickens. During 4th week PI, the Pop treatment group exhibited a slight elevation in antibody titers against NDV compared to the not-fed group, ranging from log $2^{2.2}$ at the beginning to log $2^{5.5}$ at the end. The observed difference was not statistically significant ($P>0.05$).

Cell-mediated immune responses

The maximum SI values of 4.7± 0.12 were obtained at 5 μg/mL of PHA in all experimental groups. The

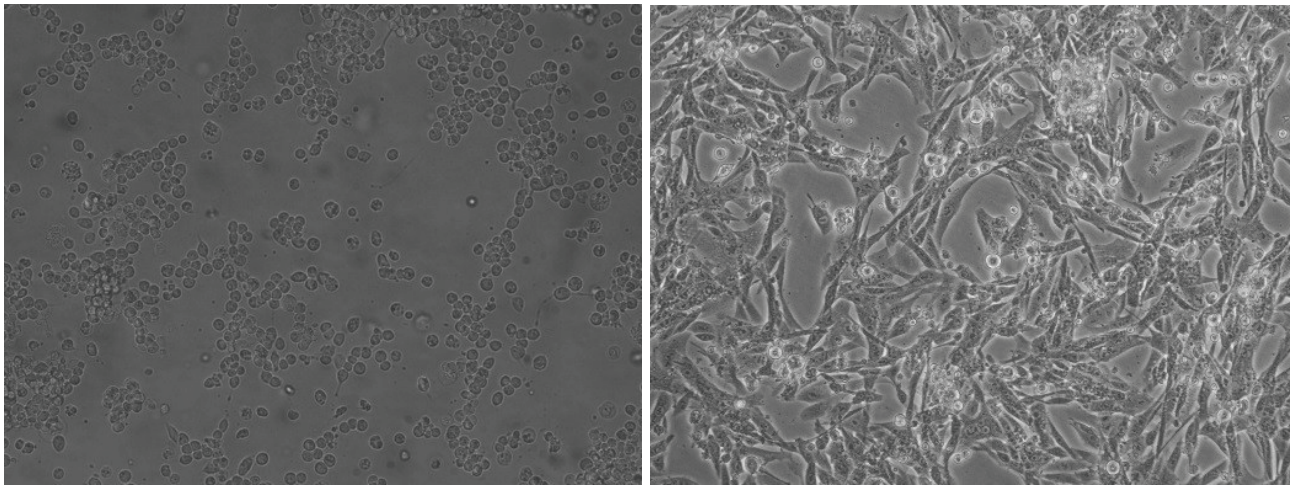
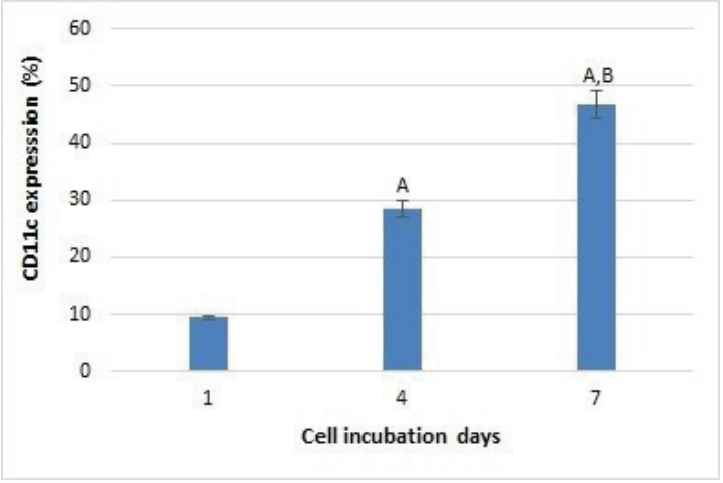
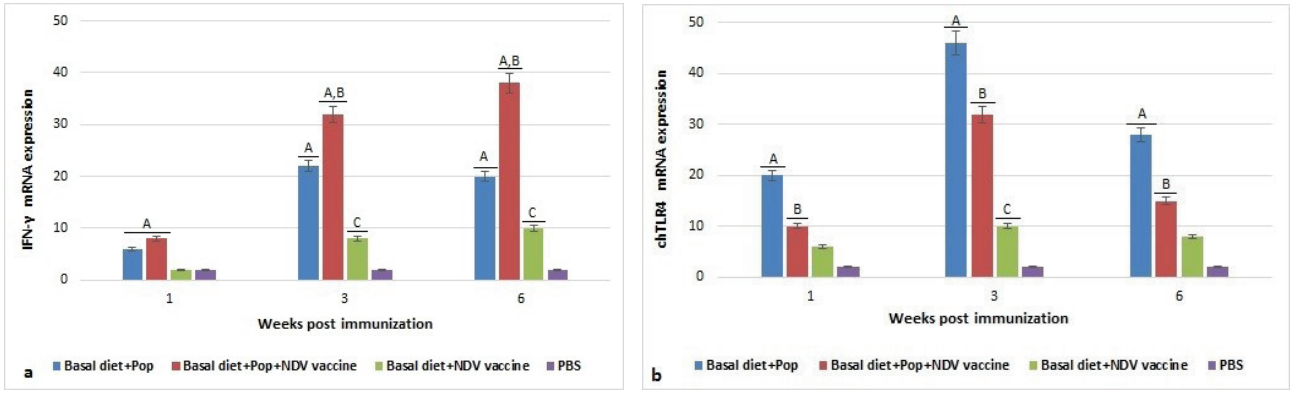


