

Effects of vitamin E on T cell subsets and immunoglobulin-containing plasma cells in the spleen of heat-stressed broiler chickens

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Summary

Given a bird's underdeveloped lymph vessels and lymph nodes, the spleen is of the utmost importance in the avian immune system. Broiler chickens can be subjected to heat stress (HS), which causes an increase in mortality, a decrease in feed intake, body weight gain, and hatchability. Vitamin E is an important antioxidant for cells, but there is limited information about how heat stress affects spleen tissue in chickens and how vitamin E can change this effect. Therefore, the aim of this study was to investigate the effects of vitamin E based on the location of subpopulations of T cells and immunoglobulin-producing plasma cells in the spleens of broiler chickens subjected to HS. The results indicated that vitamin E supplementation in the broiler chicken diet could increase the population of immune system cells in heat-stressed chickens. The findings of this study could be used as a reference for the development of management strategies for broiler chickens, especially in countries with a hot climate.

Keywords: avian, heat stress, plasma cell, spleen, T cell, vitamin E

Lymphoid tissue is an organized structure that protects against micro-organisms. In birds, the spleen is regarded as a secondary lymphoid tissue (24). Overall, the spleen makes a very important contribution to the avian immune system because of the poorly developed avian lymphatic vessels and nodes (9).

Heat stress (HS) is a very important factor in the broiler industry as it decreases feed consumption, slows weight gain, increases mortality, and lowers hatchability (21). Increasing the environmental temperature adversely affects specific immunity, the neuroendocrine system, and the antioxidant system in chickens (19). Heat stress suppresses the white blood cells (28). It has also been reported that HS reduces antibody production in young chickens (23).

The main role of vitamin E is to act as an antioxidant and it is important for the development of the immune system and its functions (7). As a fat-soluble antioxidant, vitamin E protects the cell membrane and subcellular organs from free radicals and plays an important role in cellular antioxidant defense systems (30). Dietary vitamin E has been reported to stimulate immune responses against bacterial and viral infec-

tions in poultry (16, 17). It has been observed that the antibody titers, specifically those of immunoglobulin G (IgG) and immunoglobulin M (IgM) in the liver and lymphoid organs, decreased significantly with HS, and the addition of vitamin E in the diet significantly regressed this effect at both low and high temperatures (10). Moreover, a previous study showed that HS caused oxidative damage and increased the immune response in the chicken spleen (27).

Despite this information, there is very little information about the effects of vitamin E on the defense system cells of the spleen in heat-stressed broiler chickens. Therefore, the aim of this study was to evaluate the effects of vitamin E by determining the location of T lymphocyte subpopulations and Ig-including plasma cells in the spleens of broiler chickens subjected to HS.

Material and methods

Animals. Ross 308 chickens (21 one-day-old, male) were used as material in the study. Chickens were kept in accordance with the conditions of the „Regulation Regarding the Wellness of Farm Animals” published in the Official Gazette of the Republic of Turkey in 2011. Standard broiler

chick and chicken feed sold and produced in feed factories were used in the feeding of animals. The birds were not vaccinated during rearing. The birds were kept in a constantly bright environment.

Three groups of chickens were formed as follows:

1. Control group: kept at $22 \pm 2^\circ\text{C}$ and fed *ad libitum*,
2. Heat stress group: kept at 35°C (5 hours per day), fed *ad libitum*,
3. Vitamin E group: kept at 35°C (5 hours per day), and administered vitamin E (DL- α -tocopherol acetate, Merck) with *ad libitum* nutrition.

Vitamin E in liquid form was given to birds by the gavage method.

The chickens were first fed a starter diet (*ad libitum*, from day 1 to 21) and then a growth/final diet (from day 22 to day 42). The environmental temperature was gradually reduced from $32 \pm 1^\circ\text{C}$ to $24 \pm 1^\circ\text{C}$ until the 15th day after hatching. Heat stress application (5 hours a day at 35°C) was started on the 16th day.

