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COMPARISON OF CELL DEATH RATE IN VARIOUS CHICKEN LYMPHOCYTES POPULATIONS IN A RESPONSE TO THREE TEMPERATURE TREATMENTS

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ABSTRACT

The goal of the experiment was to evaluate susceptibility of lymphocytes populations derived from bursa of Fabricius, spleen, thymus, and blood to temperature treatments *in vitro*. Temperatures used in study was 37 °C (thermoneutral), 41 °C (mild heat stress) and 45 °C (severe heat stress). Cells were incubated for 8 hours and after 3 and 8 hours were measured numbers of apoptotic, necrotic and CellROX positive cells by flow cytometry. Representation of live lymphocyte phenotypes (CD3, CD4, CD8, BU1) in cell populations were also measured. At all temperature treatments, thymus lymphocytes population had the lowest ($p < 0.05$) percentage of apoptotic cells. On the other hand, significantly highest numbers ($p < 0.05$) of apoptotic cells were found in bursal lymphocytes after 8 hours of incubation. Differences in numbers of necrotic cells were more pronounced at 45 °C, where after 3 and 8 hours of incubation were significantly highest numbers ($p < 0.05$) of necrotic cells in spleen. Increasing temperature induce production of reactive oxygen species (ROS) in cells and highest proportion ($p < 0.05$) of ROS-positive cells were found in bursal and spleen cell population after 8 hours of incubation. Transmission electron microscopy showed loss of mitochondrial matrix and disrupted mitochondrial membrane in mitochondria of lymphocytes at 45 °C as initial damage caused by ROS. From individual cell phenotypes, there were significantly decreased ($p < 0.05$) B lymphocytes in bursa of Fabricius, CD4+ and CD8+ lymphocytes in thymus and whole T lymphocytes population in spleen at 45 °C. Various susceptibility to temperature can be predictive factor of worse function in some organs in stress conditions where body temperature increase.

Keywords: broiler chicken, temperature, immunity, apoptosis, oxidative stress

INTRODUCTION

Temperature represents the biggest stressor of current poultry breeding and production. Heat stress (HS) has deleterious effects mainly in the broiler chickens due to their rapid metabolism and high density of chickens in production facilities. Many negative consequences of HS were described in broiler chickens which lead to poor performance **(Liu *et al.*, 2020)**. HS suppresses the immune system of the body and disrupts the architecture of the lymphoid tissues in chickens **(Hirakawa *et al.*, 2020)**. HS is known to induce increased levels of pro-inflammatory cytokines such as IL-1β, IL-6, IL-18, and TNF-α **(Goel *et al.*, 2021).** Chronic inflammation is a negative consequence of HS, which increase body temperature **(Quinteiro-Filho *et al.*, 2010; Cândido *et al.*, 2020).** Second negative consequence of HS is oxidative stress (OS). OS is presented by overproduction of reactive oxygen species (ROS) above the chicken antioxidant capacity **(Akbarian *et al.*, 2016)**. Overproduction of ROS induce cell death signaling pathways resulted in apoptosis and necroptosis **(Redza-Dutordoir and Averill-Bates, 2016).** Due to immunosuppressive effects of HS on lymphoid organs of the chicken, it was described increased levels of cell death in main lymphoid tissue. In bursa of Fabricius were found pathological changes and higher levels of apoptosis after exposure to HS **(Liu *et al.*, 2021)**. In chicken spleen, HS treatment induced upregulation of apoptotic genes expression **(Xu *et al.*, 2017).** Similarly, HS-induced apoptosis resulted in dysregulation of T lymphocytes development in thymus **(Li *et al.*, 2020)**. Moreover, there were described other examples of HS-induced cell death in other cell types. Laying hens exposed to HS decreased their egg production due to activation of FasL/Fas and TNF-α pathways resulting in apoptosis of ovarian follicular cells **(Li *et al.*, 2020)**. In chicken cardiomyocytes, HS induced significantly higher levels of apoptosis accompanied by increase in activity of caspase 3 and caspase 9 **(Zhang *et al.*, 2018)**. *In vitro*, hen erythrocytes viability decreased and activity of caspase 3, caspase 7 and percentage of hemolytic erythrocytes increased rapidly after short term exposure to 43 °C and 45 °C **(Szabelak *et al.*, 2021).** HS cause metabolic changes, which increase body temperature. However, we have little knowledge how temperature itself influence immune cells in chickens. Taken together, cell death related to HS is key point for disruption of growth performance, chicken health and higher susceptibility to pathogens. Cell populations of lymphoid organs play indispensable role in defensibility of organism. This study can reveal how temperature acts itself on chicken immune cells and provide new evidence about factors influence the immune response. Therefore, the goal of this study was to evaluate the susceptibility of chicken lymphoid cells isolated from bursa of Fabricius, thymus, spleen, and blood to temperature treatments *in vitro*.

MATERIAL AND METHODS

Ethics statement

The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of Faculty of AgriSciences, Mendel University, in Brno (protocol code 57199/2020-MZE-18134 and date of approval: 17.8.2022) for studies involving animals.

