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Research article

THE EFFECT OF MODERATE HEAT ON RAT PITUITARY ACTH CELLS: HISTOMORPHOMETRIC, IMMUNOFLUORESCENT AND HORMONAL STUDY

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In areas with moderate continental climate, increased average ambient temperature during the summer represents a stressogenic factor that affects the hypothalamo-pituitary-adrenocortical axis in mammals. Therefore, we wanted to examine the effects of 4 days of constant exposure to moderately elevated ambient temperature (35 ± 1°C) on the histomorphometric and immunofluorescent characteristics, as well as on the hormonal secretion of pituitary corticotropes (ACTH) cells in adult male rats. In comparison with the controls kept at 20 ± 2°C, a significant increase ($p<0.05$) of the absolute and relative pituitary weight (23.1% and 36.1%, respectively) was registered after exposure to heat. The localization, as well as the shape of the ACTH cells in the heat exposed group was not significantly altered, but their immunopositivity was weaker. After 4 days of heat exposure, a weaker signal confirmed the relative fluorescence intensity of the ACTH cells (15.3%, $p<0.05$). In heat exposed rats, an increase of the cellular and nuclear volumes of immunolabelled ACTH cells and decrease of their volume density (6.9%, 14.3% and 20.0%, respectively; $p<0.05$) was registered. Observed histomorphometric and immunofluorescent features of the pituitary ACTH cells were in accordance with the increased ($p<0.05$) value of plasma adrenocorticotropic hormone (ACTH) by 23.7% compared to the control rats. It can be concluded that the 4-day exposure to moderately elevated ambient temperature intensifies pituitary ACTH secretion in adult male rats.

Key words: moderate heat, ACTH cells, histomorphometry, immunofluorescence, rat

INTRODUCTION

One of the physical stressors that strongly influences animal homeostasis, besides cold and immobilization stress, is high ambient temperature [1,2]. Due to the global climate changes and spreading of the tropic climate boundaries to further northern latitudes,

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living organisms in the South-Eastern parts of Europe are subjected to elevated ambient temperature during the summer months, which represents an inevitable stress to all organisms. Like other stressors which are recognized as threatening factors for the survival of an individual [3], elevated ambient temperature provokes many different stress responses in a living organism, one of the first of which is the neuroendocrine response. It is well-known that the first reaction to a thermal stressor is activation of the sympatho-adrenomedullary system, but if the stimulus persists, in an attempt to regain and maintain homeostasis, the stimulation of the hypothalamic-pituitary-adrenal (HPA) axis occurs [4]. Therefore, the response of the HPA axis is thought to be an important survival element [3].

It is recognized that short-term exposure to high ambient temperature rapidly activates the HPA axis as part of a well-known physiological concept, elevating the plasma adrenocorticotropic hormone (ACTH) which leads to a subsequent rise of the blood corticosterone (CORT), both known as "stress markers" [1,5]. Acting in the hypothalamus and pituitary gland, these hormones have a crucial regulatory part in the HPA axis activity resulting in a termination of the animal response to an external stressor [6].

Most of the studies that are investigating the effect of short-term exposure of rats to elevated ambient temperature (one hour at 38°C) on the activity of the HPA axis [5,7-9], have found increased blood ACTH and CORT concentrations. On the other hand, there is very few and ambiguous data about the effect of prolonged exposure to moderately high ambient temperature on the activity of the HPA axis, showing absence or increased values of blood ACTH and CORT concentrations after 4-5 days exposure to 35-38°C [10,11]. The morphological studies are even more scarce. Our earlier data revealed diminished histomorphometric characteristics of ACTH cells, followed by decreased ACTH and CORT blood concentrations after one day of moderate heat exposure (35 ± 1°C) [12].