Figure 1. a) Maturation of dendritic cells generated from chicken peripheral blood monocytes cultured for 7 days in the presence of GM-CSF and IL-4. Stimulated cells (right) showed a typical dendritic protrusion morphology compared to the monocytes on the first day (left) in culture (40x magnification); b) Upregulation of the CD11c marker was detected during dendritic cell maturation, as indicated by uppercase letters. The absence of a letter implies no statistical difference ($P < 0.05$).





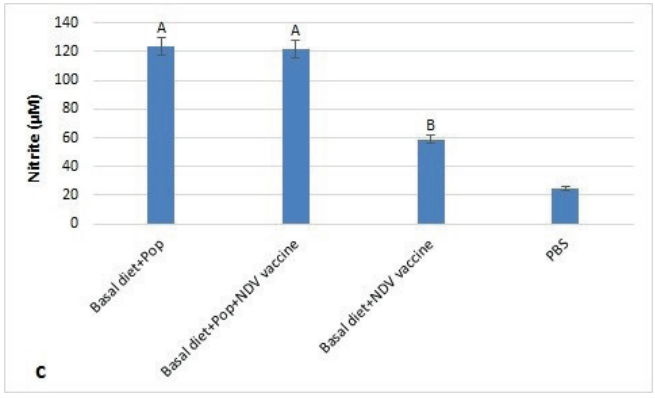


Figure 2. Cytokine gene expression analyses of chicken monocyte-derived dendritic cells from experimental groups using quantitative real-time PCR. a) The live NDV vaccine increased IFN-γ mRNA expression compared to the negative control, which was significantly upregulated in the Pop-fed group; b) chTLR4 mRNA expression was significantly upregulated in two Pop-fed groups; c) Pop significantly reduced nitric oxide production in groups fed it. The uppercase letters demonstrate significant differences ($P < 0.05$). The absence of a letter implies no statistical difference.

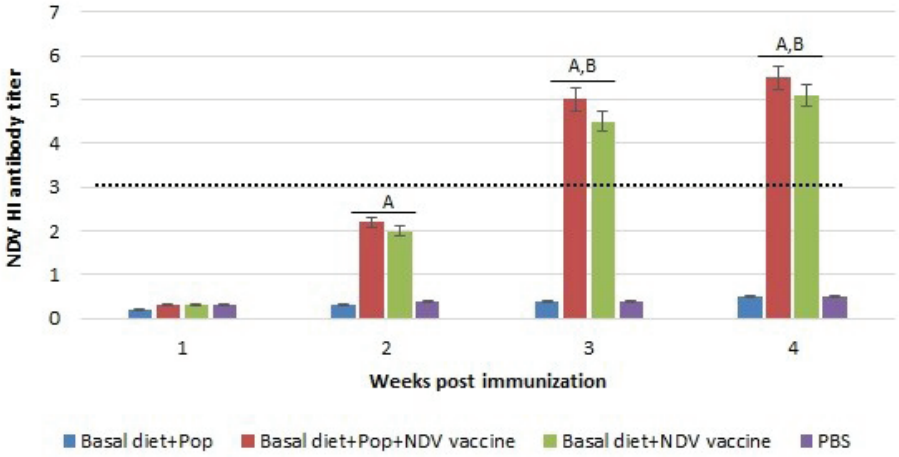


Figure 3. Serum HI antibody titers after vaccination of chickens with the live NDV vaccine and fed with a pomegranate peel regimen. Data are represent as mean ± standard deviation of antibody titters at specific weeks. The HI titer ≥ 3 log2 was considered positive (the dotted line indicates this cut-off value). The uppercase letters demonstrate significant differences ($P < 0.05$). The absence of a letter implies no statistical difference.