Animals

Clinically healthy and young (2–5 weeks old) commercial broiler male chickens (n=48) of the Cobb 500 breed were used in this study. Chickens were kept in littered floor boxes (at a breeding facility of Mendel University in Brno, Czech Republic) and feed (Broiler mini, midi and maxi forte, De Heus, Belgium) and water were provided *ad libitum*. Temperatures and light regime (1 hour dark and 23 hours of light in first week, 6 hours of dark and 18 hours of light after first week) were set according to guidelines for Cobb 500 broiler chickens. Blood and tissues were obtained from chickens sacrificed by cervical dislocation.

Experimental Design

The experimental procedure was designed to isolate and culture the cells from various sources (blood, bursa of Fabricius, spleen and thymus) of chicken body to assess the cell viability, phenotype and ultrastructure under thermoneutral (37 °C) and HS conditions (41°C and 45 °C). For every measurement were performed 6 repetitions. The blood was obtained according to **Larsberg *et al.* (2021)**. The blood

obtained from chicken wings (approximately 4 ml) was centrifuged (400 g, 12°C, 10 min.), afterward the buffy coat was collected and mixed (8 ml of phosphate buffer saline (PBS, Ca- and Mg- free; Biosera, Nuaille, France). At the same time, the spleen was dissected from chicken using sterile instruments. In a biosafety cabinet, spleen tissue was cut into small pieces and rewashed with a PBS containing antibiotics and pushed through the 40 µm cell strainer. Mononuclear cells collected from blood and spleen were isolated using Histopaque 1.077 (Sigma Aldrich, St Louis, USA) density-gradient centrifugation (867 g, 12°C, 10 min.). After centrifugation, cells at the interface were obtained, washed two times with PBS (5 min., 400 g, 12 °C) and used for subsequent analysis. Bursa of Fabricius and thymus were dissected using sterile instruments, minced into small pieces (3 mm^3) and smashed through 40 µm cell strainer and washed through by sterile PBS **(Hirakawa *et al*., 2020).** Isolated cells were counted using automated cell counter, EVE™ (NanoEnTek, Seoul, Korea).

Cell culture

Briefly, cells were then resuspended in RPMI 1640 culture medium (Thermo Fisher Scientific, Waltham, USA) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich, Gillingham, UK) and 1% of antibiotic and antimycotic solution (Thermo Fisher Scientific, Waltham, USA). Cells were plated at the density of 2 x 10^6 cells/ml into 24 well culture plates. Cultures were maintained at 37 °C, 41 °C and 45 °C in a 5% CO_2 humidified atmosphere. After 3 and 8 hours, cell aliquots from each temperature were used for the viability, ROS, phenotype and ultrastructure assessment.

Flow cytometry

The changes in the cell viability, ROS and phenotype analyses were performed using flow cytometry. In order to check the cell apoptosis and necrosis, membrane-permeant nucleic acid green-fluorescent dye YO-PRO™-1 Iodide (100 nM, Thermofisher, Wal-tham, USA) and propidium iodide (PI, 50 µg/mL, Biotium, Freemont, USA) were used. Briefly, cells (1 x 10^6) were diluted in 500 µL of phosphate buffered saline and incubated with 1 µL of YO-PRO-1 for 15 minutes in the dark at room temperature (RT). Subsequently, cells were washed by phosphate buffered saline by centrifugation (400 g, RT, 5 min.), stained with PI and analyzed. The proportion (%) of YO-PRO-1 positive cells (YO-PRO-1+/PI-) was classified as apoptotic cells and cells positive for PI (YO-PRO-1-/PI+) were recognized as necrotic cells (Figure 1g). To measure the production of ROS in all cell types, CellROX™ Deep Red Reagent (5 µM, Thermofisher, Waltham, USA) was used. Cells (1 x 10^6) were diluted in 500 µL of phosphate buffered saline and stained with CellROX™ for 15 minutes in the incubator at 37 °C. After incubation, samples were washed (400 g, RT, 5 min.) and analyzed. The proportion (%) of cells positive for CellROX was classified as ROS positive cells (Figure 1f). To observe the phenotype of cells, immunofluorescence staining was performed. The cells (1 x 10^6 cells) were stained using a primary antibody shown in Table 1. Blood and spleen leucocytes were stained by anti CD3, CD4, CD8 and BU1 antibodies. Bursal lymphocytes were stained by BU1 anti MHCII antibodies and thymic lymphocytes population were stained by anti CD3, CD4 and CD8 antibodies. To exclude the dead cells from the analysis, samples were co-stained with dead cell marker such as PI and LIVE/DEAD Fixable Green Dead Cell Stain Kit (Molecular Probes, Eugene, OR). Samples were analysed immediately after staining and washing procedure using BriCyte E6 cytometer (Mindray, Zhenshen, China). Fluorescent signals were acquired by MR Flow (Mindray, Zhenshen, China) in FL1 channel using 530 nm band pass filter, FL2 channel using 585 nm band pass filter and FL3 channel with 670 nm log pass filter. At least 10,000 events (cells) were acquired for each sample. Unstained samples were used as control samples to gate the positive cells according to the increased fluorescence intensity (Figure 1).

Table 1 List of primary antibodies used for flow cytometry.