Regarding the variable duration of exposure to high ambient temperature and its characteristic amplitude in the South-Eastern parts of Europe on one hand, as well as the specific effect of this factor on the activity of the HPA axis and its operative units on the other, a need for further research has been raised. Having in mind the general paucity of morpho-functional reports regarding the effect of prolonged heat exposure on the HPA axis, the purpose of this study was to elucidate the histological and hormone secreting changes this stressor might provoke in the rat pituitary ACTH cells. Therefore, we examined the histomorphometric and immunofluorescent characteristics of pituitary ACTH cells and conducted an adequate ACTH blood levels measurement after 4 days of constant exposure of rats to moderately elevated ambient temperature (35 ± 1°C).

MATERIAL AND METHODS

Animals and experimental protocol

The experiments were conducted on adult male Wistar rats, with body weight from 260g to 350g. The animals were divided into two groups (7 animals *per* group): control and heat exposed (experimental) group. The control group was kept at room temperature (20 ± 2°C), while the experimental group was continuously exposed for 4 consecutive days to moderately high ambient temperature (35 ± 1°C) in a special heat chamber with regulated air temperature and air humidity of 30-40%, as previously described [12,13]. Both groups were kept under a 12:12 h light-dark regime, having free access to standard laboratory food and water.

The specific temperature for the experimental group (35 ± 1°C) was chosen based on some previous investigations [13], where it was established as a moderate high environmental temperature. Furthermore, the climate region of South-Eastern Europe, which we belong to, is considered to have similar air temperatures during the summer months.

After 4 days of exposure, animals were anesthetized with ether narcosis (Diethyl ether stabil. G.R., Lach-Ner, s.r.o., 27711 Neratovice, Czech Republic) and sacrificed by a laparotomic procedure. The sacrifice was performed between 8.00-9.00 AM. Subsequently, samples of arterial blood (*a. dorsalis*) were taken and the plasma was frozen at -70°C for hormonal analysis, while the pituitary gland was extirpated for the purpose of further histological analyses.

All animal procedures are in accordance with the EU Directive 2010/63/EU and were approved by the Local Animal Care Committee of the Faculty of Veterinary Medicine-Skopje (No.0201-4506/2 from 7.11.2011).

Immunohistochemistry

The pituitaries were removed immediately after euthanasia, weighed and fixed in 4% paraformaldehyde for 24 h. After dehydration in ethanol with increasing concentration, they were enlightened in xylol and embedded in paraffin. For immunostaining, series of seven horizontal 5-μm thick sections, cut through three levels (superior, middle and inferior) of the distal part of the gland were used. The immunohistochemical localization of pituitary ACTH cells was performed using a peroxidase-antiperoxidase (PAP) method [14] as described in detail previously [12,17,20]. In general, after the section's rehydration, 0.3% H_2O_2 was used for blocking the endogenous peroxidase activity, while non-specific staining was reduced by normal porcine serum (DAKO A/S; Glostrup, Denmark). After incubation in primary antibody for 24 h (hACTH antiserum DAKO A/S, Glostrup, Denmark; Code No., Ref: N1531, Lot No. 10016800; 1:200), with strong reaction with rat ACTH (cross reaction verified by Dr. Yang from Dako Corp. [15]), sections were incubated in secondary antibody for 1 h (swine anti-

rabbit IgG; DAKO, Glostrup, Denmark; Code No. P 0399, Lot No. 20011615; 1:100), and then in rabbit PAP complex (DAKO A/S, Glostrup, Denmark; 1:100) for 45 min. Each step was followed by rinsing of the sections in PBS. The visualisation was achieved by 0.05% diaminobenzidine (DAB; Serva, Heidelberg, Germany) and 0.03% H_2O_2 and the counterstaining was performed with haematoxylin. The control sections were treated the same way, but without the primary antibody.

Digital visualization was obtained by a Leitz DM RB light microscope (Leica, Germany) with a DFC320 CCD camera (Leica Microsystems Ltd. Switzerland) and a DFC Twain Software (Leica, Germany).

