EFFECTS OF VITAMIN E ON T CELLS IN GUT-ASSOCIATED LYMPHOID TISSUE (GALT) OF BROILER CHICKENS UNDER HEAT STRESS

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Abstract. The aim of this study was to explore the effects of vitamin E on T cells number and distribution within GALT (Gut associated lymphoid tissue) in broiler chickens submitted to heat stress. For that, the CD3, CD4 or CD8 positive cells were investigated by immunohistochemistry in the oesophageal, pyloric, jejunum, ileum and caecal tonsils from 4, 5, 6 weeks old Ross 308 male broilers reared under standard temperature conditions (22 ± 2°C) (group C) or submitted to heat stress (35°C for 5 hours per day) (group HS) and eventually treated with vitamin E (DL-α-tocopherol acetate, 300 mg/kg, (group HSE), each group containing 21 birds. Heat stress markedly depleted T cell population in GALT but the cell distribution was not modified. By contrast, the vitamin E treatment has considerably increased the T cell population by acting on all T cell types in every lymphoid area (inter-follicular zones, germinal centers, epithelial crypts). These results show that vitamin E can remarkably counteract the adverse effect of heat stress on the immune function and dietary vitamin E supplementation can be recommended in broilers, especially in the summer months.

Keywords: broiler chickens, heat stress, vitamin E, Gut-Associated Lymphoid Tissue, T cells

Introduction. At present, broilers are more forced to lessen food consumption and to gain weight faster with each passing day in the poultry industry (Alarslan, 2000). Heat is the most important cause of stress for poultry. Conditions of heat stress cause an increasing concern in poultry production due to the rapid development of poultry industry in hot climate countries and to the reduced performance of poultry during summer months in temperate countries (Bonnet *et al.,* 1997; Abu-Dieyeh, 2006). High environmental temperatures affect the development of specific immune responses in chickens (Thaxton *et al.,* 1968; Thaxton and Siegel, 1972; Niu *et al.,* 2009).

Chickens do not possess any lymph nodes, but very developed various types of lymphoid tissue (Arai *et al*., 1988; Matsumoyo and Hashimato, 2000). GALT (Gut-associated lymphoid tissue) plays an important role in the mucosal immune response (Lillehoj and Trout, 1996). Due to their localisation, oesophageal tonsils are frequently exposed to environmental as well as food antigens (Olah *et al.,* 2003). Pyloric tonsil is a novel peripheral lympho-epithelial organ of the gastrointestinal tract in the chicken. According to Nagy and Olah (2007), together with the oesophageal tonsil, pyloric tonsil controls the spread of antigens to the blood via the mesenteric lymph vessels. Lymphoid tissue is present at the anti-mesenterial side of chicken jejunum and ilocaecal transition (Befus *et al.,* 1980; Casteleyn *et al.,* 2010). The caecal tonsils are two large lymphoid aggregates occurring at the caecum rectum junction (Gomez Del Moral *et al.,* 1998). Caecum is exposed to the permanent and insistent invasion of bacterial or non bacterial antigens of extra-caecal origin, since it receives the back flowing urine from the urodeum of the cloaca through the rectum. Therefore, caecal tonsils play a highly important role in immunological surveillance against foreign microorganisms (Kitagawa *et al.,* 1998; Rezaian and Hamedi, 2007).

In the past, vitamin E was approved as a vitamin against sterility. Today, it has gained great importance as an antioxidant. Due to a strong antioxidant activity, vitamin E protect from oxidation some very important functions such as protein synthesis in cells and membrane formation (Dündar and Aslan, 1999). Heat stress as a stressor stimulates the release of corticosterone and catecholamines. It also damages the structures of cell membranes throughout lipid peroxidation (Freeman and Capro, 1982; Panda *et al.,* 2008).

The aim of this study was to explore the effect of vitamin E on the localization of T lymphocyte subpopulations in oesophageal and pyloric tonsils, in jejunum and ileum as well as in caecal tonsils of broilers submitted to heat stress.

Material and Methods. In this study, 63 one day-old Ross 308 breed male broiler chickens were used. The chickens were divided into three groups [control group exposed to an ambient temperature of 22 ± 2°C, heat stress group submitted to 35°C for 5 hours per day and vitamin E treated group submitted to 35°C for 5 hours per day and treated with vitamin E (DL-α-tocopherol acetate, Merck) at the dose of 300 mg/kg by oral gavage], each containing 21 birds. The chickens were fed *ad libitum* with a starter diet from day 1 to day 21 and with a growing / finishing diet from day 22 to day 42. The ambient temperature on day one was set at 32 ± 1°C and then gradually decreased until it reached 24 ± 1°C on day 15. Application of heat stress (35°C for 5 hours per day) began on the sixteenth day. The protocol design was approved by the Institutional Animal Ethics Committee of the University of Adnan Menderes.

