# Impact of dietary supplementation of vitamin E (alpha-tocopherol acetate) on genetic expression of inflammatory cytokines and growth efficiency of broiler chickens

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## Abstract:

This research was undertaken in order to determine the impact of nutritional vitamin E on genetic expression of Interferon-gamma (IFN-γ) and Interleukin-1β (IL-1β) as pro-inflammatory and Interleukin-4 (IL-4) and Interleukin-10 (IL-10) as anti-inflammatory cytokines in spleen and liver, heamagglutination inhibition antibody titers (HI titers) against Newcastle Disease Virus (NDV), total protein, albumin, globulin and growth efficiency of broiler chickens. Seventy two one-day-old Cobb broiler chicks had been allocated randomly into two groups. Each group has 3 replicates, each with 12 chicks. The first group was given basal diet. The second group was fed the basal diet with vitamin E (100 mg/kg diet). The obtained results revealed that dietary supplementation of vitamin E significantly ($p<0.05$) increased growth efficiency, total protein, albumin, globulin and the antibody titers to NDV compared to the control group. Dietary supplementation of vitamin E significantly ($P<0.05$) decreased the expression of pro-inflammatory (IL-1β, IFN-γ) and anti-inflammatory (IL-4 and IL-10) cytokines in spleen and liver. In Conclusions: vitamin E supplementation (100 mg/kg diet) can enhance growth efficiency, serum total protein, albumin, globulin and humoral immunity, down-regulate pro-inflammatory and anti-inflammatory cytokines gene expression in broiler chickens.

**Keywords:** Broilers, Vitamin E, Cytokines, Humoral immunity, Growth efficiency.

## 1- Introduction:

Vitamin E is an antioxidant fat-soluble vitamin that protects the membranes of the cells from oxidation **(Traber and Atkinson, 2007).** Two vitamin E families exist (tocopherol and tocoterinol). Alpha tocopherol is the most biologically active source of vitamin E involved on the pathway of glutathione peroxidase and protects species against oxidative damage by responding in fat peroxidation with lipid radicals **(Shakeri *et al.*, 2020).** Dietary vitamin E has an immunomodulative effect on T-cells that can benefit the immune system and wellbeing of chicken broilers **(Min *et al.*, 2018).** The addition of vitamin E to broiler diets reduced the expression of pro-inflammatory (IFN-γ and IL-1β) cytokines in chickens that had acquired intravenous lipopolysaccharide **( Leshchinsky and Klasing, 2003). Zhang *et al.*, (2010)** found that the dietary supplementation with alphatocopherol reduced plasma protein levels of both pro- inflammatory cytokines (IFN-γ, IL-1β, and IL- 6) and anti-inflammatory cytokines (IL-4 and IL-10). **Habibian *et al.*, (2014)** confirmed that a 250 mg/kg vitamin E supplement had high titers against NDV in thermo-neutral conditions.

It is important to keep broiler chickens in good health. The immune system is critical to defend against infectious agents **(Dalia *et al.*, 2018).** The key proteins of immunity cytokines were known as endogenous signaling molecules which mediate the cellular mechanism against inflammatory responses **(Hietbrink *et al.*, 2006).** The cytokines can be classified according to their functionality in the control of inflammation and immunity into pro-inflammatory (IFN-γ & IL-1β) and anti-inflammatory cytokines (IL-4 & IL-10) **(Kogut, 2000).**

In response to pathogenic challenges, pro-inflammatory cytokines are released **(Gabler and Spurlock, 2008).** IFN-γ is a class of pro-inflammatory cytokine released by activated monocytes, macrophages, T-lymphocytes (T cells), and natural killer cells **(Ivashkiv, 2018).** IL-1β pro-inflammatory cytokine that activates lymphocytes and macrophages in response to pathogens **(Low *et al.*, 2003).**

In animal models, IL-4 is an anti-inflammatory cytokine that reduces the initiation and severity of inflammation **(Su *et al.*, 2016).** IL-10 is a pleiotropic and important anti-inflammatory cytokine developed by innate and adaptive immunity cells such as dendritic cells, macrophages, mast cells, natural killer cells, eosinophils, neutrophils, B lymphocytes (B cells), cytotoxic T

lymphocytes (CD8+ T cells), T-helper cell (TH1, TH2, TH17), and regulatory T cells **(Mollazadeh et al., 2019).**

Therefore, the aim of the current research is to examine the impact of dietary vitamin E addition on pro-and anti-inflammatory cytokines gene expression, antibody titers against NDV, total protein, albumin globulin and growth efficiency of broiler chickens.