Tissue samples were taken from each group of 7 birds when they were 47 days old. The samples were both placed in ice-cold Periodate-lysine-paraformaldehyde (PLP) and 10% NBF (Neutral buffered formalin) for fixation, and then the samples that fixed 10% NBF were taken embedded in paraffin blocks for microtome section, while the samples that fixed PLP were taken frozen for cryostat sections. The samples were placed in an optimal cutting temperature compound (Tissue-Tek; Sakura Ltd., Torrance, CA, USA) and frozen immediately by plunging them into liquid nitrogen.

Preparation of tissue sections. Paraffin and cryostat sections were cut (6 μm thickness at 50 μm intervals). Serially taken tissue sections were placed on slides coated with organosilane (3-aminopropyltriethoxy-silane, A3648; Sigma).

Histochemistry. The sections were stained with Crossman's modification of Mallory's triple stain (13) for general morphological evaluation. Methyl Green-Pyronin and James silver techniques were applied for demonstration of plasma cells and reticular fibers, respectively (13).

Immunocytochemistry. Tissue samples were immunostained by the indirect immunoperoxidase (indirect IP) techniques for the detection of immunoglobulins (16). For this

purpose, goat anti-chicken IgG, IgA, and IgM antibodies (Bethyl Lab) were used. Antibodies were diluted to 1 : 250.

Clusters of differentiation (CD) CD3, CD4, and CD8 mouse anti-chicken antibodies (Southern Biotech) were applied to the cryostat sections to determine the T cell types in the spleen. The sections were fixed in ice-cold ethanol and then stained with the indirect IP technique. Primary antibodies were diluted to 1 : 200, 1 : 100, and 1 : 100 respectively (14).

Examination of tissue sections. The tissue sections were examined under a light microscope (Leica DMLB microscope) to determine the localization of CD3+, CD4+, and CD8+ T cells. The frequency of immunoglobulin-including plasma cells in the spleen was determined semi-quantitatively (1). The slides were photographed with a camera (Leica DC-200).

Statistical analysis. All obtained data were compared using several statistical methods. The conformity of data to normal distribution was examined with the Shapiro-Wilk test, and Levene's test was used to determine the homogeneity of variance. Because the data was not normally distributed the Kruskal-Wallis analysis of variance (ANOVA) was used for comparison between groups. Tamhane's T2 test was used to compare group averages. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. Differences were considered statistically significant if $p < 0.01$ and $p < 0.001$ (5).

Results and discussion

Histological examination. Trabecular arteries, central arteries, and veins were extending from the splenic capsule into the spleen. Red and white pulp regions were prominent (Fig. 1). The periarteriolar lymphoid sheath (PALS) surrounding the central artery consisted mainly of lymphocytes, plasma cells, and reticular fibers. The red pulp contained blood cells and lymphocytes. The germinal center (GCs) enveloping capsule was rich in reticulum fibers in the heat-stressed group. Plasma cells were mostly localized around the arterioles in the GC, and were observed in the peri-ellipsoidal lymphoid tissue (PEL). The basement membrane of the penicillar capillary and the capsule