Tissue samples (oesophageal junction with proventriculus, pylorus junction with the duodenum,

jejunum-ileum and right proximal caecum) were taken from 7 birds in each group when birds were 4, 5 and 6 weeks old. Tissue samples were placed in the Optimal Cutting Temperature compound (Tissue-Tek; Sakura Ltd, USA) and immediately frozen by plunging into liquid nitrogen. The frozen samples were stored in a freezer at -20°C until used for immunocytochemistry. Cryostat sections for immunohistochemistry were cut at intervals of 50 µm and with thickness of 6 µm. Tissue sections taken serially were placed on organosilane (3-aminopropyltriethoxy-silane, A3648; Sigma) coated slides. Sections were immunstained by the Avidin-Biotin-Peroxidase Complex (ABC) method (Withanage *et al.,* 1997). They were fixed in ice-cold 100 % ethanol for 5 minutes. After washing with PBS for 5 minutes, they were placed in a moisture chamber and preincubated with 8 % normal goat serum in 100 mM phosphate-buffered saline for 30 minutes, followed by incubation overnight at 4°C with primary mouse monoclonal antibodies against chicken CD3, CD4 and CD8 T cell clusters (Southern Biotech) diluted to 1:200, 1:100 and 1:100 respectively. The sections were placed in PBS for 5 minutes after washing with PBS. The secondary antibody (1 % biotin-conjugated goat anti-mouse immunoglobulins, DAKO E0433) diluted to 1:200 was applied for 1 hour at room temperature (22 ^{0}C). The sections were held in PBS for 5 minutes after washing with PBS. They were then incubated with an ABC (Avidin-Biotin Complex) solution (Vectastain ABC kit, Vector Laboratories Inc., USA) for 1 hour at room temperature (22 ^{0}C). The sections were placed in TBS (Tris-Buffer-Saline) for 5 minutes and were finally coloured with substrate-chromogene solution (3.3-diaminobenzidine-tetrahydrochloride dehydrate D-5905; Sigma) and slightly counterstained with haematoxylin. Control sections were stained by a procedure as described above but monoclonal antibodies were not applied to the sections. Th eprepared sections were examined under a light microscope (Leica DMLB microscope), localisation and the frequency of the CD3, CD4, CD8 positive T cells in the different tissues were determined semi-quantitatively (Bianchi *et al.,* 1992; Kum *et al.,* 2013). Fifteen microscopic fields (40 X) were examined for each tissue for each antibody. Photographs of the tissues were taken with a Leica DC-200 camera. All data were checked for normal distribution with Shapiro-Wilk and homogeneity of variance with Levene's test. The data were compared among groups using Kruskall-Wallis analysis of variance (ANOVA), according to data were not normally distributed. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. The differences were considered statistically significant if $P<0.01$ and $P<0.001$ (Conover, 1980).

Results. The frequencies of CD3, CD4 and CD8 positive cells in the different parts of the GALT are reported in Table I and Fig. I.

Although the oesophageal tonsil structure was not obvious in 4 week old chickens and that lymphoid tissue was organized as lymph follicles, the density of CD3 positive cells was higher than those of the CD4 or CD8 positive cells in the control group. In chickens exposed to heat stress, the density of CD3 positive cells remained higher than the CD4 and CD8 positive cells in the oesophagus but the frequencies of CD3, CD4 or CD8 positive cells have both markedly declined in the stressed birds. On the other hand, in chickens exposed to heat stress and treated with vitamin E, the density of T cells was dramatically increased in lymphoid tissues from oesophagus whatever the age of the chickens and the densities of CD3, CD4 and CD8 positive cells were similar.

In the controls, the CD3, CD4 and CD8 positive cells were mainly seen in the *lamina propria* and in the periphery on the lymph follicle (Fig. 1A). Only few positive cells were seen in germinal centers or in intraepithelial localisation (Fig. 1B). T positive cells were also observed in inter-follicular areas (IFA) in 5 week old chickens. When they were 6 week old, positive cells were localized in lymphoid masses in the *lamina propria* underneath the surface epithelium and surrounding the mucous glands. The T cell localisation in oesophageal tonsils was not markedly modified throughout exposure to heat stress. In chickens exposed to heat stress, the CD3, CD4 or CD8 positive cells were especially seen in the subepithelial zone in the *lamina propria* as well as in the periphery of the lymph follicles (Fig. 1C). T positive cells were rarely seen in the germinal centers. In older chickens, positive cells were located in IFA and in lymphoid masses surrounding the mucous glands (Fig. 1D) as in the corresponding control birds. Positive cells occupied the periphery of the masses and few positive cells were seen in the central area of the masses.