## 2- Material and methods:

### 2.1. Chickens, Management and housing:

The current study was undertaken in the Department of Animal Wealth Development, Faculty of Veterinary Medicine, Benha University in accordance with guidelines of institutional Animal Care and Use Committee Research Ethics Broad (No. BUFVTM 02-12-20). Seventy-two one-day-old Cobb broiler chicks had been used. They were bought from El-Dakahlia Company. The chicks were allotted randomly into two groups; with each group containing three replicates of 12 chicks. The house was clean, disinfected, well-ventilated space with proper environmental temperature according to the age of chicks. Lightening was provided for 24 hours throughout the experimental period. The litter consists of fresh wood shaving. Feed and water were present all the time. The vaccination program was as the following: infectious bronchitis (IB) and Newcastle disease (ND) on day 7 (eye drops), avian influenza (H5N1) on day 9 (Sub cutaneous), Gumboro on day 14 (eye drop) and against lasota on day 18 (coarse spray).

### 2.2. Groups and treatments:

Vitamin E was obtained from AB chem Pharmaceutical Raw Materials Company, Dakahlia Governorate, in Mansoura City.

The broiler chicks were randomly allocated into two groups:

- **Group I:** supplemented with the basal diet as shown as in table 1.
- **Group II:** supplemented with basal diet + vitamin E (100 mg/kg ration) according to **(Dalia *et al.*, 2018).**

The chicks were given a well-balanced diet **(NRC, 1994)**. The experimental diets were fed in four phases: 0 to 8 day (starter), 9 to 18 day (grower), 19 to 28 day (finisher1) and 29 to 35 day (finisher2).

**Table 1: Ingredients and nutrient composition of starter, grower and finisher diets (Basal diet).**

| Ingredients | Starter | Grower | Finisher1 | Finisher2 |
|---|---|---|---|---|
| Yellow corn | 53.97 | 57.17 | 58.66 | 62.38 |
| Soya bean meal-44 | 33.40 | 32.70 | 31.50 | 22.40 |
| Corn gluten meal | 5.70 | 2.60 | 1.80 | 5.70 |
| Vegetable oil | 2.30 | 3.40 | 4.40 | 4.20 |
| Lime stone | 1.45 | 1.35 | 1.23 | 2.70 |
| Di calcium phosphate | 1.43 | 1.23 | 1.00 | 1.05 |
| L-lysine | 0.39 | 0.29 | 0.21 | 0.37 |
| DL-methionine | 0.31 | 0.31 | 0.29 | 0.24 |
| Vit&min permix | 0.30 | 0.30 | 0.30 | 0.30 |
| Sodium chloride | 0.29 | 0.29 | 0.29 | 0.29 |
| Sodium bicarbonate | 0.16 | 0.12 | 0.13 | 0.14 |
| L-therionine | 0.15 | 0.10 | 0.05 | 0.08 |
| Anticolestrdia | 0.05 | 0.05 | 0.05 | 0.05 |
| Antimycotoxin | 0.05 | 0.05 | 0.05 | 0.05 |
| Choline cloride | 0.05 | 0.05 | 0.05 | 0.05 |
| Energy enzyme | 0.02 | - | - | - |
| Phytase enzyme | 0.01 | 0.01 | 0.01 | 0.01 |
| **Nutrients** | | | | |
| MEn (Kcal/kg) | 22.02 | 20.02 | 19.06 | 17.97 |
| Linoliec Acid | 3976.22 | 3027.19 | 3101.28 | 3150.54 |
| Crude fat | 2.13 | 2.62 | 3.04 | 2.96 |
| Crude fiber | 4.98 | 6.04 | 7.03 | 6.99 |
| Lysine Dig | 3.47 | 3.46 | 3.40 | 2.87 |
| Methionine Dig | 1.22 | 1.12 | 1.03 | 0.97 |
| Methionine+ cysteine Dig | 0.62 | 0.59 | 0.55 | 0.51 |
| Threonine | 0.91 | 0.85 | 0.8 | 0.76 |
| Threonine Dig | 0.96 | 0.85 | 0.77 | 0.74 |
| Calcium | 0.83 | 0.73 | 0.66 | 0.63 |
| Available phosphorus | 0.99 | 0.90 | 0.80 | 1.35 |
| Chloride | 0.47 | 0.43 | 0.39 | 0.38 |
| Sodium | 0.23 | 0.23 | 0.23 | 0.23 |
| Acid Base Balance ( mg/kg) | 0.17 | 0.16 | 0.16 | 0.16 |