Immunofluorescence

The immunofluorescent analysis, as well as the evaluation of the relative intensity of fluorescence (RIF) was performed according to previously described data [16-17]. Briefly, pituitary sections were preincubated in normal donkey serum (Dako, Denmark; 1:10); then, after incubation for 24h with hACTH (DAKO A/S, Glostrup, Denmark; Code No., Ref: N1531, Lot No. 10016800; 1:200), they were incubated with secondary antibody (Alexa Fluor 488 donkey anti-rabbit IgG; Molecular Probes, Inc., USA; 1:200) for 2h. For the digital visualization, a confocal laser scanning microscope Leica TCS SP5 II Basic (Leica Microsystems, Germany) with the Ar-ion 488-nm laser has been used. Image analysis was achieved by a LAS AF Lite software (Leica Microsystems, Germany). Relative intensity of fluorescence (RIF) in the cytoplasm of pituitary ACTH cells was evaluated according to previously described procedures [16-17].

Morphometric analyses

Morphometric analyses were conducted as previously described in detail [17-21]. Briefly, seven immunohistochemically labelled pituitary sections were analysed (two from the superior and inferior part and three sections from the middle part of the gland). The morphometric analyses were conducted by a point counting method, using a M_{42} multipurpose test grid [22]. Counting was carried out on 50 test fields/ section at a magnification of x1000. Calculations were performed *per* animal (7 sections x 50 test fields = 350 test fields), whereas five animals were analysed *per* group. Cellular (Vc, μm^3) and nuclear (Vn, μm^3) volume, as well as a relative volume density (V_{VC}, %) of ACTH immunopositive cells were determined.

The cellular and nuclear volumes were calculated according to these formulas:

$$Vc = 1/ N_V \text{ , and}$$
$$Vn = V_{Vn/} N_V$$

where V_{Vn} represents a nuclear volume density of ACTH cell, providing an information about the nuclei attendance, while N_V indicates a numerical density of these pituitary

cells (corresponding to the number of cells *per* mm^3) and is calculated upon the formula:

$$N_V = (k/\beta)\ (N_A^{3/2} / V_{Vn}^{1/2})$$

According to previous reports [23], β represents a shape coefficient for pituitary cells, (estimated to be 1.32), k is associated with the cell distribution (k=1 for ACTH cells) and N_A represents the number of cells present in the section plane.

Relative volume density (V_{VC}) of ACTH-immunopositive cells was expressed as their percentage in a volume unit. This parameter was calculated using the formula:

$$Vvc = \Sigma(Pn + Ptc) / 50 \times 42$$

where the relative volume density of ACTH cells (Vvc) actually represents the ratio between the sum of points on nuclei (Pn) and cell bodies (Ptc) in all 50 measured test fields. As the test system has 42 points and we have measured 50 fields, the total number of points is calculated as: 50 x 42.

Hormonal analysis

For conducting the hormonal analysis, plasma samples were used and stored at -70°C until assayed. The plasma levels of ACTH in both groups (experimental and control) were determined by the IMMULITE method (Diagnostic Products Corporation; Los Angeles, CA, USA), in duplicate samples within a single assay. The intra-assay coefficient of variation was 9.6%, while the analytical sensitivity of the assay was 9 pg/ml.

Statistical analysis

The statistical analysis was conducted by STATISTICA® version 5.0 (StatSoft, Inc). The hormonal, morphometric as well as RIF data were evaluated by the Student's t-test. A $p<0.05$ level of confidence was assumed as the statistically significant result. All data were shown as means ± SD.

RESULTS

Body mass and pituitary weights

The body mass and pituitary weights are presented in Table 1. In comparison to the control group, the body mass in the heat-exposed group was significantly ($p<0.05$) decreased by 19.8%. Also, heat exposure caused a significant ($p<0.05$) increase of the pituitary gland volumes (both absolute and relative) by 23.1% and 36.1% respectively, compared to the control group.

Table 1. Body mass and pituitary weights in control and heat exposed rats

Experimental group	Body mass (g)	Absolute pituitary weight (mg)	Relative pituitary weight (mg%)
Control	337.5 ± 26.9	6.5 ± 0.6	2.5 ± 0.1
Heat exposed	270.6 ± 11.7* (-19.8%)	8.0 ± 0.4* (+23.1%)	3.4 ± 0.3* (+36.1%)

The values are the means ± SD (n=7/group), *p<0.05 *vs.* Control.