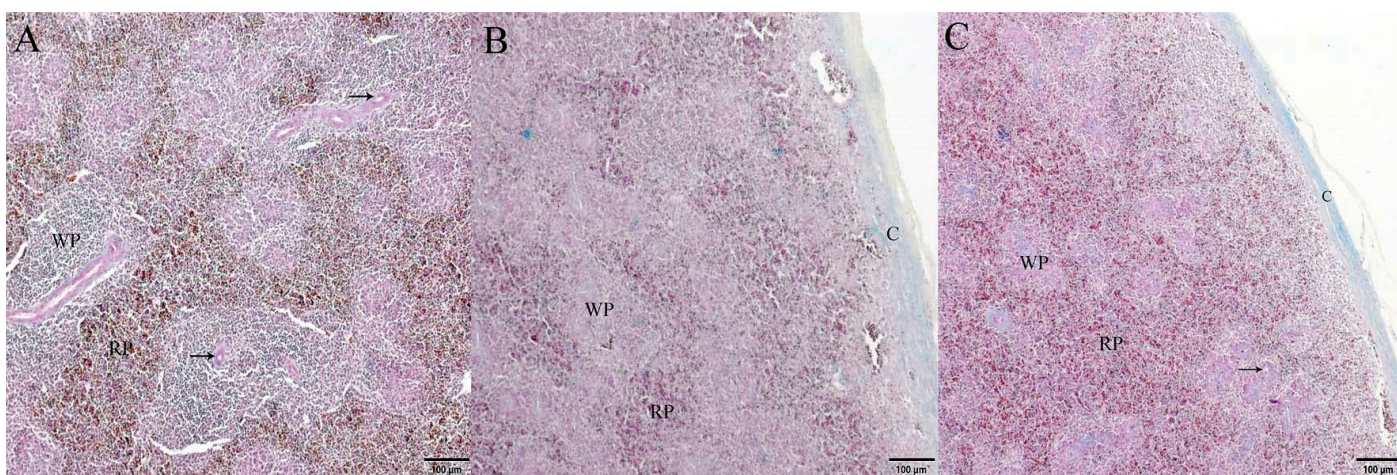


Fig. 1. Spleen histological view: A – Control, B – Heat stress, C – Heat stress + E vit

Explanations: WP – white pulp; RP – red pulp; arrow – central arteriole; C – capsule. Trichrome stain $\times 10$

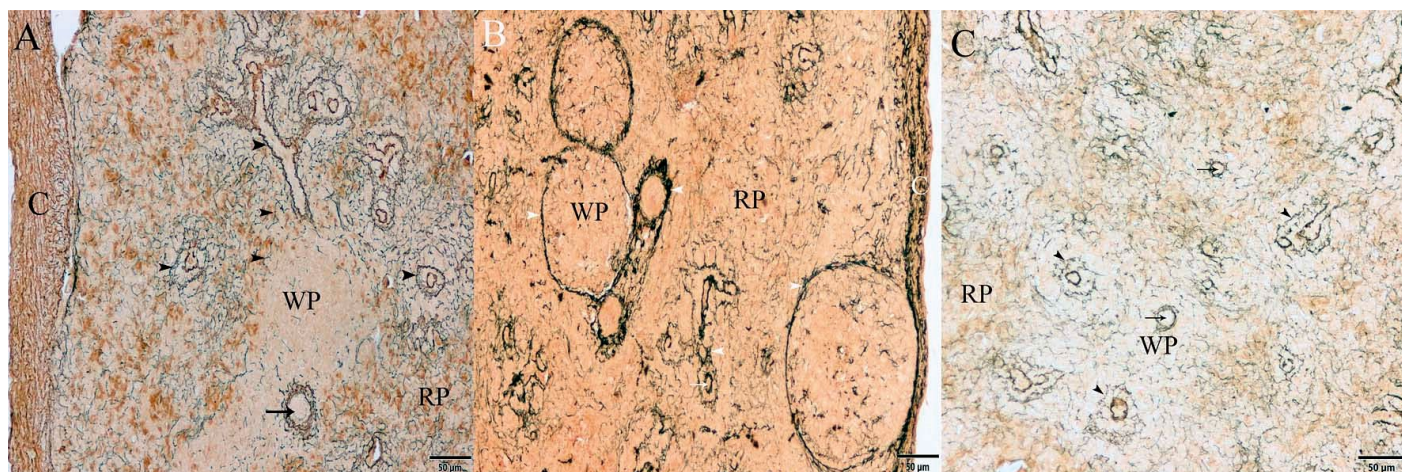


Fig. 2. Spleen histological view: A – Control, B – Heat stress, C – Heat stress + E vit

Explanations: WP – white pulp; RP – red pulp; arrow – central arteriole; arrowhead – reticular fibres; C – capsule. Metyhl Green-Pyronin and James silver techniques stain $\times 20$

of the Schweiger-Seidal sheath (SSS) were clearly observed with silver impregnation (Fig. 2).