[1] Premix provides Vit A (13000 Iu), vit D (5000 Iu), vit E (80 mg), vit K3 (3 mg), vit B1 (3 mg), vit B2 (9 mg), vit B6 (3 mg), vit B12 (0.02 mg), Niacin (60 mg), Pantothinic acid (15 mg), folic acid (2 mg), biotin (0.15 mg), iron (40 mg), copper (15 mg), zinc (100 mg), manganese (100 mg), iodine (1 mg), selenium (0.3 mg) per 1 kg diet.

**2-3- Determination of pro-inflammatory and anti-inflammatory cytokines gene expression:**

**2-3-1- Samples collection:**

At day 35, 18 representative birds (selected randomly as three birds/ replicate) had been slaughtered for sampling**.** Samples of spleen and liver had been collected and saved at -80 °C for further analysis.

**2-3-2- RNA isolation and Real-time PCR for cytokines gene expression:**

Extraction of overall RNA was performed by using the manufacturer's procedure with Trizol Reagent (Invitrogen, Korea). Concentration and purity of RNA was tested by Spectro Star Nanodrop (BMG Lab Tec.GmbH, Germany) at 260/280 nm absorbance. Then by using 2X Reverse Transcriptase Master Mix (Applied Bio system, USA) according to manufacturer instructions, approximately 2 µg of total RNA had been reverse transcribed to cDNA. With the support of NCBI Primer-BLAST software, primers had been planned. Table 1 illustrates primers used for quantitative real time PCR (qRT-PCR).

**Table 2: primers used for qRT-PCR**

| **Primer name** | **Sequence** | **Accession number** |
|---|---|---|
| β-actin | F- ACCCCAAAGCCAACAGA<br>R- CCAGAGTCCATCACAATACC | EU309690 |
| IFN-γ | F- CTGAAGAACTGGACAGAGAG<br>R- CACCAGCTTCTGTAAGATGC | FJ788637 |
| IL-1β | F- GTGAGGCTCAACATTGCGCTGTA<br>R- TGTCCAGGCGGTAGAAGATGAAG | HM179638 |
| IL-4 | F- TGTGCTTACAGCTCTCAGTG<br>R- ACGCATGTTGAGGAAGAGAC | GU119892 |
| IL-10 | F- AGCAGATCAAGGAGACGTTC<br>R- ATCAGCAGGTACTCCTCGAT | EF554720 |

**Real-Time PCR Quantitative Analysis:**

The mRNA quantification was performed by using Applied Biosystem 7500 Fast Real time PCR, USA. The SYBER Green Master Mix was used for the quantitative PCR in 20 µL reaction mix (TOPreal $^{TM}$ qPCR 2X PreMIX). For qPCR the thermal conditions were: 95 °C for 10 min; 40 cycles of 95 $^{o}$ C for 15 sec and 60 $^{o}$ C for 1min. The comparative quantification of gene expression has been determined using the $2^{-\Delta\Delta ct}$ method **(Livak and Schmittgen, 2001).**

## 2-4. Determination of HI antibody titers against NDV:

Haemagglutination inhibition (HI) method was applied to determine antibodies to NDV. Samples of blood have been obtained from five birds from each group at 7$^{th}$, 14$^{th}$, 21$^{th}$, 28$^{th,}$ 35$^{th}$ day of age. Clotted blood samples had been centrifuged in order to extract pure serum at 3000 r.p.m for 15 minutes. The serum samples were kept in labled sterile eppendorf tubes and stored at - 20 °C till used, using microtitre U-shape plate of 96 wells **(Majiyagbe and Hitchner, 1977).**

## 2.5. Determination of overall protein, albumin and globulin:

Overall proteins (g/dl) were detected at day 35 with 10 serum samples (five samples from each group) according to the method designated by **Weichselbaum (1946).** The colorimetric approach for the identification of plasma albumin (g/dl) as defined in **Doumas *et al.*, (1971).** For calculation of globulin, make subtraction of serum albumin from serum whole protein.

