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Effects of vitamin C and early-age thermal conditioning on pituitary adrenocorticotropic hormone cells in broilers chronically exposed to heat stress: an immunohistomorphometric and hormonal study

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Abstract

The aim of this study was to examine the effects of heat stress (HS) on the pituitary-adrenal axis and whether the treatments with early-age thermal conditioning (ETC) and vitamin C, alone and in combination, could have a beneficial effect in alleviating these effects. For the experiment, 400 one day-old broilers (both sexes) were used, being divided into four groups. The first group was the control (K), the second group (C) consisted of broilers which received vitamin C from the 22nd to the 42nd day *via* water in the amount of 2.00 g L^{-1}, in the third group (T), broilers were exposed to ETC for a period of 24 hr at a temperature of 38.00 ± 1.00 ˚C and the fourth group (T + C) was the combination of T and C groups. Immunohistochemically positive adrenocorticotropic hormone (ACTH) cells of broilers in all groups were irregular or stellate and distributed in the periphery and central parts of the pituitary gland, as solitary cells or in clusters. In the T + C group of broilers, a significant increase in the area of ACTH cells (18.91%) and their cores (22.75%), and cortisol level in serum compared to the control group was observed. This reaction of broilers in the T + C group facilitated their adaptation to unfavorable consequences of HS. These results suggest that hypothalamic-pituitary-adrenal axis is stimulated after the exposure to chronic HS, enabling successful adaptation of broilers to adverse conditions.

Introduction

The global mean temperature increased by 0.80 - 1.70 ˚C during the late 19th and 20th centuries,[1] causing numerous problems in the functioning of the living world, especially in homeothermic animals such as chickens.[2] An increase in body temperature in birds above the regulated range, in conditions of elevated ambient temperature and/or excessive metabolic heat production, can lead to irreversible fatal consequences for the birds.[3] Heat loss in birds can occur through respiratory-evaporative mechanisms,[4] skin evaporation mechanism[5] and sensible heat loss through radiation and convection.[6] Acclimatization of birds to heat includes autonomously controlled physiological mechanisms, working together to improve the thermal endurance[7] at all levels of the body in order to achieve homeostasis, effectively improving thermotolerance in a new hot environment. Heat stress (HS) in chickens causes reduced growth, poor meat quality, reduced feed consumption and increased mortality.[8,9]

The first response to a thermal stressor is the activation of the sympatho-adrenomedullary system,[10] and in the case of a long-term stimulus, the hypothalamic-pituitary-adrenal (HPA) system is activated in order to alleviate physiological and structural changes in the birds, as well as other vertebrates.[11-13] After exposure to high ambient temperature, the concentrations of adreno-corticotropic hormone (ACTH) in plasma and circulating glucocorticoids increase as a part of HPA system activation in broilers and mammals.[12-14] The function of gluco-corticosteroids is important during the stress response because by increasing the levels of amino acids, free fatty acids and glucose in the blood,[15] energy-rich sources are made available to the cells that animals may need for an adequate "fight or flight" response or other responses to a stressful situation. Under normal conditions, as well as

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during stress reactions, the secretion of glucocorticosteroids is regulated by a negative feedback mechanism involving the hypothalamus (corticotropic releasing hormone), pituitary ACTH and adrenal glands.[12,13,16]

Recently, there has been an increasing number of works on the importance of cortisol in birds, and the attitude that it is only found in very low concentrations is now obsolete. Namely, researchers[17] came to the result that there were very high values of this hormone in the blood of broiler chickens. This could be explained by a very high concentration of this hormone in the pre-natal and early post-natal periods, being especially important for broiler chickens because the production cycle lasts 42 days.

In a previous work, Ružić *et al.*[18] have showed that the synergistic effect of vitamin C during early-age thermal conditioning (ETC) has a beneficial effect on production characteristics under the conditions of HS in terms of reduced food conversion and increased volume of individual parts of the body in relation to the entire body mass, as well as an increased number of breaths, leading to the release of a greater amount of heat into the environment; thus, producing lower body temperatures predominantly in the T + C group. It is precisely the beneficial use of vitamin C and ETC as a method of reducing the harmful effects of HS that was an incentive for us to examine the response of the pituitary-adrenal axis in such circumstances, which was the goal of this research.