Immunohistochemical examination. The frequencies of cluster of differentiation $CD3^+$, $CD4^+$, and $CD8^+$ cells in the spleen are shown in Table 1. Immunoglobulin A (IgA) $^+$ IgG $^+$, and IgM $^+$ cells in the spleen are shown in Table 2. In the spleen, clusters of $CD3^+$ cells were observed in the PALS of the white pulp and venous sinusoids (VS) of the red pulp (Fig. 3). The $CD4^+$ cells were seen in both the PALS, VS, and GCs (Fig. 4), while $CD8^+$ cells were observed in the red pulp (Fig. 5). Although the number of $CD3^+$ cells generally decreased in the spleen, they were con-

centrated around the PALS of the white pulp in the heat-stressed group. Similarly, the number of $CD4^+$ and $CD8^+$ cells decreased in the heat-stressed group and increased in the vitamin E-supplemented group. Immunoglobulin-including plasma cells (IgA, IgG, and IgM) were observed beneath the capsule, around the white pulps, PALS, and GC and outside the GC capsule. Immunoglobulin A-positive cells were seen in the PALS (Fig. 6), IgG-positive cells were observed in the GC (Fig. 7), and IgM-secreting plasma cells were found outside the GC capsule (Fig. 8). The numbers of IgG- and IgM-positive cells were higher than that of IgA-positive cells in all the chicks. The popula-

Tab. 1. Frequencies of $CD3^+$, $CD4^+$, $CD8^+$ T cells in different groups

	Control	Heat stress	Vit E + Heat stress
CD3	++++	+++	+++
CD4	++++	++	+++
CD8	++++	++	+++

Explanations: Staining intensity is indicated by +++++, very strong; +++, strong; ++, moderate; +, weak; $\times 10$ magnification

Tab. 2. Frequencies of IgA $^+$, IgG $^+$, IgM $^+$ plasma cells in different groups

	Control	Heat stress	Vit E + Heat stress
IgA	++	+	++
IgG	+++	++	+++
IgM	+++	++	+++

Explanations: Staining intensity is indicated by +++++, very strong; +++, strong; ++, moderate; +, weak; $\times 10$ magnification