Globulin = overall protein – albumin.

### 2.6. Growth parameters:

#### 2.6.1. Body Weight (BW):

The chicks had been weighed individually (in gram) at day 1, and then the live body weight was recorded every week till 5$^{th}$ week **(Omar, 2014).**

#### 2.6.2. Body weight gain (BWG):

Body weight gain was calculated by subtracting the body weight between two successive weights every week.

**2.6.3. Feed Intake (FI):**

Weekly feed intake was estimated by subtracting the amount of feed remained from total amount offered in each group (in grams).

**2.6.4. Feed Conversion Ratio (FCR):** according to **Lambert *et al.*, (1936).**

FCR = Feed intake (g/chick/week) / Body Weight Gain (g/chick/week).

## 2-7.Statistical analysis:

Data analysis was carried out using the SPSS statistical software package **(version 23; SPSS Inc., Chicago, IL, USA).** The results achieved were found by the independent sample T-test study to be mean ± SE. Meaningful significance ($P<0.05$).

## 3-Results:

Table 3 indicated that there was a significant ($p<0.05$) improvement of growth efficiency (BW, BWG, FI and FCR) in group enriched with vitamin E in relative to control one.

Results of total protein, albumin and globulin as influenced by dietary supplementation of vitamin E were shown in table 4. The current results indicate that serum total protein, albumin and globulin for chicks enriched with vitamin E have considerably ($p<0.05$) higher values than those of their control group.

Table 5 showed results of dietary vitamin E supplements on antibody titers for NDV. This result showed that there was no significance difference between control group and group supplemented with vitamin E at week 1 and week 2, whereas the vitamin E uptake group significantly ($p<0.05$) increased antibody titers against NDV at week 3, week 4 and week 5 compared to control one.

The effect of dietary vitamin E addition on pro-inflammatory (IFN-γ, IL-1β) and anti-inflammatory (IL-4, IL-10) gene expression cytokines in spleen and liver was shown in fig 1, 2. These findings revealed the significant ($p<0.05$) decrease in expression of both pro- and anti-inflammatory cytokine genes in the vitamin E group ($p<0.05$) compared to the control group.

**Table (3): Effect of dietary supplementation of vitamin E on BW, BWG, FI and FCR of broiler chickens.**

| | parameter | Group supplemented with basal diet | Group supplemented with vitamin E |
|---|---|---|---|
| | | LSM ± SE | LSM ± SE |
| **BWt (g/chick)** | Initial wt | 47.11 ± 0.48 | 48.22 ± 0.52 |
| | 1st week | 140.22 ± 1.43[b] | 155.56±1.44[a] |
| | 2nd week | 326.89 ±1.86[b] | 363.11 ± 1.42[a] |
| | 3rd week | 697.33 ± 1.56[b] | 753.56 ± 1.79[a] |
| | 4th week | 1088.67 ± 2.26[b] | 1205.56 ± 2.42[a] |
| | 5th week | 1672.22 ± 2.53[b] | 1840.00 ± 2.43[a] |
| **BWG (g/chick)** | 1st week | 93.11 ± 1.46[b] | 107.33 ± 1.33[a] |
| | 2nd week | 186.67 ± 2.19[b] | 207.56 ± 2.13[a] |
| | 3rd week | 370.44 ± 1.56[b] | 390.44 ± 1.48[a] |
| | 4th week | 391.33 ± 1.41[b] | 452.00 ± 1.20[a] |
| | 5th week | 583.56 ± 1.69[b] | 634.44 ± 1.56[a] |
| | Final BWG | 1625.11 ± 2.81[b] | 1791.78 ± 2.50[a] |
| **FI (g/chicks)** | 1st week | 119.44 ± 0.22[b] | 127.09 ± 0.43[a] |
| | 2nd week | 372.90 ± 0.76[b] | 365.13 ± 0.38[a] |
| | 3rd week | 656.17 ± 0.46[a] | 619.51 ± 0.33[b] |
| | 4th week | 772.12 ± 1.28[a] | 745.12 ± 1.09[b] |
| | 5th week | 960.28 ± 2.10[a] | 912.07 ± 1.22[b] |
| | Total FI | 2880.91 ± 1.15[a] | 2768.93 ± 1.02[b] |
| **FCR** | 1st week | 1.39 ± 0.04[a] | 1.22 ± 0.02[b] |
| | 2nd week | 2.07 ± 0.05[a] | 1.77 ± 0.02[b] |
| | 3rd week | 1.79 ± 0.03[a] | 1.59 ± 0.02[b] |
| | 4th week | 2.01 ± 0.05[a] | 1.68 ± 0.01[b] |
| | 5th week | 1.67 ± 0.03[a] | 1.50 ± 0.02[b] |
| | Final FCR | 1.78 ± 0.02[a] | 1.56 ± 0.01[b] |