Immunohistochemical and immunofluorescent findings

The characteristic findings observed during the analysis of immunohistochemically identified ACTH cells in control male rats showed their stellate shape with long cytoplasmic processes spreading towards bordering (mostly somatotrophic) cells. The nuclei were following the shape of the ACTH cell bodies, while the specific fields with strong immunohistochemical staining (reflecting the presence of secretory granules) were spreading mostly at the cytoplasmic periphery (Figure 1A). In the heat-exposed group, we did not notice any change of the form or the localization of ACTH immunoreactive cells. Nevertheless, the immunopositivity of ACTH cells in heat exposed group was weaker (Figure 1B). Compared to the control group, the immunofluorescent appearance/glow of ACTH cells was less pronounced, while the RIF quantification suggested a significantly ($p<0.05$) weaker signal of 15.3%, after 4-day heat exposure (Figure 1C,D,E).

Morphometric results

A significant increase of the cellular and nuclear volumes of immunolabelled ACTH cells and decrease of their volume density (by 6.9 %, 14.3 % and 20.0%, respectively; $p<0.05$) was registered in heat exposed group in comparison with the same parameters in control group (Figure 2 A,B,C).

Hormonal analysis

Compared to the controls, plasma concentration of ACTH in the rats exposed to moderate heat was increased ($p<0.05$) by 23.7% (Figure 3).