When vitamin E treatment was applied, positive T cells were also found in the subepithelial zone of the *lamina propria*, particularly in the IFA in 5 week old birds (Fig. 1E) and around the mucous glands in the older chickens. They were also seen peripherally in the follicles and were observed only sporadically in the center (Fig. 1F). In the 5 and 6 weeks old birds treated with the vitamin E, the CD4 positive cells were more often seen in the germinal center (Fig. 1G) than CD8 positive cells whereas these last ones were more abundant in the intraepithelial zone.

As shown in Table I, similar densities in CD3, CD4 or CD8 positive T cells were found in the pyloric tonsils whatever the age of birds. However, the T cell counts appeared lowered in birds submitted to heat stress whereas co-treatment with vitamin E has restored the T cell frequencies to those found in the control chickens.

In addition, the localisation of T cells in the pyloric tonsils was similar among the 3 groups: CD3, CD4 and CD8 positive T cells were accumulated in the inter-follicular regions (Fig. 2A), and appeared scarcely in the germinal centers. In birds treated with vitamin E, many CD4 + cells were seen around the crypts (Fig. 2B).

The frequencies of CD3, CD4 and CD8 positive cells were similar in the jejunal and ileal lymphoid structures and gradually increased with the age of the control chickens as reported in Table I. No differences in the T cell densities were evidenced between chickens submitted

to heat stress and the controls until birds were 6 week old: at this time, the T cell densities remained low in the stressed birds. By contrast, the vitamin E treatment has markedly and precociously increased the T cell counts in the jejunal and ileal structures since chickens were 4 week old. Again, the T cell localisation was not strictly affected by heat stress and/or vitamin administration compared to the not stressed and not treated chickens: CD3, CD4 and CD8 positive cells were distributed in the *lamina propria*, around the intestinal glands and core of the villi in the jejunum and ileum (Fig. 3A). Nevertheless, it was noted accumulation of the CD8 positive cells around the jejunal crypts in vitamin E treated birds (Fig. 3B) and T cells appeared as more dispersed in villi and lymph follicles in the stressed birds (Fig. 3C).

Table 1. *Frequencies of CD3, CD4, CD8 T cells in different groups, olds and tissues*

			Oesophageal tonsils	Pyloric tonsils	Jejunum	Ileum	Caecal tonsils
4-week-old	Control	CD3	++++	+++	++	++	++++
		CD4	+++	+++	++	++	++
		CD8	+++	+++	++	++	+++
	Heat stress	CD3	+++	++	++	++	+++
		CD4	++	++	++	++	++
		CD8	++	++	++	++	++
	VitE+Heat stress	CD3	++++	++++	+++	+++	++++
		CD4	++++	+++	+++	+++	++++
		CD8	++++	+++	+++	+++	++++
5-week-old	Control	CD3	++++	+++	++	++	+++
		CD4	+++	+++	++	++	++
		CD8	+++	+++	++	++	+++
	Heat stress	CD3	+++	++	++	++	+++
		CD4	++	++	++	++	++
		CD8	++	++	++	++	++
	VitE+Heat stress	CD3	++++	+++	+++	+++	++++
		CD4	++++	+++	+++	+++	++++
		CD8	++++	+++	+++	+++	++++
6-week-old	Control	CD3	++++	+++	+++	+++	+++
		CD4	+++	+++	+++	+++	++
		CD8	+++	+++	+++	+++	+++
	Heat stress	CD3	+++	++	++	++	+++
		CD4	++	++	++	++	++
		CD8	++	++	++	++	++
	VitE+Heat stress	CD3	++++	++++	++++	++++	++++
		CD4	++++	+++	+++	+++	++++
		CD8	++++	+++	+++	+++	++++

++: little, +++: moderate, ++++: marked, Vit E: Vitamin as DL-α-tocopherol acetate.

In caecal tonsils, CD3 positive cells in controls were abundantly distributed within the lymphatic nodules whereas the density of the CD4 positive cells was less. Few CD3, CD4 and CD8 positive cells were also present throughout the germinal centers. The CD8 positive cells were mainly found in the peripheral zone of the germinal centers (Fig. 4A) and in the epithelium of the crypts (Fig. 4A). No differences in the overall density of T cells or in their localisation were observed according to the age of the control chickens (Table I). In birds exposed to heat stress, the T cell counts were lowered and they were especially found in the subepithelial diffuse lymphoid tissue and scarcely in the germinal centers (Fig. 4B) while in birds co-treated with vitamin E, the density of T cells was markedly increased: they were distributed within the *lamina propria* and the crypt epithelium was loaded with

CD3 positive cells (Fig. 4C). Many of them were CD8 positive cells (figure 4D) and some CD4 positive cells were observed around the tonsils and in the epithelium of the crypts (Fig. 4E). As in the other groups, few positive cells were seen in the germinal centers.