Materials and Methods

Day-old chicks sex determination. In earlier works, the feather sexing method for determining the sex of day-old chicks was described.[19]

Animals and experimental design. For the experiment, 400 one-day-old (both sexes) Cobb 500 broilers were used. The broilers were divided into four groups, each with four repetitions of 25 individual animals; 100 chickens *per* group divided into pens, all in the same production facility. The first group (K) consisted of controls, the second group (C) included broilers that received vitamin C (Veterinary Institute Subotica, Subotica, Serbia) from the 22nd to the 42nd day through water in the amount of 2.00 g L^{-1} (1.00 g of vitamin C contained 100 mg of active substance), the third group (T) included chickens exposed to ETC and the fourth group (T + C) included those exposed to ETC and also received vitamin C, as described in detail by Ružić *et al.*[9] Ambient temperature in the facility was measured from 08:00 to 20:00 hr in 2-hr intervals. Figure 1 shows the average temperature in the facility for the study period between the 29th and 42nd days of breeding and such high temperatures were the result of natural environmental conditions. All groups (including K) were subjected to HS conditions for 14 days at the maximal temperature of 30.68 ˚C (Fig. 1). These temperatures corresponded to chronic stress. Relative humidity remained between 40.00 and 70.00%. The composition of the food consumed by the chickens was commercial, as it was also shown in the previous work.[18] This paper is a part of the doctoral dissertation research approved by the Ethics Committee on the Protection of Animals Used for Scientific Purposes of the University of Novi Sad, Serbia (EK: II- 2018-02).

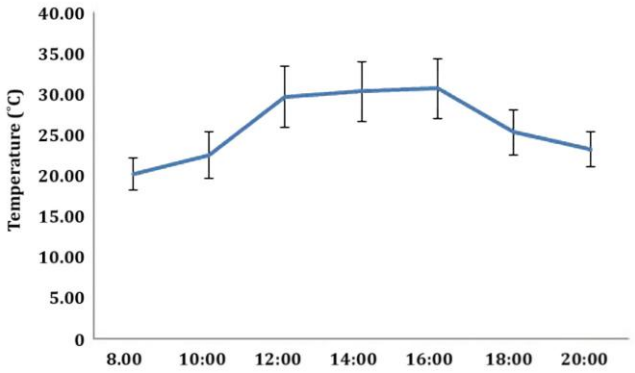

Fig. 1. The facility air temperature in the last two weeks of production.

Pituitary gland extraction. At the slaughter line, the pituitary gland was taken for immunohistochemical examination of ACTH cells. The sampling was done immediately after 42 days of breeding. Initial incision was made to remove the skin from the head. The next step was decapitation in the area of the 1st cervical vertebra. The tip of the scissors was then introduced into the foramen magnum and a superficial incision of the skull was made in the laterocranial direction, just above the eye orbit. This cut was made from one side and the other, and with it the skull cap was obtained. After removing this bony structure, the brain was visible and accessible. Pulling out the brain in the caudal direction revealed the optic chiasm, which needed to be cut with a careful incision. Immediately below the optic chiasm is the floor of the 3rd cerebral ventricle and the *sella turcica*. The pituitary gland was slightly raised with scissors and the infudibulum was cut. If the pituitary gland got stuck in the bony structure, it was necessary to perform a gradual and careful preparation, along with cutting the bones of the base of skull. The whole process was extremely slow and very demanding due to the consistency of the brain mass.

Immunohistochemical staining. Isolated pituitaries were prepared for histological analysis by the standard procedure of dehydration in a series of increasing concentrations of alcohol, enlightened in xylene and embedded in paraplast (Histolab Product AB, Gothenburg, Sweden). The ACTH cells were labeled immunohistochemically on 5.00 µm-thick pituitary sections (hACTH antiserum DAKO A/S, Glostrup, Denmark). The detailed procedure of immunohistochemical labeling of ACTH cells was previously described in detail.[12]

Morphometric analysis. Morphometric analysis was performed on digital images of labeled pituitary sections taken with a LEITZ DM RB light microscope (Leica, Wetzlar, Germany) acquired with a digital CCD camera (DFC320; Leica Microsystems, Heerbrugg, Switzerland). Thirty fields of view were selected for each analyzed case. Image analysis was performed using the ImageJ Software (National Institutes of Health, Bethesda, USA). Regarding ACTH immunoreactive cells, our analysis included the measurement of their area and nuclear area. Nucleocytoplasmic ratio was calculated as the quotient of the nuclear area and cytoplasmic area, where the cytoplasmic area was obtained as a difference between the area of the upper cells and the area of their nuclei. The analysis was performed using a Multi-purpose Test System M_{168} (length of one test line = 17.88 μm, area of one point of the test system = 15.49 μm^2, surface of the test field = 2601.54 μm^2 and length of test lines = 1501.92 μm), superimposed over the analyzed digital image of histological sections. Volume density of ACTH cells was obtained as the quotient of the number of points in the test system hit by immunopositive cells and the total number of dots in the system number of test points was 168 *per* each analyzed field.[20]