Antibody	Host/Isotype	Conjugate	Company
Mouse Anti-Chicken CD3-FITC (CT-3) (Mature T lymphocytes)	Mouse/ IgG1κ	FITC	Southern biotech
Mouse Anti-Chicken CD8α-FITC (CT-8) (Cytotoxic T lymphocytes)	Mouse/ IgG1κ	FITC	Southern biotech
Mouse Anti-Chicken CD4-PE (EP96) (Helper T lymphocytes)	Mouse/ IgG1κ	PE	Southern biotech
Mouse Anti-Chicken Bu-1-PE (AV20) (B lymphocytes)	Mouse/ IgG1κ	PE	Southern biotech
MHC Class II Monoclonal Antibody (2G11) (Mature bursal B lymphocytes)	Mouse/ IgG1	PE	Invitrogen

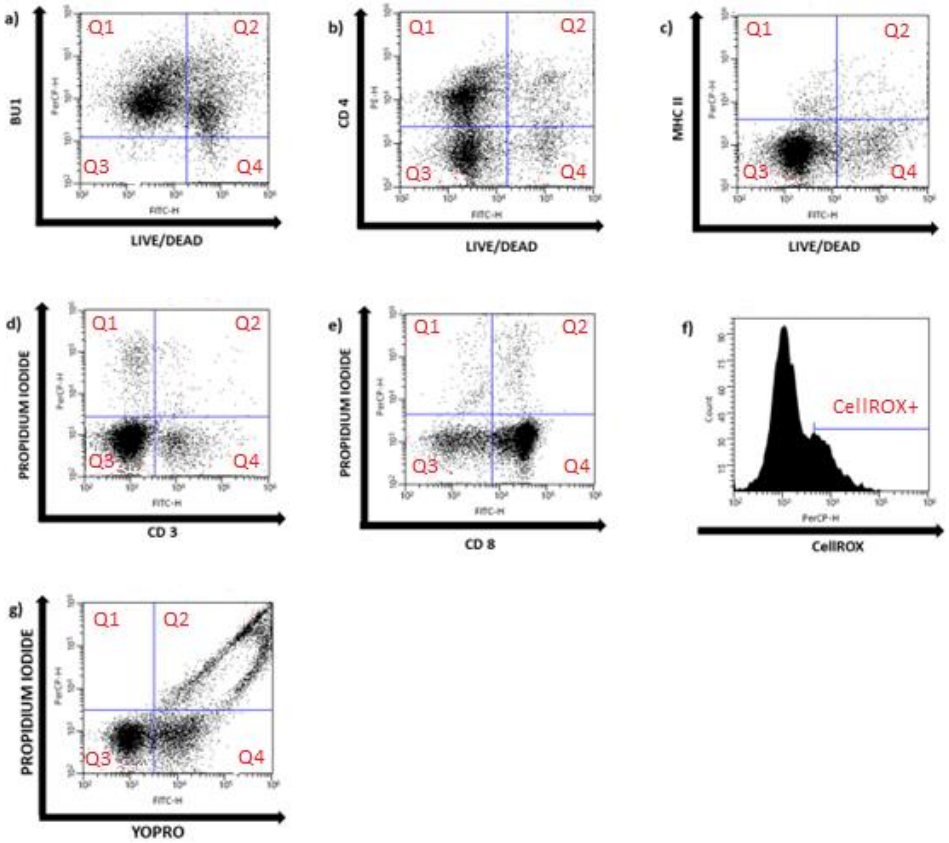

Figure 1 Representative dot plots revealing gating strategy of viability, phenotype and CellROX analyses; a) BU1 antibody for labeling B lymphocytes. BU1+ and LIVE/DEAD- cells in the Q1 quadrant were selected for evaluation; b) CD4 antibody for helper T lymphocytes detection. CD4+ and LIVE/DEAD- cells in the Q1 quadrant were selected for evaluation; c) MHC II for labeling of antigen presenting cells. MHC II+ and LIVE/DEAD- cells in the Q1 quadrant were selected for evaluation. d) CD3 antibody marking mature T lymphocytes. CD3+ and PI- cells in quadrant Q4 were selected for evaluation; e) CD8 antibody marking cytotoxic T lymphocytes. CD8+ and PI- cells in the Q4 quadrant were selected for evaluation; f) CellROX+ cells were treated as cells under oxidative stress. CellROX+ cells were selected for evaluation based on comparison with a negative control. g) Assessment of cell death. Live cells were treated with PI- and YOPRO- cells in the Q3 quadrant. YOPRO+ and PI- cells in quadrant Q4 were treated as apoptotic. YOPRO + and PI+ in the Q2 quadrant were selected as necrotic.

Transmission electron microscopy

To observed lymphocytes pathological changes, transmission electron microscopy was used, according to **Svoradova *et al*. (2018)**. Briefly, cells were collected after 3 hours of incubation due to find initial pathological changes caused by oxidative stress. The cells after collection were immediately fixed in Karnovsky fixative and then washed in a cacodylate buffer. Cell pellets were embedded into 2% agar and postfixed in 1% osmium tetraoxide. Samples were then dehydrated by acetone, and embedded into Poly/Bed (Polysciences Inc., Warrington, USA). The blocks of individual samples were cut into semithin sections and examined under JEOL JEM-2100 transmission electron microscope (JEOL, Tokyo, Japan) and visualized with a digital camera Gatan Orius Sc600a CCD (Gatan, Inc., Pleasanton, CA, USA).