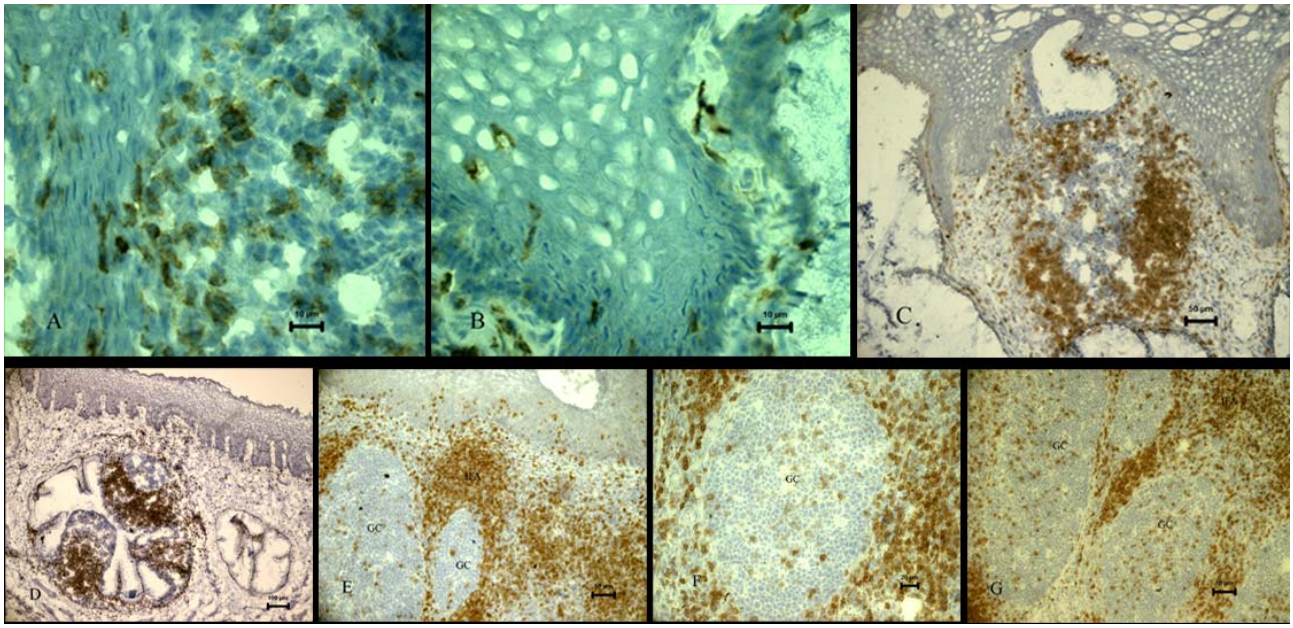

Figure 1: A. The CD3 positive cells were mainly seen in the *lamina propria* of the oesophageal tonsils in 4-week-old control chicken, Bar: 10 µm. **B.** The CD3 positive cells were also scarcely found an in intraepithelial localisation in 4-week-old control chicken, Bar: 10 µm. **C.** The CD4 positive cells were observed in periphery on the lymph follicles of the oesophageal tonsils from 5-week-old chickens exposed to heat stres. Bar: 50 µm. **D.** The CD4 positive cells were found in lymphoid masses surrounding the oesophageal mucous glands in 6-week-old chickens exposed to heat stress. Bar: 100 µm. **E.** The CD3 positive cells were mainly found in the inter-follicular areas (IFA) of the oesophageal tonsils in 5-week-old chicken exposed to heat stress and treated with vitamin E. Bar: 100 µm. **F.** Few CD3 positive cells were seen in the germinal center (GC) of the oesophageal lymph follicles in 5-week-old chicken exposed to heat stress and treated with vitamin E. Bar: 20 µm. **G.** CD4 positive cells were relatively abundant in the germinal center (GC) of the oesophageal lymph follicles in 5-week-old chicken exposed to heat stress and treated with vitamin E. Bar: 50 µm.