Values are means ± standard error. Mean values with different letters at the same row significantly P≤0.05.

**Table (4): Effect of dietary supplementation of vitamin E on serum total protein, albumin and globulin of broiler chicks.**

| | Group supplemented with basal diet | Group supplemented with vitamin E |
|---|---|---|
| **Total protein** | $3.04 \pm 0.02^{b}$ | $4.44 \pm 0.01^{a}$ |
| **Albumin** | $1.39 \pm 0.01^{b}$ | $1.58 \pm 0.01^{a}$ |
| **Globulin** | $1.65 \pm 0.04^{b}$ | $2.86 \pm 0.05^{a}$ |

Values are means ± standard error. Mean values with different letters at the same row significantly P≤0.05.

**Table (5): Effect of dietary supplementation of vitamin E on serum HI titers (Log2) against NDV of broiler chicks.**

| | Group supplemented with basal diet | Group supplemented with vitamin E |
|---|---|---|
| Day7 | $5.60 \pm 0.51^{a}$ | $6.20 \pm 0.37^{a}$ |
| Day14 | $2.60 \pm 0.25^{a}$ | $3.00 \pm 0.32^{a}$ |
| Day21 | $4.60 \pm 0.25^{b}$ | $6.80 \pm 0.66^{a}$ |
| Day28 | $5.60 \pm 0.40^{b}$ | $7.40 \pm 0.60^{a}$ |
| Day35 | $4.80 \pm 0.37^{b}$ | $5.80 \pm 0.37^{a}$ |

Values are means ± standard error. Mean values with different letters at the same row significantly P≤0.05.