Statistical analyses

Obtained data were evaluated using GraphPad Prism version 9.3.1 (GraphPad Software, San Diego, CA, USA). Normality of data distribution was tested by Shapiro-Wilk W test. One-way ANOVA followed by Dunnett's test for control versus experimental group comparisons was used. Differences among the experimental group were evaluated with two-way ANOVA followed by Sidak test. The data are expressed as the means ± SD; difference is statistically significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

RESULTS

Differences between temperature treatments

Viability of fresh cells after isolation were in blood cells: 85%, spleen cells 84%, thymic cells 92%, bursal cells 88%. Generally, levels of apoptosis positive cells were higher than necrotic cells. At higher temperatures, higher numbers of apoptotic cells were found after 3 hours compared to 8 hours of incubation. Levels of apoptosis were more balanced between heat treatments at 8 hours of incubation. Necrotic cells increased with temperature in every lymphoid cells. In blood leukocytes, apoptosis levels were significantly higher at 45°C ($p < 0.01$), after 3 and 8 hours (Fig. 2a). Similarly, necrosis levels were significantly higher at 45°C than at 41°C and 37°C ($p < 0.05$) after 3 and 8 hours of incubation (Fig. 2b). In spleen, there were increased levels of apoptosis ($p < 0.01$) after 3 hours (Fig. 2c) with increased temperature. However, apoptosis levels were almost the same after 8 hours. With increased temperature, necrosis increased gradually after 3 and 8 hours (Fig. 2d). Apoptosis levels in lymphoid cells isolated from bursa of Fabricius increased with temperature after 3 hours ($p < 0.01$). On the other hand, significant difference ($p < 0.05$) was observed only between 37°C and 45°C groups after 8 hours of incubation (Fig. 2e). At 45°C, there were significantly higher levels ($p < 0.001$) of necrosis compared to 41°C and 37°C after 8 hours (Fig. 2f). In thymus, apoptosis levels were almost the same among the groups. Only at 45°C, there was higher levels ($p < 0.01$) of apoptotic cells with compared to 37°C after 3 hours (Fig. 2g). However, necrotic cells levels increased gradually with temperature after 3 and 8 hours (Fig. 2h).

Comparison of apoptosis, necrosis and reactive oxygen species production between the cells derived from spleen, bursa of Fabricius, thymus, and blood.

Levels of apoptotic cells after 3 and 8 hours at all temperature treatments were shown in Figure 3 a, b respectively. Thymic lymphocytes seem to be least susceptible to apoptosis because they had significantly ($p < 0.05$) lowest proportion of apoptotic cells at all heat treatments. On the other hand, blood and bursal lymphocytes showed significantly ($p < 0.05$) highest proportion of apoptosis at all temperatures after 3 hours. However, blood lymphocytes apoptosis decreased after 8 hours and bursal lymphocytes apoptosis levels remained unchained, therefore B cells population seems to be most susceptible to apoptosis, especially at higher temperatures ($p < 0.05$). Higher temperature had more pronounced effect on necrosis levels in cells (Figure 3 c, d). Generally, temperature increased incidence of necrosis in cells. At 37 °C and 41 °C there were no significant differences between groups. However, levels of necrosis significantly increased ($p < 0.05$) in spleen lymphocytes after 3 and 8 hours at 45 °C. After 8 hours of incubation, there were observed significant difference where lowest levels ($p < 0.05$) were observed in bursal lymphocytes compared to other cell populations at 41 °C. CellROX positive cells levels were strongly induced with increasing temperature (Figure 3 e, f). Lowest production of ROS ($p < 0.05$) were observed in thymic lymphocytes at all temperature treatments. At 41 °C, there were observed highest levels of CellROX positive cells in spleen and bursal lymphocytes after 3 and 8 hours, respectively. In bursal and spleen populations, there were found significantly highest production ($p < 0.05$) of ROS after 8 hours at 45 °C from all temperature treatments, therefore these cells seem to be more susceptible to heat induced oxidative stress then blood and thymic cells.