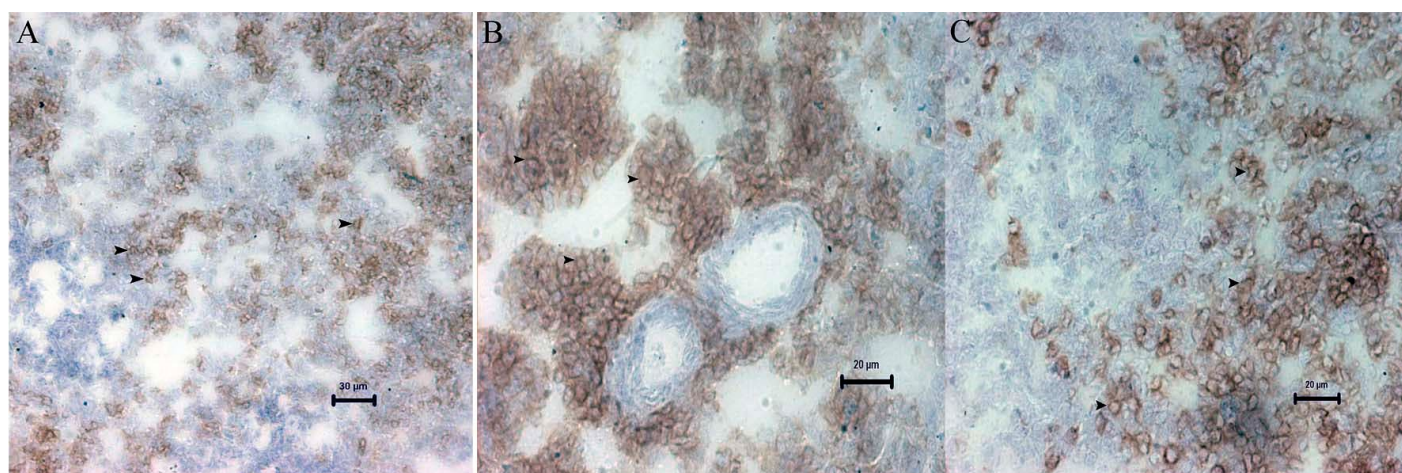


Fig. 3. Spleen CD3: A – Control, B – Heat stress, C – Heat stress + E vit

Explanation: arrowhead – CD3 positive cells $\times 40$

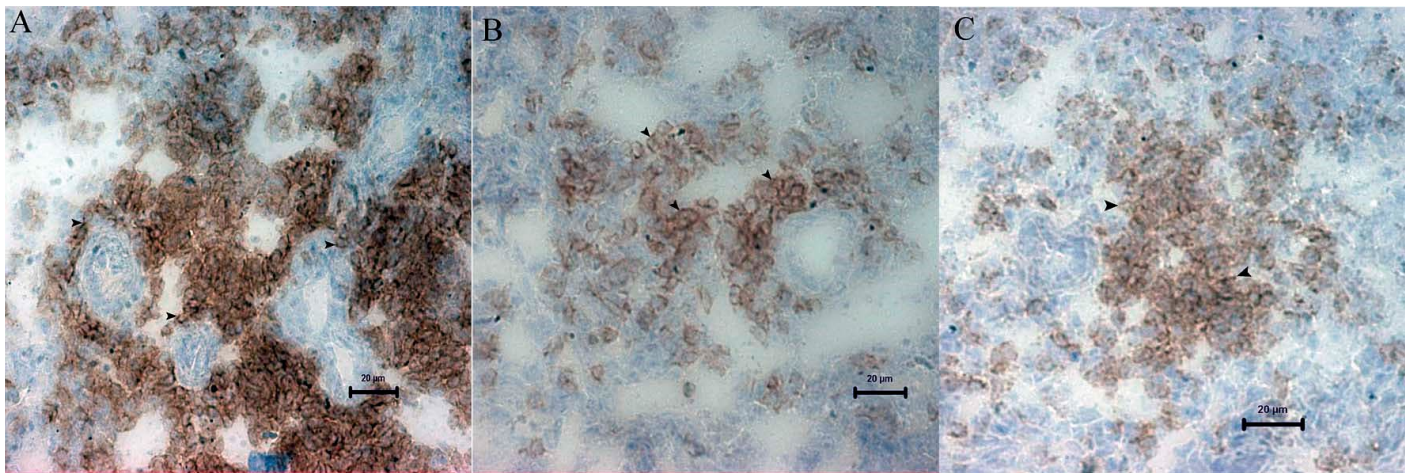


Fig. 4. Spleen CD4 A – Control, B – Heat stress, C – Heat stress + E vit
Explanation: arrowhead: CD4 positive cells $\times 40$

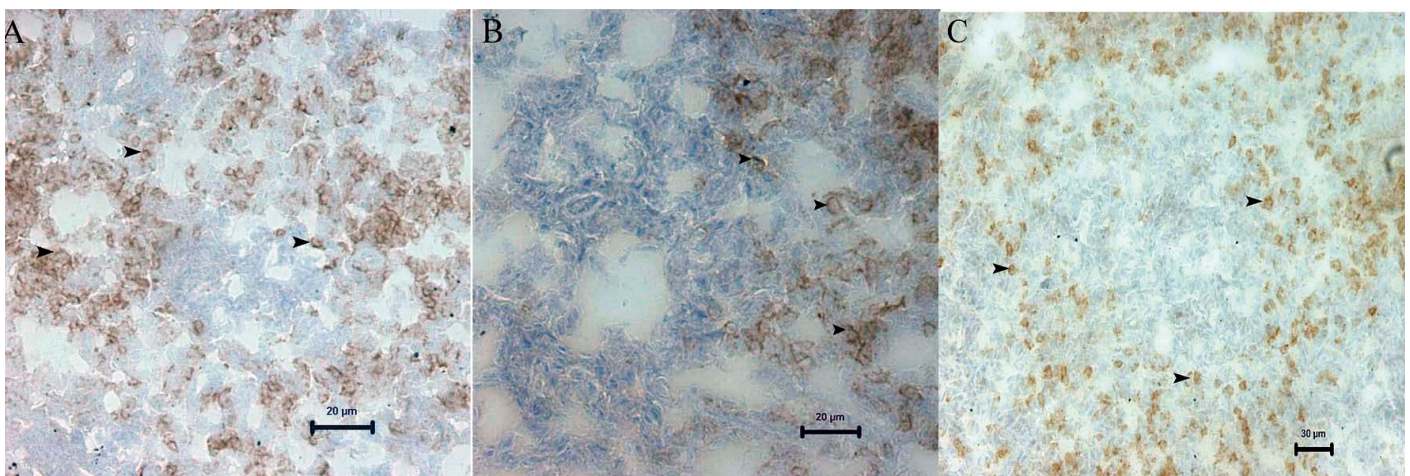


Fig. 5. Spleen CD8 A – Control, B – Heat stress, C – Heat stress + E vit
Explanation: arrowhead: CD8 positive cells $\times 40$

