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Research article

THE EFFECTS OF LONG-TERM EXPOSURE TO MODERATE HEAT ON RAT PITUITARY ACTH CELLS: HISTOLOGICAL AND HORMONAL STUDY

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Global warming causes an increased ambient temperature and prolonged heatwaves during the summer, which represent stressogenic factors affecting the hypothalamo-pituitary-adrenocortical (HPA) axis in mammals. The aim of this study was to investigate the effects of long-term (7-60 days) exposure to moderately elevated ambient temperature (35 ± 1°C) on the histological aspect and secretory ability of pituitary adrenocorticotropic (ACTH) cells, as well as on the corticosterone output, in adult rats. Stereological parameters of ACTH cells were estimated upon immunohistochemistry. The blood concentrations of ACTH and corticosterone were determined by immunoassays. The volume of ACTH cells in rats exposed to moderately high temperature for 7, 14, 21, 30 and 60 days decreased ($p<0.05$) by 18.1%, 14.5%, 13.5%, 8.6% and 14.2% respectively, compared to the same parameter in the controls. The volume density of ACTH cells in the groups exposed to elevated temperature for 7, 14, 21, 30 and 60 days decreased ($p<0.05$) by 40.0%, 33.3%, 26.7%, 13.3% and 26.7% respectively, in comparison with control rats. The plasma concentration of ACTH varied differently ($p<0.05$) with the duration of exposure to the elevated temperature. The serum concentration of corticosterone was decreased ($p<0.05$) by 54.9%, 24.4%, 29.9%, 21.1% and 24.4% in groups subjected to moderately high temperature for 7, 14, 21, 30 and 60 days respectively, all compared to the control value. Despite some signs of functional recovery of ACTH cells during the treatment, the impression is that the long-term character of this stressor overcomes the capacity of the HPA axis for resistance.

Key words: ACTH cells, elevated temperature, pituitary, rats, stereology

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INTRODUCTION

The topic of global warming is currently one of the most discussed problems in the world. According to the World Meteorological Organization (WMO) observations, 2011-2020 will be the warmest decade on record [1]. The Community Earth System Model (CESM), a global climate model that *inter alia* provides state-of-the-art computer simulations of the Earth's future climate, projects an increase of the daily maximum temperatures during summer by 1-4°C by year 2050 and 2-7°C by 2095, across the United States [2]. These projections that include the occurrence of the long-term series of the average annual air temperature are applicable for the Western Balkans region as well, suggesting increased values of the air temperatures in this part of Europe. The beginning of the warming process in this part of South-Eastern Europe is reported to have started 30 years ago, resulting with an increase of the average annual air temperatures by 1°C [3,4]. Additionally, long-term observations and climate modeling indicate an expansion of the subtropical climate to the northern hemisphere, as well as a global increase of heatwaves frequency and intensity [1,5,6]. A heatwave represents a period of excessively hot weather, when the air temperature exceeds a threshold value for a particular geographical region in several consecutive days [7,8].

Ambient temperature is one of the essential environmental factors that primarily determines the physiological processes in living organisms. Thus, the temperature extremes may strongly influence animal homeostasis and trigger numerous physiological, autonomic, and behavioral responses [4,9]. The "animal thermal comfort zone" is an ambient temperature range in which animal metabolic processes are stable and directed to the storage of carbohydrates, proteins and fat [10]. Temperatures above this range induce thermal stress in the organism and the activation of the sympatho-adrenomedullary system, as the first response to the thermal stressor, or in the case of a persistent stimulus, the activation of the hypothalamic-pituitary-adrenal (HPA) axis [11]. An increase in the plasma adrenocorticotropic hormone (ACTH) and circulating glucocorticoids, as part of the HPA axis activation upon exposure to high ambient temperature, stimulates numerous metabolic processes (glycogenolysis, lipolysis, and proteolysis) aimed to increase energy availability [4,9,12,13]. The majority of the studies, focused on the effect of short exposure of rats to high ambient temperature (one hour at 38°C), have found increased blood ACTH and corticosterone concentrations [13-16]. On the other hand, our earlier research suggested reduced immuno-histomorphometric parameters of ACTH cells, with concurrently decreased blood ACTH and corticosterone concentrations in rats after one-day exposure to moderate heat (35 ± 1°C) [17]. Four days of continuous exposure to the same ambient temperature (short-term exposure) caused an active resistance of rat ACTH cells, manifested by their weaker immuno-positivity/fluorescence, increased cellular and nuclear volumes and intensified ACTH secretion [9,18]. Furthermore, the adrenocortical volume and number of cells in all cortical zones, as well as the serum levels of aldosterone and corticosterone, were significantly increased in the rats subjected to elevated temperature (35 ± 1°C) for four days [4].