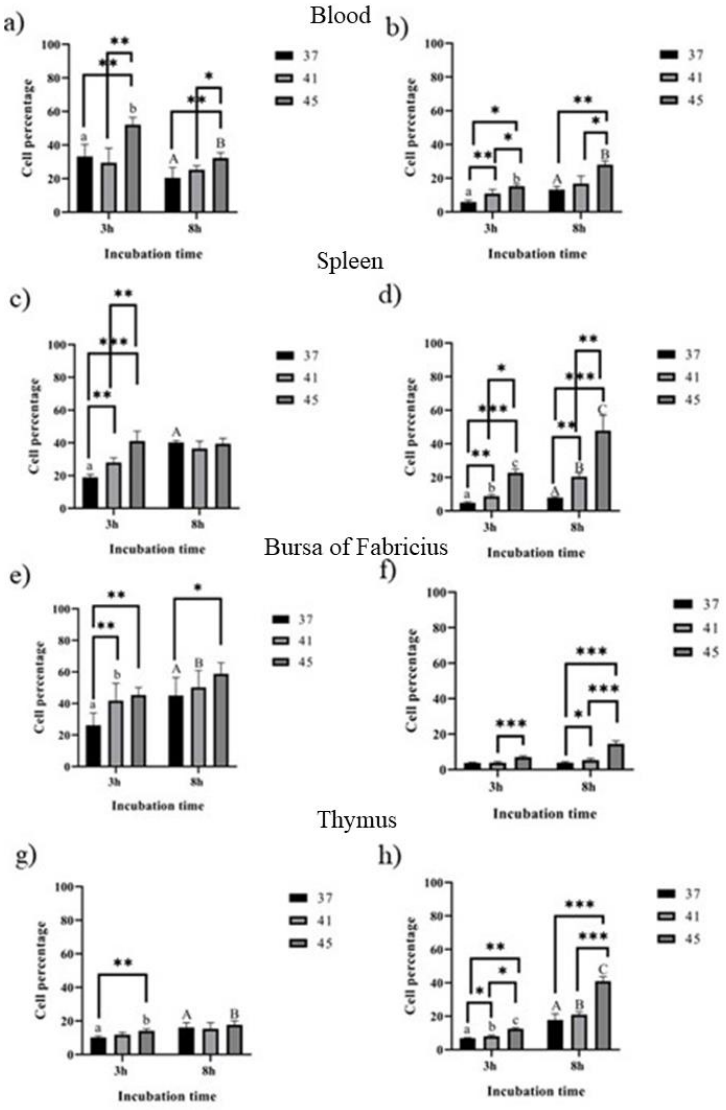

Figure 2 Apoptosis and necrosis levels in blood, spleen, bursa of Fabricius and thymus; a, c, e, g) apoptosis levels; b, d, f, h) necrosis levels; a, b) apoptosis and necrosis levels in blood leukocytes after 3 hours and 8 hours incubation at different temperatures, respectively; c, d) apoptosis and necrosis levels in spleen lymphoid cells after 3 hours and 8 hours incubation at different temperatures, respectively; e, f) Apoptosis and necrosis levels in bursa of Fabricius lymphoid cells at different temperatures, respectively; g, h) Apoptosis and necrosis levels in thymus at different temperatures, respectively. The data are expressed as the means ± SD; difference is statistically significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Phenotype of lymphoid cells in response to temperature treatments

Only prolonged exposure to temperatures caused significant changes in representation of individual phenotypes. Therefore, there were observed no significant changes (Figure 4 a, c, e, g) after 3 hours. In blood lymphocytes, temperature treatment didn´t influence representation of phenotypes, after both 3 and 8 hours (Figure 4 a, b). In other cell populations, there were seen significant changes after 8 hours where there were seen significant decrease of some phenotypes at 45 °C. In spleen, there were significantly decreased T lymphocytes population (CD3+, CD4+ and CD8+) at 45 °C compared to 37 °C ($p < 0.05$) after 8 hours (Figure 4 d). In bursa of Fabricius, there was seen gradual decrease in B lymphocytes with increasing temperature ($p < 0.05$) after 8 hours (Figure 4 f). At 45 °C, there were significantly decreased helper CD4+ and cytotoxic CD8+ lymphocytes in thymic lymphocytes population (Figure 4 h).

Transmission electron microscopy

Splenic lymphocytes were selected for demonstration of pathological changes. Observation by transmission electron microscopy showed normal lymphocyte morphology at 37 °C. At 37 °C, most of the cells showed no pathological changes connected with oxidative stress (Figure 5 A). Mitochondria of splenic lymphocytes had clearly visible cristae (Figure 5 C). On the other hand, at 45 °C, an apoptotic splenic lymphocyte in a stage of forming apoptotic bodies were seen (Figure 5 B). Oxidative stress induced pathological changes in splenic lymphocytes

mitochondria at 45 °C, represented by disruption of mitochondrial membrane and loss of mitochondrial matrix and cristae (Figure 5 D).