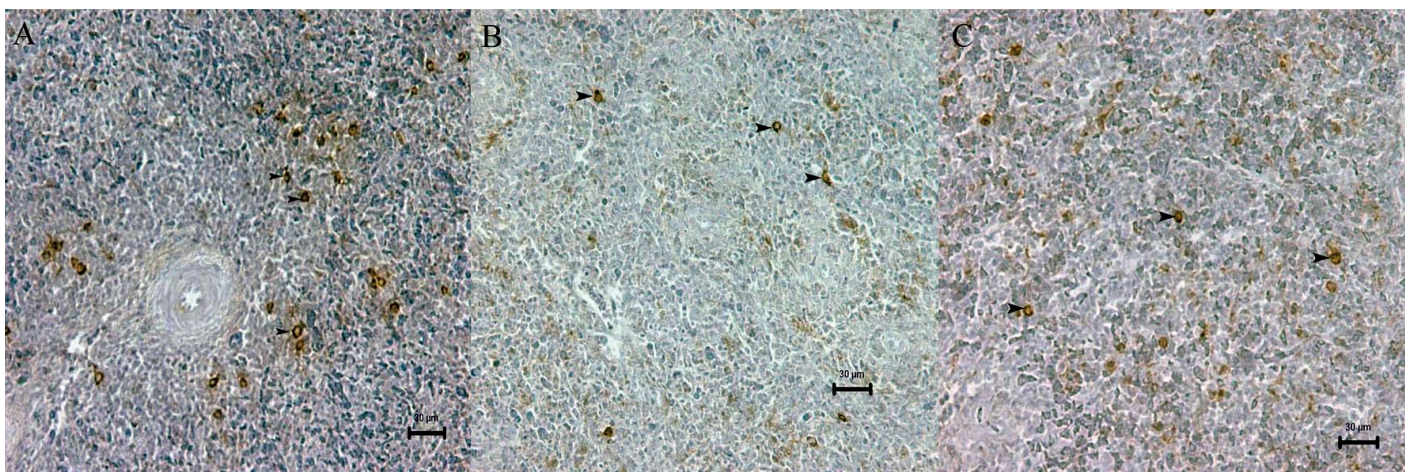


Fig. 6. Spleen IgA A – Control, B – Heat stress, C – Heat stress + E vit
Explanation: arrowhead: IgA positive cells $\times 40$

tions of IgA-, IgG-, and IgM-including cells in the heat-stressed group were increased in the spleen of the vitamin E-supplemented group compared with those of the control group (Tab. 1).

During prenatal life, the spleen is surrounded by a thin capsule, which gradually becomes thicker at later stages of life (15). Avian spleens have well-developed

ellipsoids, which are known as THE SSS (29). The findings of the spleen in this study were similar to those of other fowl spleen. Ellipsoids develop in the circumference of the penicillar arterioles, which are the terminal portions of the arteriole branches. In this study, reticular cells were observed around the ellipsoids. White and red pulp were observed in the