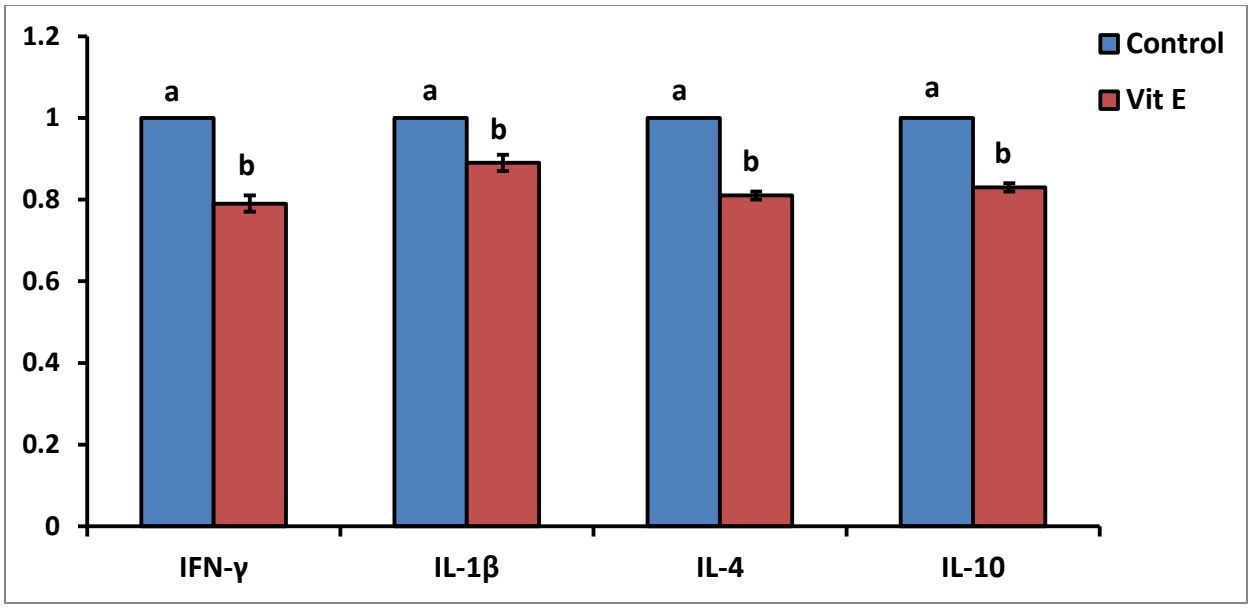


**Fig (1): Effect of dietary supplementation of vitamin E on pro-inflammatory (IFN-γ, IL-1β) and anti-inflammatory (IL-4, IL-10) cytokines gene expression in spleen of broiler chicks.**

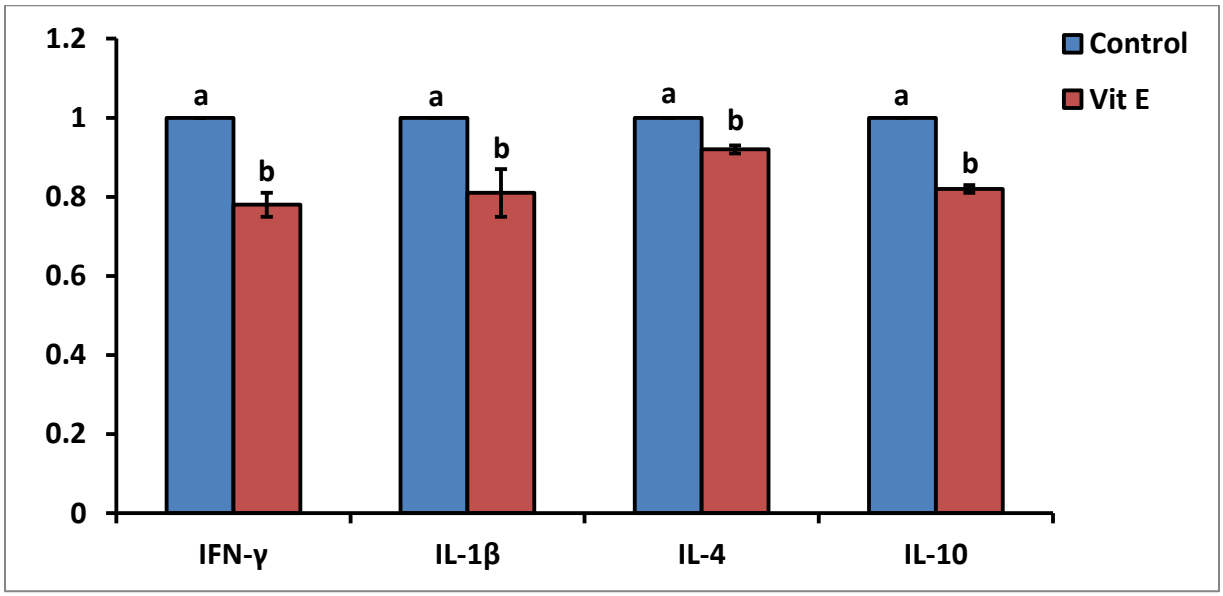


**Fig (2): Effect of dietary supplementation of vitamin E on pro-inflammatory (IFN-γ, IL-1β) and anti-inflammatory (IL-4, IL-10) cytokines gene expression in liver of broiler chicks.**

## 4-Discussion:

This study showed that addition of vitamin E in the diet of broilers resulted in a significant improvement in growth efficiency compared to control group. This result is consistent with **Maini *et al.*, (2007)** who noted that supplementation of 200 mg/kg of vitamin E in broiler diet cause an increase of body weight compared to the basal diet. Using of vitamin E as a supplement revealed a significant ($P<0.05$) and better FCR (**Abd El-Hack *et al.*, 2017)**. Also, **Hedayati *et al.*, (2021)** explained that vitamin E enriched group had a better growth performance than control one.