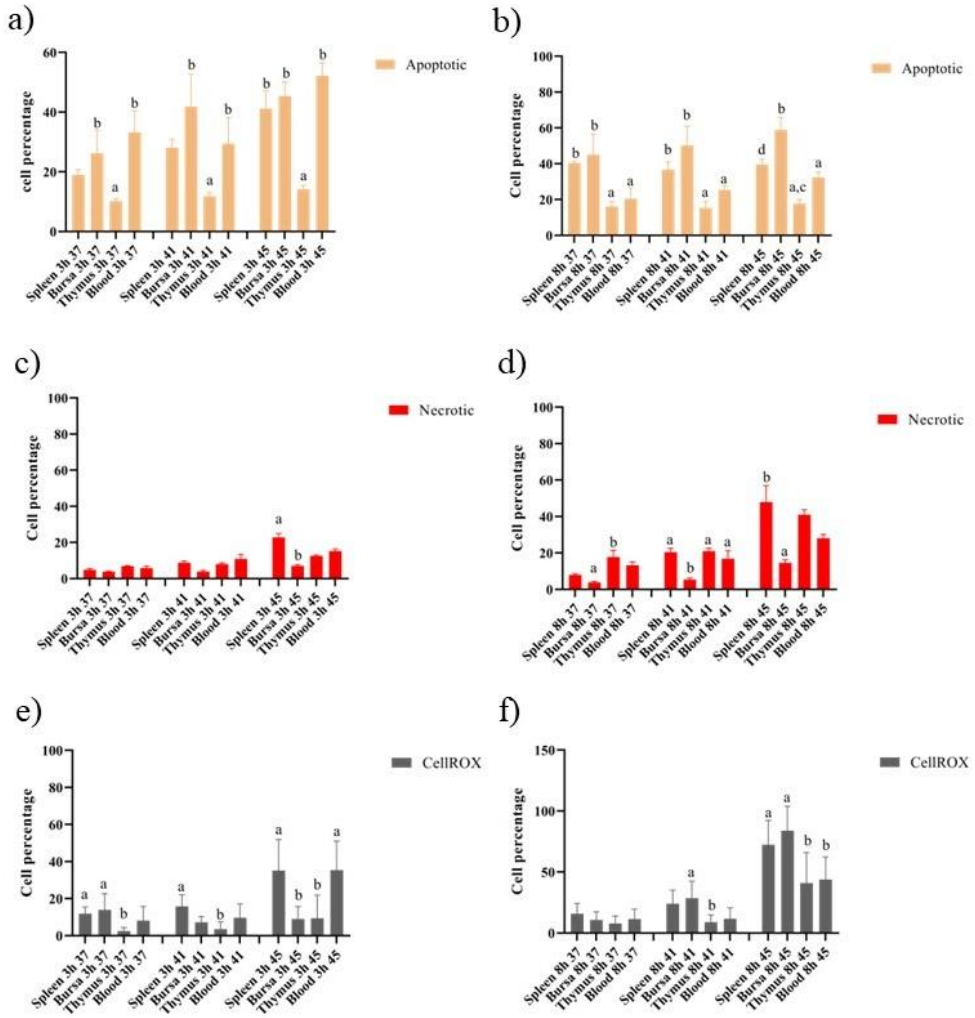

Figure 3 Comparison of apoptosis, necrosis and ROS production in lymphoid cells derived from spleen, bursa of Fabricius, thymus and blood at 3 temperature treatments; a, b) Percentage of apoptotic cells after 3 and 8 hours, respectively; c, d) Necrotic cells levels in response to temperature treatments after 3 and 8 hours, respectively; e, f) ROS production evaluated by CellROX in cells after 3 and 8 hours, respectively. The data are expressed as the means ± SD; difference between cell populations is statistically significant at $p < 0.05$.

DISCUSSION

The main goal of the study was to determine effects of heat treatments on incidence of cell death in chicken lymphocytes. In the study, increased temperatures were shown to induce both apoptosis and necrosis in chicken lymphoid cells. In past, there were described mechanism of cell death induction in HS. Generally, HS led to decrease mitochondrial membrane potential, loss of cytochrome c from mitochondria and activation of caspase 3 and caspase 9 apoptosis pathway **(Gu *et al*., 2014)**. Transcription factor p53 modulate expression of various genes responsible for apoptosis, cell cycle arrest DNA maintenance in response to stress **(Beckerman and Prives, 2010)**. P53 penetrate through mitochondrial membrane and play a crucial role in induction of apoptosis. In HS treated human umbilical endothelial cells, p53 mitochondrial translocation inhibition caused significant alleviation of cytochrome c release and caspase 9 activation **(Gu *et al*., 2014)**. Cytoplasmic Ca^{2+} elevation is involved in the process of apoptosis induction and ROS play a role as an upstream regulator **(Li *et al*., 2014)**. Interestingly, tissue specific tendency to apoptosis or necrosis after heat treatments were observed in our study. Apoptosis as an energy dependent process requires enough levels of adenosine triphosphate (ATP). Therefore, after longer time of incubation, there were observed higher numbers of necrotic cells because their exhausted energy resources **(Fink and Cookson, 2005)**. Moreover, it was described mechanisms of OS-induced apoptosis if the overproduction of ROS is enormous. ROS inhibit the caspases activation and trigger the necrosis **(Samali *et al*., 1999; Galán *et al*., 2001)**. In stimulation of necrosis, it seems to be crucial the length of period to exposure to high temperature. In this study, with prolonged period of incubation significantly increased proportion of necrotic cells. Cells death was reported previously as consequence responsible for decreased performance of chickens **(Li *et al*., 2020)**. However, there is lack of information about the susceptibility of lymphocytes populations to temperature-induced cells death in chickens. Interestingly, bursal and spleen lymphocytes seem to be more susceptible to heat induced apoptosis and ROS production then thymic and blood lymphocytes as observed in our study. Similarly, **Xu *et al*. (2017)** observed strong upregulation of pro-apoptotic genes and higher percentage of apoptotic cells in spleen of heat stressed chickens. HS induced apoptosis in bursa of Fabricius was noticed by TUNEL (Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling assay staining) on immunohistochemical sections **(Liu *et al*., 2021)**. Both organs play an indispensable role in antibody based immune response. Bursal B lymphocytes population play crucial role in antibody immune response in broiler chickens and in our study their numbers decreased significantly with increasing temperature. Additionally, in our study, CD4+ lymphocytes levels decreased significantly in spleen and thymus after 8 hours at 45 °C. Negative impact of HS on CD4+ lymphocytes were observed in another studies **(Mashaly *et al*., 2004; Jahanian and Rasouli, 2015)**. CD4+ lymphocytes help B lymphocytes to generate stronger and longer antibody response **(Swain *et al*., 2015)**. It is well known that HS diminished antibody response which was mentioned in many studies **(Bartlett and Smith 2003; Mashaly *et al*., 2004; Jahanian and Rassouli, 2015; Hirakawa *et al*., 2020).** IgY, IgA and IgM against bovine serum albumin decreased in heat stressed chickens **(Hirakawa *et al*., 2020).** Total antibody production was significantly reduced in laying hens exposed to HS **(Mashaly *et al*., 2004)**. In another study, HS disrupted the production of IgM and IgG against sheep red blood cells **(Bartlett and Smith 2003)**. Similarly, antibody response against Newcastle disease virus and infectious bronchitis virus were significantly diminished in heat treated broiler chickens **(Jahanian and Rasouli, 2015)**. Therefore, sensitivity of bursal B lymphocytes to increased temperatures can be one from the reasons of disrupted antibody response in heat stressed chickens. Decrease of splenic and thymic CD4+ and CD8+ lymphocytes numbers at 45 °C is consistent with **Hirakawa *et al*., (2020)**. In their study, broiler chickens exposed to HS significantly declined their CD4+ and CD8+ levels in spleen and thymus. However, if we compare whole cell death incidence and ROS production, thymic lymphocytes population seems to be more resistant to higher temperatures then others, which can be explained by several ways. Tissue specific sensitivity of lymphatic tissue to cell death could be associated with various sensitivity to OS or antioxidant capacity of each organ. Phenomenon of tissue specific sensitivity to