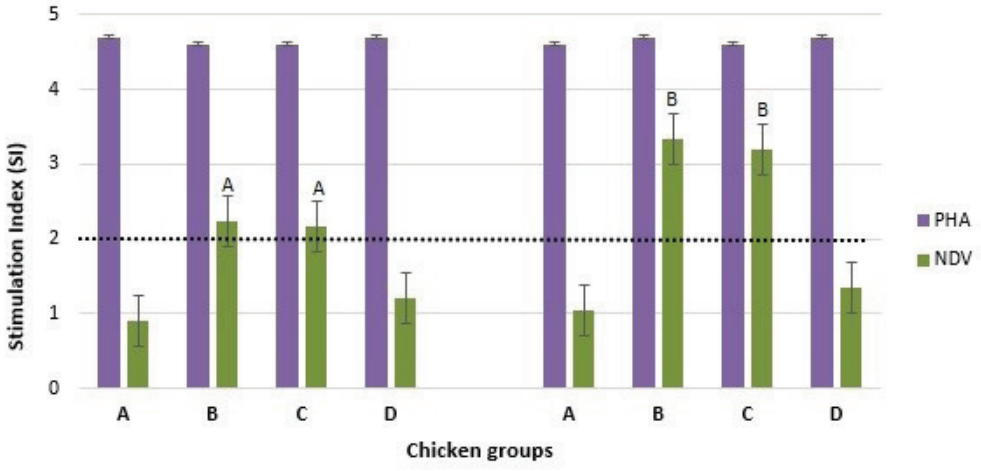


Figure 4. NDV-specific cell-mediated immunity in the peripheral blood of chickens vaccinated with the live NDV vaccine and fed with a pomegranate peel regimen. Data are presented as mean ± standard deviation of stimulation at specific weeks. A stimulation index ≥ 2 was considered positive (the dotted line indicates this cut-off value). PHA, as a general mitogen, exhibited the highest stimulation index across all groups. The uppercase letters demonstrate significant differences ($P < 0.05$). The absence of a letter, except for PHA, implies no statistical difference.

NDV vaccine significantly ($P<0.05$) induced lymphocyte proliferation in the two immunized groups compared with the PBS-negative control, especially in the fourth week PI (Figure 4). The stimulation capacity of the NDV antigen in these groups was higher than the threshold (SI= 2); however, compared to group A, an increase in the lymphoproliferative response was observed in group B. Although this difference was not significant, it indicates that feeding with Pop can further stimulate the cell-mediated immune response to NDV vaccination.

DISCUSSION

Plants with immunomodulatory properties can stimulate and improve both innate immunity and the immune response of birds to vaccination. Innate immunity is the most efficient protective response to any early-life stressful infectious factors. Improving poultry innate immune response and health parameters provides an alternative approach to further reduce the likelihood of infectious disease outbreaks and prevent the failure of a vaccination strategy in breeding farms. Feed additives including probiotics, herbal medicine, and natural plant-derived compounds as immune system stimulators or growth promoters are considered as alternative to eliminate the use of some subtherapeutic antibiotics (Gong et al., 2014). Studies are in progress concerning the potential of herb bioactive compounds to strengthen host innate and adaptive immune responses to avian pathogens. Plant-originated biomaterials which include antioxidants have better biocompatibility, bio-degradability, and bio-safety properties than synthetic compounds. A diet supplemented with natural antioxidants may improve poultry immune function due to their cofactor role in cytokine regulation (Giannenas et al., 2018). We showed Pop, a potential source of polyphenolic compounds with high antioxidant properties, provided potential innate immune modulation in chickens by stimulating chTLR4 activation.

ChTLR4 is an effective innate immune response member activated by bacterial lipopolysaccharide (LPS) and orchestrates intracellular signaling cascades through MyD88 or TRIF adaptor proteins. ChTLR4 signaling links innate and adaptive immunity by promoting macrophage activation, enhancing antigen presentation, and regulating Th1/Th2 balance (Keestra & van Putten, 2008). The expression of the chTLR4-LPS-MD2 complex induces a cascade of signaling pathways upon downstream activation of NF-κB resulting in the overproduction of IFN-γ, pro-inflammatory cytokines, and inflammatory mediators in response to infection (He et al., 2006; Iqbal et al., 2005; Kogut et al., 2005). In particular, IFN-γ, IL-1β, IL-6, iNOS, AvBDs, CXCLi2, and IL-18 have significantly expressed in chickens infected with the