GC: Germinal center, IFA: Inter-follicular areas

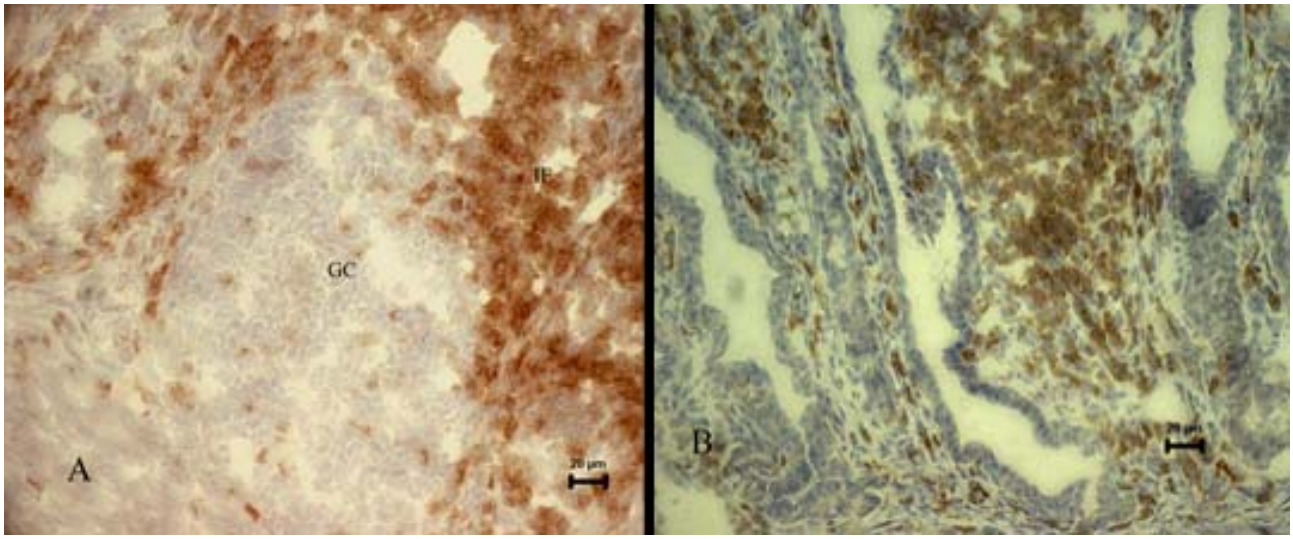

Figure 2: A. CD3 positive T cells were observed in the interfollicular (IF) regions of the pyloric tonsils in 5-week-old chicken exposed to heat stress and treated with vitamin. Bar: 20 µm. **B.** CD4 positive T cells were seen around the crypts of the pyloric tonsils in 5-week-old chicken exposed to heat stress and treated with vitamin E. Bar: 20 µm.

GC: Germinal center, IF: Interfollicular regions.

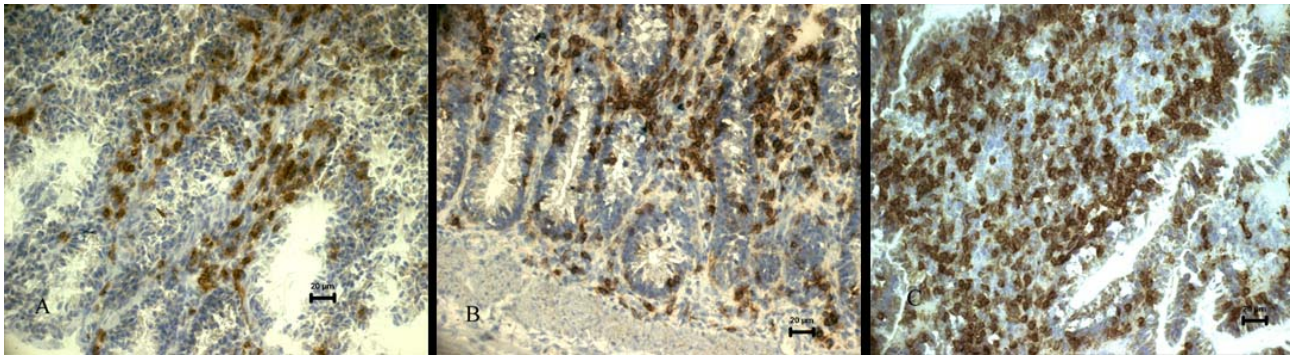

Figure 3: A. CD8 positive T cells were found in the *lamina propria*, around the intestinal glands and core of the villi of ileum in 6-week-old chicken exposed to heat stress and treated with vitamin E. Bar: 20 µm. **B.** CD8 positive T cells were found abundant around the crypts of the jejunum in 6-week-old chicken exposed to heat stress and treated with vitamin E. Bar: 20 µm. **C.** CD4 positive T cells were dispersed in the villi and lymph follicles of ileum in 5-week-old chicken exposed to heat stress. Bar: 20 µm.