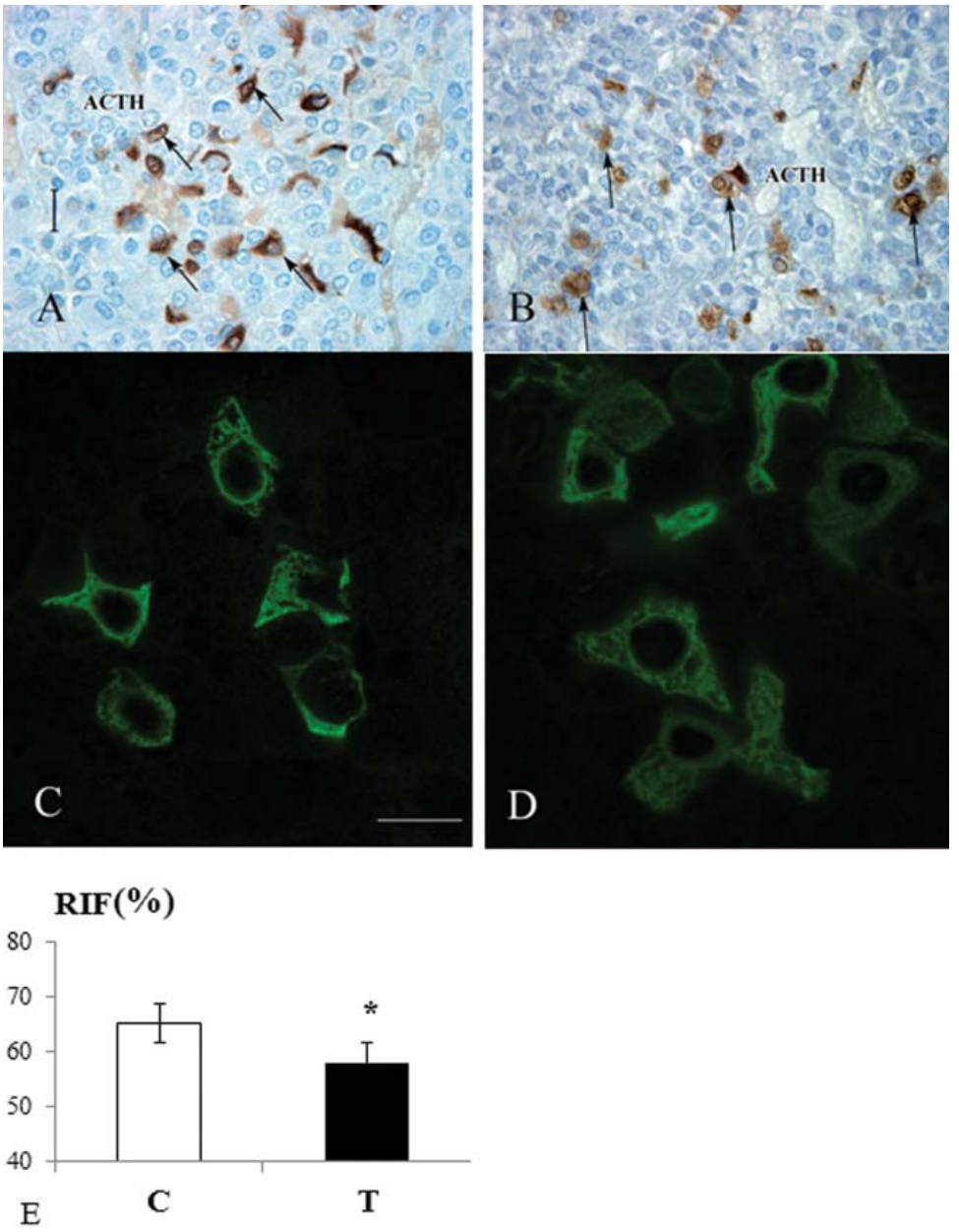

Figure 1. Immunopositive ACTH cells (black arrows) in the distal part of the pituitary gland from **A)** control and **B)** heat exposed male rats (bar = 16μm); Immunofluorescent appearance of ACTH cells in the distal part of the pituitary gland form **C)** control and **D)** heat exposed male rats (bar = 50 μm, region of interest (ROI) 9.5x); **E)** relative intensity of fluorescence (RIF, %) measured in ACTH cells from control (C) and heat exposed (T) groups. The values are the means ± SD (n=7/group), *p<0.05 *vs.* C.