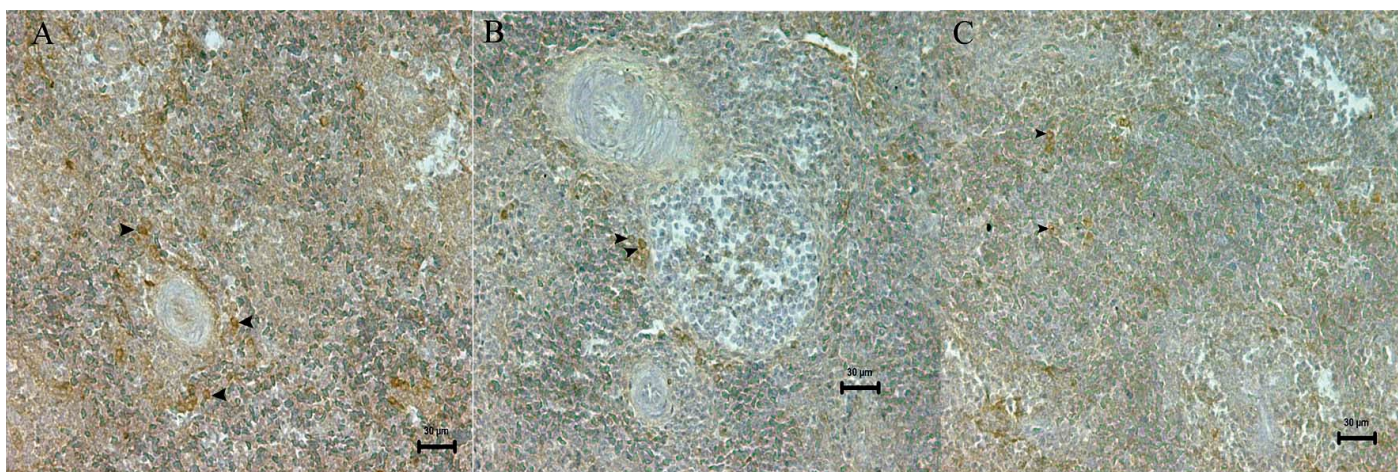


Fig. 7. Spleen IgG A – Control, B – Heat stress, C – Heat stress + E vit
Explanation: arrowhead: IgG positive cells $\times 40$

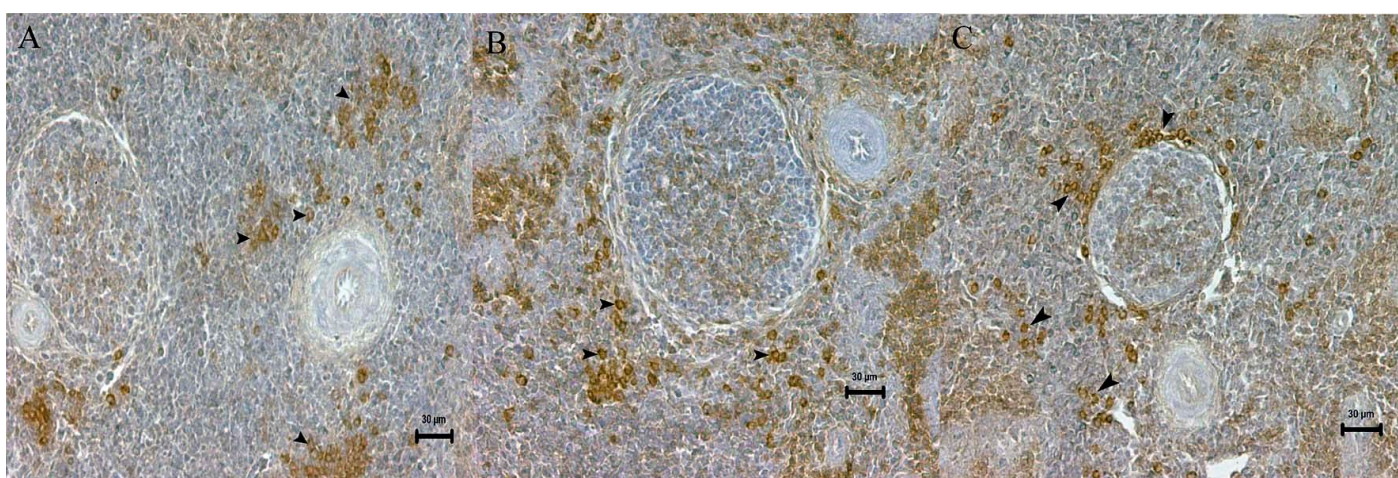


Fig. 8. Spleen IgM A – Control, B – Heat stress, C – Heat stress + E vit
Explanation: arrowhead: IgM positive cells $\times 40$

chicken spleen at seven weeks of age in the control and experimental groups, and the GC was prominent.