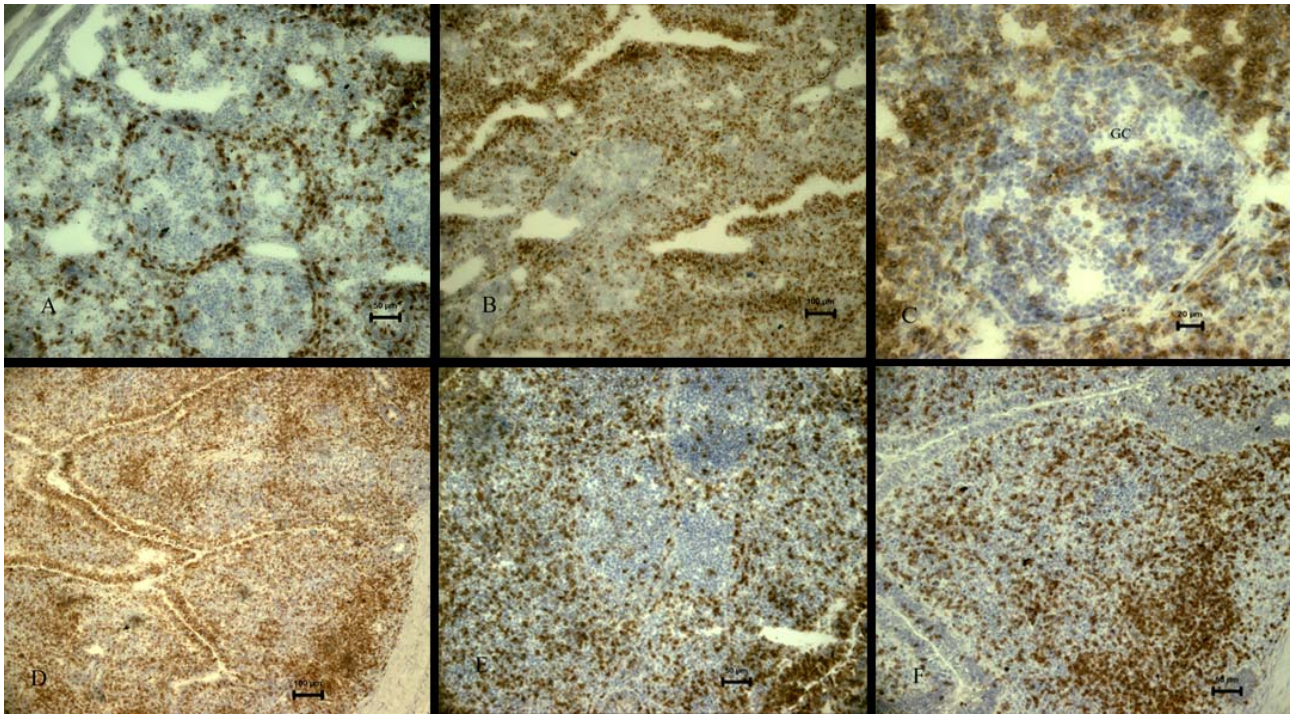

Figure 4: A. CD8 positive T cells were found in the peripheral zone of the germinal centers and within epithelium in the caecal tonsils in 5-week-old control chicken, Bar: 100 µm. **B.** CD3 positive cells were seen in the subepithelial diffuse lymphoid tissue and scarcely in the germinal centers in the caecal tonsils in 6-week-old chicken exposed to heat stress. Bar: 20 µm. **C.** CD3 positive cells abundantly loaded the crypt epithelium in the caecal tonsils in 6-week-old chicken exposed to heat stress and treated with vitamin E. Bar: 100 µm. **D.** CD8 positive cells were seen in the crypt epithelium in the caecal tonsils in 5-week-old chicken exposed to heat stress and treated with vitamin E. Bar: 50 µm. **E.** Few CD4 positive cells were seen in the crypt epithelium in the caecal tonsils in 6-week-old chicken exposed to heat stress and treated with vitamin E. Bar: 50 µm.

GC: Germinal center.

Discussion. Environmental conditions are extremely important for poultry production. Heat is a significant stressing factor for broilers. Bonnet et al. (1997) found that food digestibility of broilers was significantly decreased in chronic heat exposure. Kussaibati et al. (1982) reported that it rose at thermoneutrality, but a decreased lipid digestibility with saturated fats was observed in young chickens. Zuprial et al. (1993) found that chronic heat stress significantly decreased protein digestion and Wolfenson et al. (1987) observed reduced blood flow in the upper gastrointestinal tract after chronic heat stress. Mashaly et al. (2004) indicated that exposure to heat stress can reduce both the number and activities of leukocytes in laying hens. Thaxton et al. (1968) demonstrated that high environmental temperatures affect the development of specific immune responses in young

chickens. Oznurlu et al. (2010) found that long term heat stress for embryos (40.1-40.6°C egg temperature) induced a delayed development of thymus and bursa of Fabricius. The same researchers observed that peripheral blood ACP- ase (acid phosphatase) - and ANAE (alpha-naphtyl acetate esterase)-positive lymphocyte counts were lower in heat stressed animals than in the controls (Oznurlu *et al.*, 2010).