different strains of NDV (Zhang et al., 2019). The host's inflammatory response to infection is a double-edged sword because it enhances immune defenses and induces a specific immune response. On the other hand, the overexpression of innate immune responses (cytokine storm) may lead to severe tissue damage. It has been found that the high levels of NO produced by activated macrophages in response to pathogens can have toxic effects on the host (Palmer et al., 1987). Similarly, an attenuated virus mimics natural infection in the live vaccine immunization process creating strong and long-lasting immune responses using Th1 and Th2 cytokines (Liu et al., 2012; Zhang et al., 2019). In our experiment, the evaluated maximum lymphoproliferative response and the IFN-γ mRNA expression level indicate induction of cell-mediated immunity were observed in NDV-vaccinated chickens than in the controls.

In addition to innate immunity, we showed the impact of Pop on the immune response of chickens vaccinated with NDV. The trial findings revealed that following feeding of Pop the humoral immune response against NDV was not boosted, but the cell-mediated immune response was further stimulated. Research on plants with potential effects on the poultry immune system, specifically those containing phytochemicals like flavonoids, alkaloids, and terpenoids, has yielded varying results. For instance, administering thyme and ginseng, either separately or together, to NDV-vaccinated broilers results in higher HI antibody titers than vaccinated chickens who had not received these supplements (Hassanin et al., 2024). Additionally, ginseng stem-and-leaf saponin has been shown to increase levels of intestinal intraepithelial lymphocytes and IgA+ cells, leading to significant improvements in both systemic and intestinal mucosal immunity in poultry vaccinated against NDV (Zhai et al., 2011). Broilers that received ginger-supplemented drinking water also exhibited high NDV antibody titers and increased CD3, CD4, and CD8 lymphocyte levels (Abdel-Maksoud et al., 2023). Moreover, curcumin supplementation has been linked to significant increases in NDV antibody titers and stimulation of B lymphocyte proliferation in chickens (Rajput et al., 2013). In contrast, other studies have reported that varying levels of thyme extract (Talazadeh et al., 2015), Mentha spicata (Ghoudarzi et al., 2016), and sumac powder (Toghyani and Faghan, 2017) did not influence the antibody response to the NDV vaccine.

In addition to boosting immunity to NDV vaccination, our findings revealed a remarkable increase in NO production in chickens that consumed Pop-supplemented diets. The low NO level observed in the control chickens can be attributed to the inclusion of essential amino acids in their basal diet. The findings demonstrate a notable increase in NO production levels in vaccinated chickens that received the live vaccine; however, this elevation was still lower than that observed in the group receiving Pop and vaccinated against NDV. The antioxidant capacity of pomegranate protects NO from oxidative destruction and further supports immune response induction. The live NDV vaccine is a potent platform for enhancing immune responses by activating T-cells. Activated T cells secrete IFN-γ as the important inducer of iNOS gene transcription and protein production, leading to NO production. Data on NO production in chickens that did not consume Pop and were immunized against NDV indicate the level of NO was increased compared to the control chickens.

Different parts of the pomegranate, especially the peels, have been shown to possess potent anti-inflammatory effects and antioxidant activity. However, these bioactive compounds vary depending on the type of pomegranate and the climatic and geographical cultivation conditions. Pop contains rich sources of polyphenols, especially hydrolyzable tannins (ellagitannins and gallotannins) and condensed tannins (proanthocyanidins), but it is mostly a by-product or waste of juice and paste factories. The ability of tannins to scavenge free radicals depends on the number and polymerization degree of hydroxyl groups. Tannins have strong antioxidant activity because their hydroxyl groups are easily oxidized (Ismail et al., 2012; Saad et al., 2012). The total peel tannin of 30 Iranian pomegranate cultivars has been estimated at 173 to198 mg/gr dry materials. The maximum antioxidant activity of the Saveh red-skinned (93.60%) and the lowest in the Emberti (69.90%) can be linked to the genetic diversity of these cultivars in tannin biosynthesis (Tehranifar et al., 2010). In our study, the purchased Pop from GiyahKala exhibited a remarkable DPPH-scavenging capacity (83.95%) suggesting its promising antioxidant activity. We do not know whether the used powder is prepared from one or possibly several pomegranate cultivars.

CONCLUSION

Supplementation with Pop positively affected the development of Th1 and Th2 immune responses to NDV vaccination. This effect was more evident

in the improvement of innate immune indices and significant increases in acquired immune responses to the vaccine were not observed. Further studies will be necessary to verify the immunomodulatory activity of Pop on monovalent or multivalent poultry vaccines in commercial broilers.

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Conflicts of interest

The authors declare no conflict of interest.

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