CD5 (2), CD8, and CD4 have been used as markers to identify T and B cells for over 30 years. Yasuda et al., (1998) reported that the PALS, also known as the T-cell area, are predominantly composed of T cells. Mature T cells have four major compartments: CD8 (Lyt-2) and CD4 (L3T4). T progenitor cells express CD4⁺8[−], but the role of CD4⁺8⁺ cells, which carry both CD4 and CD8 antigens, in thymic differentiation is unclear (18). However, it is known that the CD3 antigen receptor complex is important for thymic differentiation. CD3 is expressed in the cytoplasm of pro-thymocytes and in the membrane of later-stage thymocytes. CD3 binds to all mature T cell membranes, and to those of a small amount of Purkinje cells. CD3 is present at all stages of T-cell development (6). In the present study, CD3⁺ T cells were found in the PALS of the white pulp and in the VS of the red pulp. However, the number of CD3⁺ cells in the heat-stressed group generally decreased around the PALS of the white pulp, indicating that HS causes a decrease in the number of late-stage and mature T lymphocytes in the spleen. However, the use of vitamin E seemed to reduce this decrease.

CD4 is found on the surface of T helper cells, macrophages, monocytes, and dendritic cells, and it is a co-receptor that communicates with antigen-presenting cells (APCs) (9). The CD4 molecule recognizes and binds to major histocompatibility complex (MHC) class II molecules carrying antigens and is a marker of T helper cells (3). Zhu et al. (31) reported that CD4⁺ cells were detected in the light zone of GCs. The percentage of CD4⁺ cells was higher in chickens supplemented with 87 mg/kg of vitamin E than those of the other groups (supplemented with 0, 17, 46 mg/kg of vitamin E), but vitamin E had no effect on the percentage of CD8⁺ cells in the seven-week-old chicks (7).

CD8 is a transmembrane glycoprotein predominantly found on the surface of cytotoxic T cells, which plays an important role in T cell signaling. Unlike CD4, CD8 binds to MHC class I molecules and is a marker for cytotoxic T cells (8). In this study, the number of CD4⁺ and CD8⁺ cells decreased in the heat-stressed group, and increased in the vitamin E-supplemented group compared to the control group. This finding indicates that HS leads to a decrease in the number of both helper and cytotoxic T lymphocytes. It was also observed that vitamin E treatment reduced this effect.

IgA is the most prevalent antibody subclass in the human body and plays an important role in mucosal defense (22, 25). IgA is expressed on myeloid cells (macrophages, Kupffer cells, etc.) and is considered to be a non-inflammatory antibody, but IgA can inhibit or initiate inflammatory responses passively and actively (11). IgG is produced and expressed by plasma B cells and is one of the major components of humoral immunity. Some of the important functions of IgG, which have many different functions, are as follows: opsonization (coating of pathogen surface), pathogen elimination, and toxin neutralization, among others (26). Immunoglobulin G levels are measured during the diagnosis of many diseases (4). Immunoglobulin M is the first and largest antibody to appear against antigens in the body. Immunoglobulin M, which is known as a natural antibody, is released from B-1 cells. Unlike classical B cells, B-1 cells develop during the fetal and neonatal periods (12). The absence of natural IgM has been reported to increase local bacterial infections, causing widespread inflammation and high mortality (20).

Khan et al. (16) reported that selenium supplementation caused a sudden reduction of the number of IgM-including plasma cells in splenic tissue, and this condition was restored by vitamin E supplementation in chickens. Vitamin E may have a particular target effect on IgM-including plasma cells in comparison with IgA- and IgG-positive cells (16). Vitamin E protects the cells involved in the immune response against oxidative damage and enhances the function and proliferation of these cells (17). In this study, the number of IgG- and IgM-positive cells were higher than those of IgA-positive cells in all the chicks. Moreover, the population of IgA-, IgG-, and IgM-including cells decreased in the spleen of the heat-stressed group and increased in the spleen of the vitamin E-supplemented chickens, compared with the control group.

The findings of this study indicate that HS affects humoral immunity. However, the active and passive inflammatory responses and mucosal defense are also affected.

In countries with hot climates, heat stress causing poor performance in poultry during the summer months is a cause of concern. The aim of this study was to identify the effects of dietary vitamin E on HS in the spleen of chickens. Overall, from the findings of this study it can be concluded that vitamin E supplementation to broiler chicken diets could increase the population of immune system cells in heat-stressed chickens. Therefore, the findings of this study could be used as a reference for the development of management strategies for broiler chickens.

Ethical approval: Ethical approval for this study was given by the Institutional Animal Ethics Committee of Adnan Menderes University (64583101/2021/179).

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