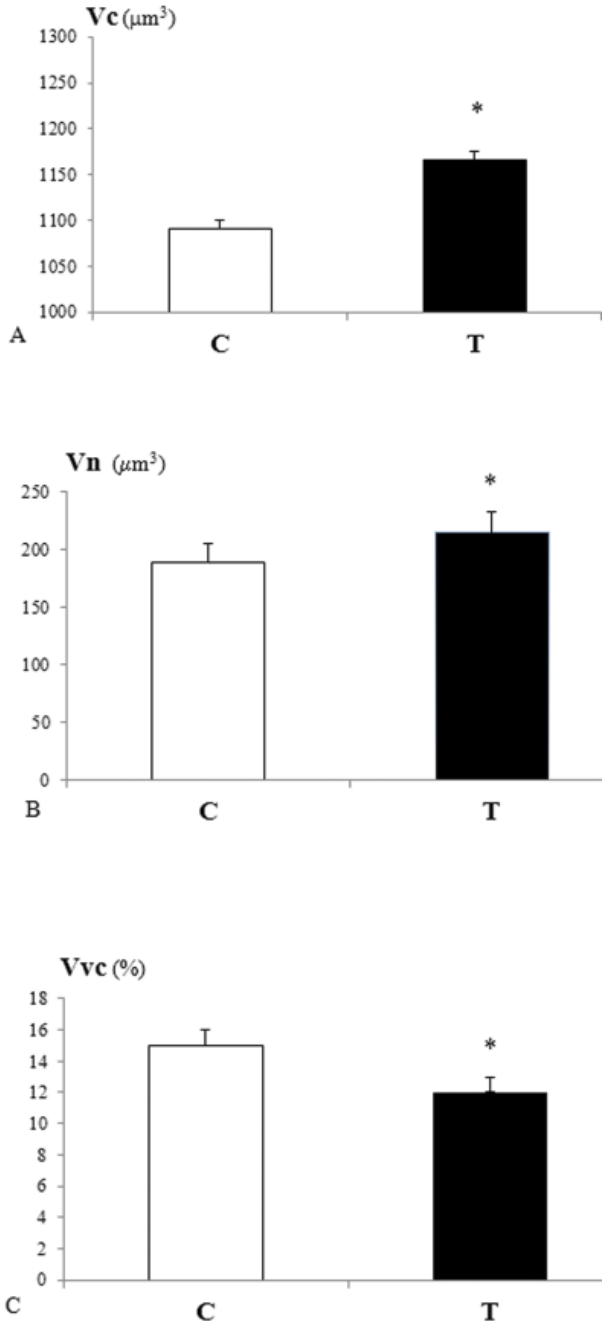

Figure 2. A) Cellular volume (Vc; μm³), **B)** nuclear volume (Vn; μm³) and **C)** relative volume density (Vvc; %) of pituitary ACTH cells in control (C) and heat exposed (T) rats. All values are the means ± SD (n=7/group), *p<0.05 *vs.* C.

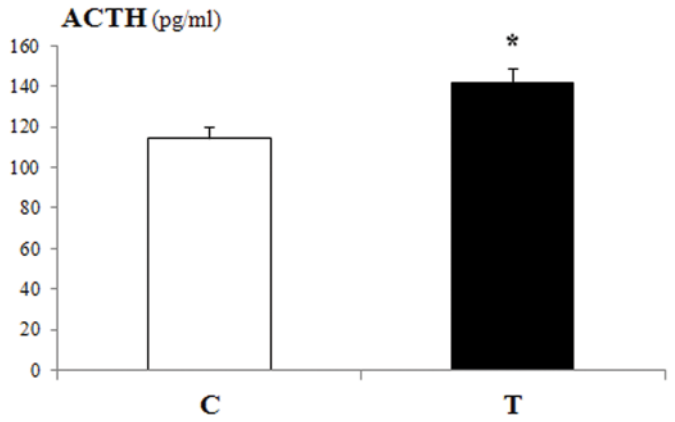

Figure 3. Plasma concentration of ACTH (pg/ml) in control (C) and heat exposed (T) rats. Values are means ± SD (n=7/group), *p<0.05 *vs.* C.

DISCUSSION

The main objective of this study was to evaluate the histological and hormone secreting changes of the rat pituitary ACTH cells after 4 days of constant exposure to warm environment, considering the important role of circulating ACTH and related glucocorticoids in a successful acclimatization [24]. In line with this, the areas with moderate continental climate are characterized by prolonged periods with high ambient temperature during the summer months, representing a persistent stressogenic factor, which is very conducive to our research.

The decreased body mass of our rats after 4-day exposure to elevated ambient temperature is in line with data observed by other authors in rats [25] and pigs [26], chronically exposed to high ambient temperatures (33-36°C). This decrease might be a result of decreased food intake and increased water consumption in animals subjected to moderately high ambient temperature [27,28]. Our findings of increased pituitary weights are in coherence with the literature data. Namely, Koko et al. [7] showed elevated pituitary weights in rats subjected to acute heat stress (38°C). The observed elevation appears to be a result of increased intermediate and posterior pituitary lobe weight, due to dilation of small blood vessels and thickened hypothalamic axons in the latter [7]. Presumably, the increase of the relative pituitary weight found in our study is primarily the consequence of evidently decreased body weight, *i.e.* decreased divisor in the adequate formula (quotient) for relative pituitary weight calculation.