Total protein, albumin and globulin results affected by dietary vitamin E supplementation revealed higher values of total serum protein, albumin and globulin for vitamin E supplemented chicks than control groups ($p<0.05$). These results are similar to **Rashidi *et al.*, (2010)** who found that vitamin E supplementation improved plasma total protein, albumin and globulin. Also, **Gouda *et al.*, (2019)** who found that supplementation of vitamin E (200 IU/kg diet) increase the total plasma protein and globulin values ($p<0.05$) at 42 day old chicks. Moreover, **Attia *et al.*, (2020)** explained the same result. The increase in gamma-globulins is caused by the increase in immunoglobulin production **(Gružauskas *et al.*, 2014).**

Regarding the HI titers against NDV, the result showed there was a significant ($p<0.05$) increase of antibody titers against NDV at week 3, week 4 and week 5 in the group supplemented with vitamin E relative to control group. These results were acceptable with **Swain *et al.*, (2000)** who found that broiler chicks, which were fed with vitamin E, have considerably enhanced antibody titers against NDV. In the community fortified with Vitamin E (200 mg/kg)**, Desoky, (2018)** documented a significantly improved humeral immune response against Newcastle. **Sheikh *et al.*, (2020)** found that at week 3 of vitamin C (500 mg/) and vitamin E (200 mg/) significantly enhance ($P<0.05$) antibody titer. Vitamin E treatment showed the highest titer while the lowest titer was in the control.

The current study showed that the gene expression of pro- and anti-inflammatory cytokines in the vitamin E enriched group was significantly ($p<0.05$) lower compared to the basal dietary group. This finding is in agreement with **Leshchinsky and Klasing, (2003)** who noted that the addition of Vit E decreased the expression of proinflammatory cytokines in

lipopolysaccheride-receiving chickens. Also, **Zhang *et al.* (2010)** observed a reduction in plasma protein levels of both inflammatory cytokines (IFN-γ, IL-1β) and (IL-4 and IL-10) in alpha-tocopherol dietary supplementations. **McCary *et al.*, (2011) f**ound that the expression of some cytokines, such as the IL-10, is decreased by higher doses of tocopherols**.** The same trend was reported by **El-Senousey *et al.*, (2018)** who found that the dietary addition of vitamin C, vitamin E or alpha lipoic acid (ALA) had greatly decreased the mRNA expression levels of IL-1β, IL-6 and IFN-γ in the spleens of broilers in relative to the control group. Moreover, **Khatun *et al.*, (2020)** indicated that the IFN-γ pro-inflammatory cytokine was decreased by 0.25 percent L-Arginine and 50–150 mg/kg vitamin E supplementations. The reason of our observation for the down-regulation of both pro- and anti-inflammatory cytokines may be due to the increase vitamin E level in broiler diet cause maintaining the (T-helper cell1/T-helper cell2) balance leading to increase balance of inflammatory response **(Kaiser *et al.*, 2012)**. When the Th1/Th2 equilibrium is disrupted, the cytokines secreted by Th1/Th2 cells are abnormally expressed, causing the inflammation to develop **(Zhao *et al.*, 2020).** Alteration the expression of cytokines in broiler chickens that could have a beneficial impact on immune function **(Khatun *et al.*, 2020).** This explains the immunomodulatory effect of vitamin E.

## 5- Conclusion:

From these results, it is concluded that supplementation of vitamin E (alpha-tocopherol acetate 100 mg/kg diet) in the diet of broiler chickens may cause a down-regulation of inflammatory cytokines (pro and anti-), as well as an increase of HI titers against NDV, total protein, albumin, globulin and growth efficiency of broiler chickens.

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