OS was described in the past. **Kim *et al*. (2018)** tested transcriptional properties of 101 OS-related genes in 10 various tissues in healthy mice in physiological conditions. They described various levels of expression of glutathione peroxidases and superoxide dismutase in tissues. Similarly, antioxidant enzymes activity was different in liver and pectoralis major muscle in chickens under HS **(Habashy *et al*., 2019)**. Higher susceptibility of B lymphocytes population with compared to thymic population could be also explained by different sensitivity to DNA damage of lymphocyte populations. It is described that B lymphocytes are less resistant to DNA damage induced by low dose gamma radiation with compared to T lymphocytes **(Felgentreff *et al*., 2021)**. It is well known that high temperatures cause DNA damage **(Habibi *et al*., 2022)**. Therefore, B lymphocytes population is much susceptible to heat induced DNA damage and subsequently higher rates of apoptosis are triggered in B lymphocytes. In avian cell biology, a debatable topic is what temperature is ideal for cell culture. In birds, there were described body temperature in range from 38.53 °C to 43.85 °C, in resting and active birds respectively **(Prinziger *et al*., 1991)**. Interestingly, it is recommended culture temperature 41 °C for avian dendritic cells derived from bone marrow **(Wu *et al*., 2010)**. Similarly, it was used 41 °C for chicken peripheral blood mononuclear cells culture **(Larsberg *et al*., 2021)**. For chicken monocyte derived dendritic cells culture is recommended 37 °C **(Kalaiyarasu *et al*., 2016)**. Finally, based on methodology for chicken mesenchymal stem cells culture, optimal temperature is 37 °C **(Adhikari *et al*., 2019)**. Our experiment showed usability of both temperatures for short term lymphocytes culture. However, better viability and lower numbers of CellROX positive cells was observed at 37 °C, so, it could be recommended to culture chicken lymphocytes at 37 °C. Cell type seems to be important for setting the optimal temperature because there were observed better viability at 41 °C in comparison with 37 °C in chicken satellite cells **(Siddigui *et al*., 2021)**.