Hormonal analyses. Blood sampling to obtain serum performed on the 42nd day of age according to the 24 individuals from each group and an equal number of male and female individuals (three male and three female chicks from each replicate). Blood samples were taken by cardiac puncture and collected into sterile test tubes. The extracted blood was allowed to coagulate at room temperature for 2 hr, after which it was centrifuged for 20 min at 3,000 revolutions *per* min. The resulting serum was placed at – 80.00 ˚C temperature until the hormone concentration analysis. The level of cortisol hormone was determined without diluting the serum using the radioimmunoassay cortisol diagnostic kits (Institute for the Application of Nuclear Energy, Zemun, Serbia) intended for quantitative determination of total cortisol concentration. Radioactivity was measured with a gamma scintillation counter (CompuGamma LKB, Brussels, Belgium) at the Institute for the Application of Nuclear Energy in Zemun, Serbia.

Statistical analysis. The results were processed by standard statistical methods using the statistical software package R programming language (version 4.3.2; R Core Team Vienna, Austria) and the programming environment for statistical calculations. Determination of the degree of statistical significance of differences between the groups was performed using the method of analysis of variance; while, further analysis was performed using the Tukey's *post hoc* test for multiple comparisons between groups for a significance level of 95.00% ($p < 0.05$). The mean and standard deviation were calculated and the values were reported as such.

Results

Qualitative histological findings. In control broilers, immunohistochemically positive ACTH cells were irregular or star-shaped and distributed throughout the periphery and central parts of the pituitary gland. Cytoplasmic processes tended to encompass adjacent cells or spread between them. The nuclei were oval in shape, with visible nucleoli. In these cells, dark secretory granules were visible in the peripheral ACTH cells. In the experimental broilers, the shape and distribution of ACTH cells were not significantly changed compared to controls; but, it could be noticed that they were located near the dilated capillaries. In T + C group broilers, ACTH cells were slightly larger compared to the controls (Fig. 2).

Morphometric findings. Morphometric analysis of the area of immunopositive ACTH cells in broiler chickens in group C showed a significant increase by 12.70%, compared to the controls ($p < 0.05$). In broilers of T + C group, this parameter was also significantly increased by 18.91, 5.51 and 15.47%, compared to the control, C and T groups, respectively ($p < 0.05$; Fig. 3A). The ACTH cells area in T group broilers was significantly reduced by 8.63%, compared to the C group ($p < 0.05$; Fig. 3A). The nuclear area of ACTH immunoreactive cells in C group broiler chickens was significantly increased by 22.75, 15.02 and 15.29%, compared to the control, T and T + C groups, respectively ($p < 0.05$; Fig. 3B).

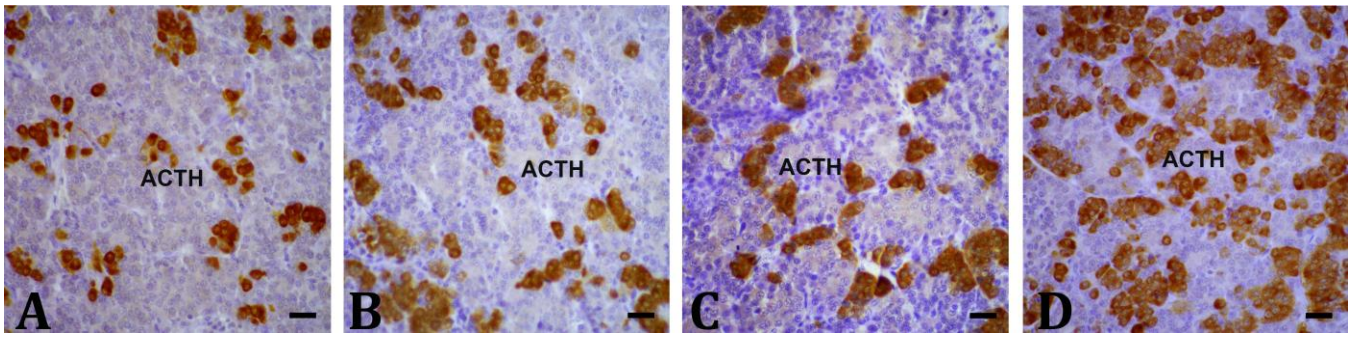

Fig. 2. Representative photographs of immunopositive adrenocorticotropic hormone (ACTH) cells in the pituitary glands from **A)** control , **B)** vitamin C , **C)** early-age thermal conditioning (ETC)-exposed and **D)** ETC + vitamin C groups. Brown colour: 3,3'-diaminobenzidine chromogen; Scale bar: 30.00 μm.