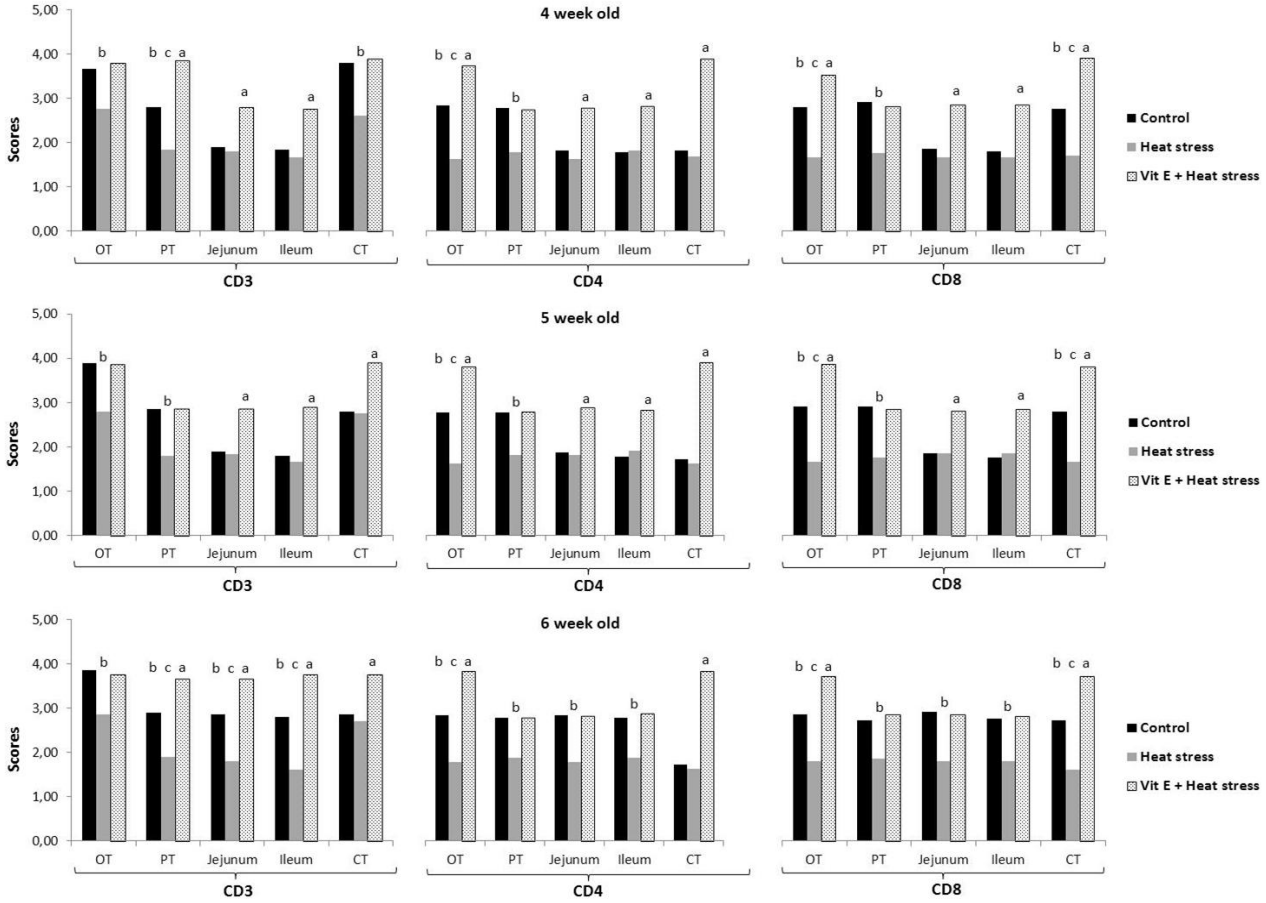

Figure I. *Scores of CD3, CD4, CD8 T-cells in different groups, olds and tissues. OT-Oesophageal tonsils, PT-Pyloric tonsils, CT-Caecal tonsils, Vit E-Vitamin as DL-α-tocopherol acetate.*
[a, b, c] Different letters indicate statistically significant differences (P<0.001.) in the same row.

This study was conducted to determine the effects of heat stress and application of vitamin E against heat stress in oesophageal, pyloric and caecal tonsils as well as in jejunum and ileum lymphoid structures.

Olah et al. (2003) reported that T lymphocytes in the oesophageal tonsils occurred mainly in the inter-follicular (IFA) space but that they were also found in the germinal centers. In the present study, positive cells were mainly observed in inter-follicular areas (IFA), and scarcely in intraepithelial areas and in germinal centers. In 6 week old chickens, positive cells localized in lymphoid masses in the *lamina propria* underneath the surface epithelium and surrounding the mucous glands. Similarly, positive T cells in the pyloric tonsils were mainly seen in inter-follicular regions, and few positive cells were also encountered in the germinal centers. Nagy and Olah (2007) found that the dense inter-follicular regions were filled with $CD3^+$ T cells in pyloric tonsils and that many of them expressed CD4 and were considered as helper T cells whereas few cells were CD8 cytotoxic T cells. Vervelde et al. (1998) described that a great number of leukocytes were $CD3^+$ lymphocytes, especially CD4 or CD8 positive T cells, located in the epithelium and *lamina propria* of chick intestine. The number of intra-epithelial leukocytes was greatest in the duodenum and jejunum, and decreased in the proximal part of the caecum and in the colon (Vervelde and Jeurissen, 1993). Lillehoj and Chung (1992) found that jejunum intraepithelial lymphocytes expressing the CD8 antigen increased gradually until 4-6 weeks of age and subsequently declined when chickens were more aged while $CD4^+$ cells represented a minor subpopulation among the intestinal lymphocyte subpopulations. In this study, CD3, CD4 and CD8 positive cells in the jejunum and ileum of chicken were distributed in the *lamina propria*, around the intestinal glands and core of the villi. Yasuda et al. (2002) observed that many CD4 positive cells were seen in the inter-follicular areas of the caecal tonsils. On the contrary, Lillehoj and Trout (1996) claimed that CD4 positive T cells occurred in the central and deep zone of the tonsils. Gomez Del Moral et al. (1998) reported that CD8 positive cells predominate as intraepithelial lymphoid cells as well