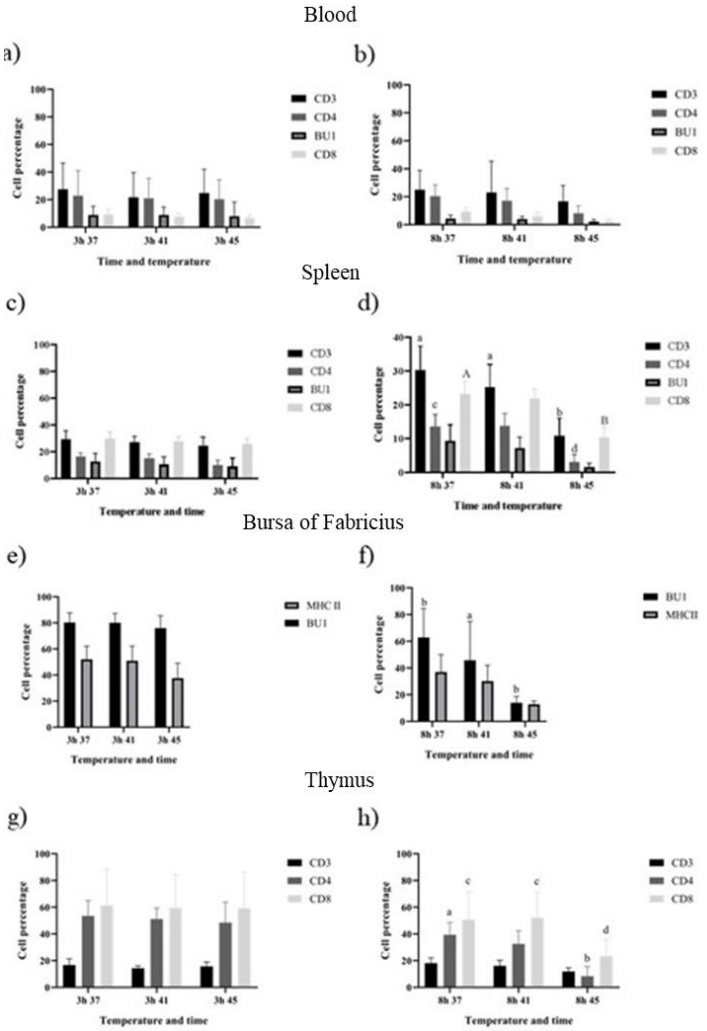

Figure 4 Percentage of live cells of each phenotypes in blood, spleen, bursa of Fabricius and thymus in response to 37 °C, 41 °C and 45 °C. a, b) Percentage of live blood lymphocytes after 3 and 8 hours respectively; c, d) Live spleen lymphocytes cells after 3 and 8 hours long heat treatments respectively; e, f) Live bursa of Fabricius-derived lymphocytes percentage after 3 and 8 hours respectively; g, h) Live thymus lymphoid cells after 3 and 8 hours temperature treatments respectively. The data are expressed as the means ± SD; difference between temperatures is statistically significant at $p < 0.05$.

Taken together, OS induced apoptosis in lymphocytes, whereas prolonged exposure to ROS triggered necrosis particularly in bursal and spleen lymphocytes. Bursa of Fabricius and spleen were found to be susceptible to HS in previous studies. Our results support these findings by higher levels of cell death observed in spleen and bursa of Fabricius at 45 °C. Our results further showed that disrupted humoral immunity in heat stressed chickens could be attributed with sensitivity of CD4+ lymphocytes and B lymphocytes to increased body temperature.

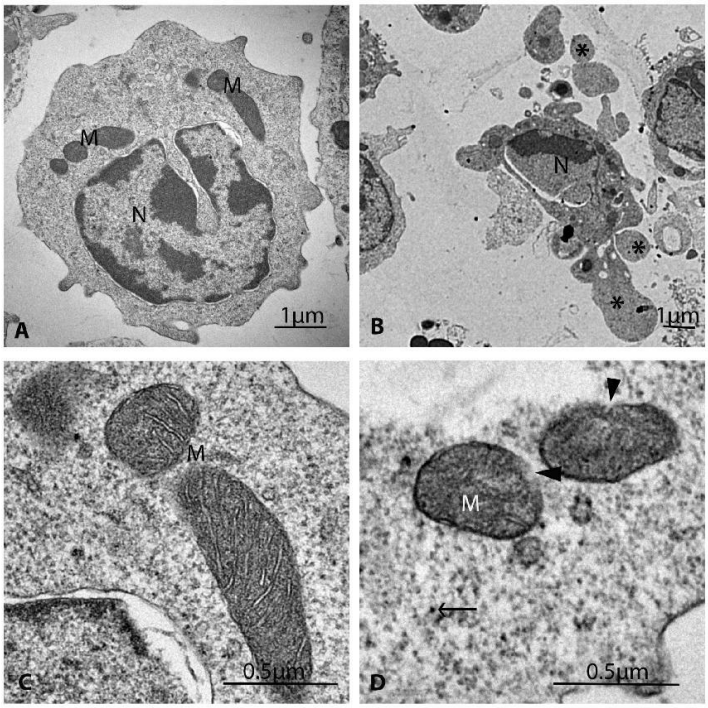

Figure 5 Comparison of pathological changes demonstrated in splenic lymphocytes: A, C pictures at 37 °C and B, D from 45 °C after 3 hours of incubation in different temperatures. A. Normal Lymphocyte, surface with membrane folds. Large articulated nucleus (N) with active nucleolus. Euchromatin and heterochromatin are well differentiated. In cytoplasm abundant mitochondria (M). Magnification: 2500x B. A cell in an advanced stage of apoptosis. Condensed dark cytoplasm, compact chromatin remnants (N) are present. The cell breaks down into apoptotic bodies (*). Magnification: 2500x C. In detail mitochondria (M) with cristae and abundance of ribosomes in the cytoplasm. 10000x D. In detail damaged mitochondria (M), with broken membrane (arrowhead) and loss of mitochondrial matrix. Ribosomes in cytoplasm (arrow). Magnification: 10000x.

CONCLUSIONS

In summary, heat treatments induced apoptosis and necrosis in chicken lymphocytes. However, the levels of apoptosis and ROS production were more pronounced in bursa of Fabricius and spleen with compared to blood and thymus which can imply their higher susceptibility to heat.

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