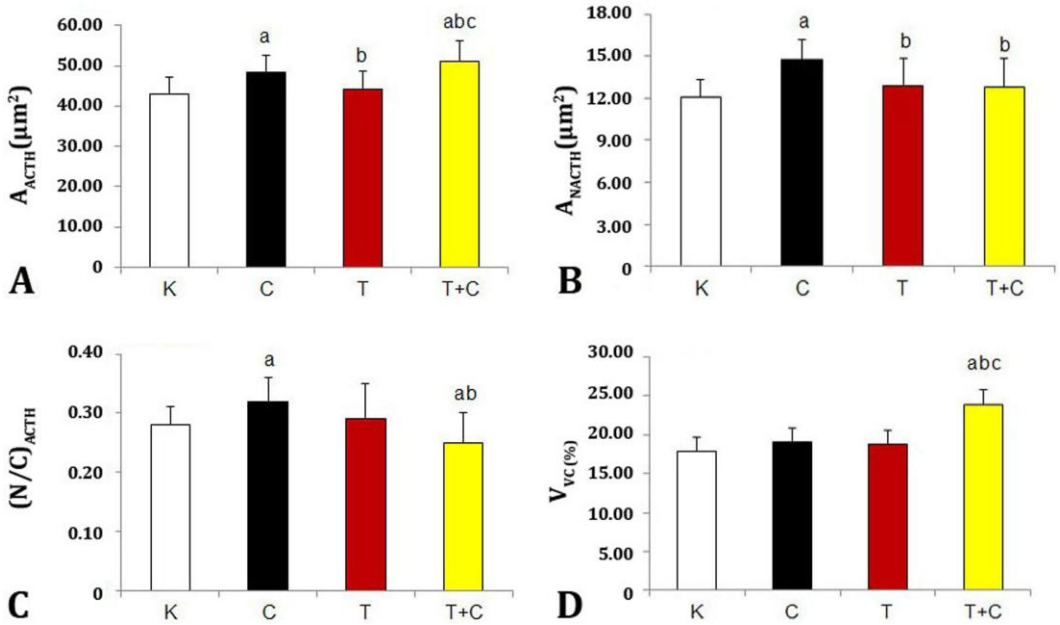

Fig. 3. Morphometric parameters of immunopositive adrenocorticotropic hormone cells in the pituitary glands from control (K), vitamin C (C), early-age thermal conditioning (ETC)-exposed (T) and ETC + vitamin C (T + C) groups. **A)** A_{ACTH}: Area of corticotropic cells; **B)** A_{NACTH}: Area of corticotropic cell nuclei; **C)** $(N/C)_{ACTH}$: Nucleocytoplasmic ratio of corticotropic cells; **D)** Vvc: Volume density of corticotropic cells. [a] $p < 0.05$ *vs.* group K; [b]$p < 0.05$ *vs.* group C; [c]$p < 0.05$ *vs.* group T.

The results of the ACTH immunoreactive cells nucleo-cytoplasmic ratio showed that it was significantly changed in C (+14.28%) and T + C (– 13.79%) groups compared to the control group ($p < 0.05$; Fig. 3C). In T + C group, nucleo-cytoplasmic ratio of ACTH cells was significantly ($p < 0.05$) decreased by 21.88 and 13.79%, in comparison with C and T groups, respectively (Fig. 3C). Volume density of the immunopositive ACTH cells in T + C group broilers was significantly increased by 33.20, 25.56 and 27.65%, compared to the control chickens ($p < 0.05$), as well as C and T groups chickens, respectively (Fig. 3D).

Hormonal findings. Cortisol concentration in the serum of broiler chickens was measured on day 42. It was found that in all experimental groups (C, T and T + C); it was statistically significantly increased by 12.86, 9.46 and 5.30%, compared to the control group ($p < 0.05$). In the comparison of groups T and T + C with group C, there were no statistically significant differences ($p > 0.05$; Fig. 4).