as in the subepithelial zone although some CD8 positive cells were found in the diffuse lymphoid tissue. In this study, numerous CD3 positive cells were distributed within the lymph nodules but the density of CD4 positive cells were less. Few CD4 and CD8 positive cells were also present throughout the germinal centers. CD8 positive cells were also found in the peripheral zone of the germinal centers and in the epithelium.

Heat stress has markedly reduced the counts of T cells in GALT in the present study but no change in the T cell distribution was observed. These results agree with previous findings (Thaxton *et al.,* 1968; Wolfenson *et al.,* 1987; Mashaly *et al.,* 2004; Panda *et al.,* 2008; Oznurlu *et al.,* 2010). This effect could indirectly be related to increase in release of corticosteroids under heat stress. Heat stress stimulates release of corticosterone and catecholamines and initiates lipid peroxidation in membranes of cells including T and B lymphocytes (Panda *et al.,* 2008). Chronically high corticosterone concentrations decrease disease resistance (Gross, 1992) as corticosteroids released in response to stress have strong immunosuppressive effects (Panda *et al.,* 2008).

It is reported that vitamin E can reduce the negative effects of corticosterone induced by stress (Watson and Petro, 1982; Panda *et al.,* 2008) and acts as a potent biological chain breaking antioxidant that protects cells and tissues from lipid peroxidation damage induced by free radicals (Mc Dowell, 1989). In parallel, vitamin E provides protection against oxidative damage for cells involved in the immune response and enhances proliferation and functions of these cells (Panda *et al.,* 2008; Meydani and Blumberg, 1993). It is suggested that vitamin E has positive effects on cellular and humoral immune function and may enhance the resistance of broilers to infectious diseases (Abdukalykova *et al.,* 2008) Ig-containing plasma cells in the GALT of the broilers were decreased or not affected by heat stress but increased by vitamin E supplementation (Kum *et al.,* 2013). However, no beneficial effects from higher concentrations of dietary vitamin E (125 or 250 IU/kg) on egg quality parameters were observed in White Leghorn laying hens (Panda *et al.,* 2008). In this study, the densities of T positive cells were higher when broiler chickens were treated with vitamin E despite exposure to heat stress compared to the exposed birds or to the not stressed controls: CD4 and CD8 positive cells were more often seen in intraepithelial position in the well-organized oesophageal tonsils since chickens were 5 week old, than in the other groups; CD3 positive cells, and especially $CD8^+$ cells, were very abundant around the crypts and in the *lamina propria* of the villi in jejunum and ileum and many CD8 positive cells were observed around the tonsils and the epithelium of the tonsillar crypts. In agreement with this positive effect of vitamin E on the immune response, Erf et al. (1998) reported that percentages of CD4 positive splenocytes decreased in two week old chicks whereas these cells gradually proliferated in vitamin E treated chickens, especially when they were 7 week old. Additionally, Parmentier et al. (1995) found that functional capacities of the CD4 and CD8 positive cells were increased in 7 week old chicks. Erf et al. (1998) also reported that dietary vitamin E supplementation significantly affected T cell differentiation in the thymus and spleen of broiler chickens. Puthpongsiriporn et al. (2001) demonstrated that vitamin E supplementation at 65 IU/kg diet may enhance proliferation and activation of the lymphocyte proliferation induced by Concavalin A and *Salmonella typhimurium* lipopolysaccharide (LPS) *in vitro*, and that the antioxidant properties of egg yolks and plasma of White Leghorn hens were increased during heat stress (Puthpongsiriporn *et al.,* 2001)

As a conclusion, although heat stress induced a severe depletion of T cells in GALT in broiler chickens, the vitamin E administration has promoted and exacerbated the proliferation of T cells in oesophageal, pyloric, jejunum, ileum and caecal lymphoid structures. This positive immune effect may be related to the action of vitamin E as major lipid soluble antioxidant present in all cellular membranes. For this reason, especially in the summer months, vitamin E supplementation can be recommended in diets of broilers.

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