Results of the histomorphometric, immunofluorescent and hormonal analyses, pertinent to ACTH cells in our study, point towards stimulation of these pituitary cells after 4-day exposure to moderately high ambient temperature. More precisely, the ACTH cell and nuclei volumes were increased, while the immunohistochemical and immunofluorescent findings suggested their appearance of 'emptied entities', followed

by increased circulating ACTH concentrations. The hypothalamic corticotrophin-releasing hormone (CRH) is considered as a principal factor of the pituitary ACTH cell activity and, together with another hypothalamic hormone vasopressin (VP), was found to stimulate ACTH production and release in the circulation [29-30]. Some previous thermal stress-related studies suggest elevated plasma ACTH concentrations in rats after 4-day or 7-day exposure to moderate heat [4,11]. Actually, it was found that heat exposure provoked the rise of the plasma VP content and the pituitary VP receptor level, suggesting that VP could also be an important factor for ACTH cell activity regulation during prolonged thermal stress [4]. Furthermore, Jasnic et al. [8] asserted that decreased density of ACTH cells upon heat exposure is not necessarily connected with their number changes, but it is rather a result of cells' degranulation, which is regularly followed with secretion of pre-synthesized hormone. Fillipa and Mohamed [31] also reported total or partial degranulation of ACTH cells after secreting the ACTH hormone.

In conclusion, 4 days of continuous exposure of rats to moderately elevated ambient temperature modulated the histomorphometric and immunofluorescent characteristics of pituitary ACTH cells in a manner that indicated emptying of their hormonal content, which was confirmed by the elevation of blood ACTH concentrations. Obviously, ACTH cells as a HPA axis operative component manifest some active resistance to a prolonged stressor and successfully meet the increasing demand for their hormonal answer.

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Authors' contributions

PPF, MV and AV are the manuscript concept creators, have processed the experimental material, performed the morphometric measurements, written majority of the manuscript, designed the figures, analyzed and interpreted results of the research group in a broader context of literature. PPF an PL have organized and conducted the experiment (work in the animal unit, daily care and treatment, sacrifice, pituitary glands extraction, morphometric analyses etc.). RN and TS have performed the immunohistochemistry and light microscopy analysis/photography. JI has performed the immunofluorescence, confocal microscopy/imaging and relative intensity of fluorescence measurements. MV and AV have carefully read and critically revised the manuscript for its scientific merit and intellectual content and have supplemented the discussion and literature survey. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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EFEKTI UMERENE TOPLOTE NA ACTH ĆELIJE HIPOFIZE PACOVA: HISTOMORFOMETRIJSKA, IMUNOFLUORESCENTNA I HORMONALNA STUDIJA

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U područjima sa umereno kontinentalnom klimom, povećavanje prosečne temperature tokom leta predstavlja stresogeni faktor koji utiče na hipotalamo-hipofizno-adrenokortikalnu osovinu u sisara. Stoga, cilj ovog istraživanja je bio da se ispita uticaj 4 dana neprekidnog izlaganja umerenoj toploti (35 ± 1°C) na histomorfometrijske i imunofluorescentne karakteristike, kao i hormonsku sekreciju hipofiznih adrenokortikotropnih (ACTH) ćelija kod odraslih mužjaka pacova. Nakon izlaganja toploti, apsolutne i relativne mase hipofize su povećane ($p<0,05$) za 23,1% odnosno 36,1%, u poredjenju sa kontrolom držanom na 20 ± 2°C. Oblik i lokalizacija ACTH imunoreaktivnih ćelija u grupi pacova izloženih umereno povišenoj toploti nisu se značajno promenile u poređenju sa kontrolama, ali su bile slabije obojene. Relativan intenzitet fluorescencije ACTH ćelija potvrdio je slabiji signal (15,3%, $p<0,05$) posle 4 dana izlaganja umereno povišenoj toploti. Volumen ACTH ćelija i jedara je bio povećan ($p<0,05$) za 6,9% odnosno 14,3%, dok je volumenska gustina ovih ćelija bila značajno ($p<0,05$) smanjena za 20,0%, u poredjenju sa kontrolom. Uočene histomorfometrijske i imunofluorescentne promene ACTH ćelija hipofize su u skladu sa značajnim povećanjem vrednosti ACTH u plazmi za 23,7% u odnosu na kontrolne pacove. Može se zaključiti da je 4 dana izlaganja umerenoj toploti pojačalo hipofiznu ACTH sekreciju kod odraslih mužjaka pacova.