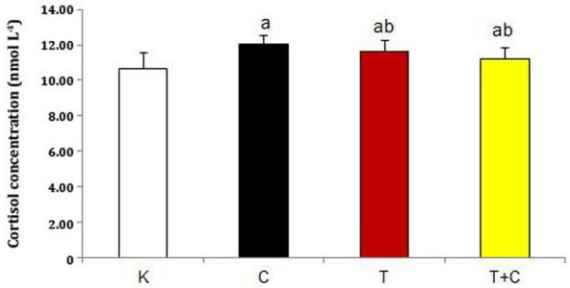

Fig.4. Concentration of cortisol in the blood of chickens on day 42 in control (K), vitamin C (C), early-age thermal conditioning (ETC)-exposed (T) and ETC + vitamin C (T + C) groups. [a] $p < 0.05$ *vs.* group K; [b]$p < 0.05$ *vs.* group C.

Discussion

According to the data from the National Climate Center in the United States[1], the global mean surface temperature has increased by 0.80 - 1.70 ˚C during the late 19th and 20th centuries, and further increases of 0.60 - 2.50 ˚C are expected over the next 50 years. This situation, in which there is a global increase in temperature every year, requires effective and economical means to overcome HS in domestic poultry more easily. Increased environmental temperature leads to the activation of the HPA axis in chickens, being accompanied by an increased concentration of glucocorticoids in the blood, regulating metabolism to enable easier adaptation and survival of chickens in HS conditions.[15]

Our findings of immunohistochemical analysis of ACTH cells showed that they were oval or irregular in shape, single or in groups, in all examined animals. The color intensity of the secretory granules was almost identical in all groups. The irregular shape of ACTH cells was associated with their increased activity. In T + C group, the cells were star-shaped, with cytoplasmic extensions directed towards the vascular network. In this group, morphometric measurements demonstrated hypertrophy and hyperplasia of the examined cells compared to the controls. The ACTH cells with increased cytoplasm were also observed in Japanese quail exposed to chronic HS,[21] and changes in the shape of these cells were also noted in rats exposed to the chronic[12] and acute HS.[10]

Hypertrophy and hyperplasia of pituitary basophilic cells have been described in White Leghorn chickens

after exposure to a high ambient temperature of 38.00 °C.[22] On electron microscopy, it was found that ACTH cells in Japanese quail had a well-developed endoplasmic reticulum and small rounded and poorly developed mitochondria, and the Golgi apparatus was moderately developed and localized around the nucleus.[23]

Histomorphometric changes in the ACTH level of pituitary cells were followed by the physiological response of the adrenal gland. The lowest cortisol values were recorded precisely in the K group on the 42nd day, when ACTH cells with reduced intensity of cytoplasmic staining could be seen, and the highest values were observed in the C group, where ACTH cells were larger and more numerous with a modified form. Similar histological changes of ACTH cells were also recorded after chronic HS in White Leghorn chickens[22] and rats.[12,16]

Various studies have shown a positive correlation between morphological changes of ACTH cells and their functional state.[13,16,24,25] In the treated groups, the intensity of cytoplasmic staining increased, as well as the level of cortisol in the serum, indicating that the HPA axis activation was the main neuroendocrine mechanism in responses to stress.[24] Corticosterone is primarily released from the adrenal glands of birds; but, it is also synthesized, although in much lower concentrations. Cortisol is especially intensely synthesized in birds during the embryonic and early post-natal periods, as well as in HS.[26] It has been observed that in early embryonic period, the concentration of corticosteroids from the adrenal gland (coricosterone, cortisol and cortisone) equalizes in the circulation and these hormones reach their peak values just before hatching.[27,28]

This research showed that the concentration of cortisol in all experimental groups was significantly increased compared to the controls after 42 days of fattening. It is assumed that the increased surface area and number of ACTH cells with cellular extensions extending into the blood vessel bring about a rapid secretion of ACTH into the blood flow, leading to an increase in circulating cortisol in the serum, which has been shown in earlier studies.[29,30] In this way, the proliferation of adrenal cortex cells is controlled and steroidogenesis is stimulated by melanocortin 2 receptor activation,[31,32] being extremely important for a successful adaptation. The stimulatory effect of ACTH on adrenocortical zona fasciculata cells was registered by hypertrophy and an increase in the activity of steroidogenic enzymes.[33]

The presented results showed that the HPA axis was stimulated after the effect of chronic stress, being especially conspicuous in T + C group. In this way, it was possible to achieve a successful adaptation and acclimatization of the broiler to unfavorable conditions caused by long-term effects of elevated temperature. In earlier studies,[9,18] it was also shown that broilers from this group adapted best to HS, being reflected in improved breathing as a way of getting rid of excess heat, as well as better meat quality. The production results were therefore markedly improved, which was of great importance for the economy.

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Conflict of interest

No conflicting interests and no funding in connection with this paper are applicable.

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