Hence, the current hypothesis was focused on two crucial aspects: the existing experimental data pertinent to the stress response and the insufficiently elaborated functional histology of ACTH cells as the operative module of the HPA axis, following long-term exposure to elevated ambient temperature (a heatwave). Therefore, this research is aimed to assess the effect of moderately increased ambient temperature (35 ± 1°C) in adult rats during a prolonged time (7-60 days), on the histological parameters and secretory ability of pituitary ACTH cells (along with the corticosterone output). The temperature set-up used in this study exceeds the thermal comfort range in rats and reflects the real weather conditions during summertime in the Western Balkan region [4].

MATERIAL AND METHODS

Animals and experimental design

All animal procedures were approved by the Local Animal Care Committee of the Faculty of Veterinary Medicine, University in Skopje (No. 0201-4506/2 from 7.11.2011) and followed the instructions provided in the EU Directive 2010/63/EU. Animals did not suffer unnecessarily at any stage of the investigation.

The experiments were conducted on adult (2.5 months old) male Wistar rats, with body mass in the range between 260g and 350g. Before the start of the experiment, all animals were pre-acclimated at room temperature (20 ± 2°C) for 14 consecutive days, under standard conditions (12:12h light-dark regime, with free access to standard laboratory feed and water). At the onset of the experiment, rats were divided into six groups (7 animals *per* group, one group *per* one large cage): the control group, as well as 6 experimental groups, were exposed to moderately high temperature (35 ± 1°C) for 7 days, 14 days, 21 days, 30 days and 60 days. The animals in the control group were kept at room temperature (20 ± 2°C), while the rats continuously exposed to elevated ambient temperature were placed in a special heat chamber, with regulated air temperature and air humidity at 30-40%, as previously described [17,19]. The specific temperature for the experimental groups (35 ± 1°C) was exploited in our previous studies and established as a moderately high environmental temperature [4,9,17]. In line with this, the mode of continuous exposure to the elevated temperature was proposed in other studies [19,20]. In order to further support the chosen temperature range, as an experimental design for thermal exposure, it should be mentioned that the climate region of Western Balkans we belong to is well known to have similar air temperatures during the summer months [4,9,17,19]. After 7 days, 14 days, 21 days, 30 days and 60 days of exposure to moderately high temperature (reflecting the exposure to a heatwave), the animals were weighed and subsequently sacrificed by a laparotomic procedure under ether narcosis (Diethyl ether stabil. G.R., Lach-Ner, s.r.o., 27711 Neratovice, Czech Republic). 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 analyses. The pituitary glands were extirpated, and

prepared for imunohistochemical staining and quantitative histological (stereological) analysis.

Immunohistochemistry and light microscopy

Upon sacrifice, the removed pituitary glands were weighted (pituitary weight to body mass relative ratio was calculated) and fixed in 4% paraformaldehyde for 24 h. After dehydration in ethanol with increasing concentration, they were enlightened in xylol and embedded in paraplast (Histolab Product AB, Göteborg, Sweden). For immunostaining, series of seven horizontal 5-μm thick sections were obtained using a rotational microtome (RM 2125RT Leica Microsystems, Wetzlar, Germany). They were cut through three levels (superior, middle and inferior) of the distal part of the gland. The immunohistochemical labelling of pituitary ACTH cells was performed using the peroxidase-antiperoxidase (PAP) method [21] as described in detail previously [9,22]. In essence, after rehydration of the sections, 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 for 24 h with primary antibodies (hACTH antiserum DAKO A/S, Glostrup, Denmark; Code No., Ref: N1531, Lot No. 10016800; 1:200), with a strong reaction with rat ACTH, the sections were incubated with secondary antibodies 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. Visualization was achieved by 0.05% diaminobenzidine (DAB; Serva, Heidelberg, Germany) and 0.03% H_2O_2 and counterstaining was performed with haematoxylin. The negative control sections were treated the same way, but without primary antibodies.

Digital images of immunohistochemically labelled pituitary sections were taken using a LEITZ DM RB light microscope (Leica Mikroskopie & Systems GmbH, Wetzlar, Germany), and a LEICA DFC320 CCD camera (Leica Microsystems Ltd., Heerbrugg, Switzerland) and the Leica DFC Twain Software (Leica, Germany).

Quantitative histology

The quantitative histological (stereological) analysis was conducted as previously described in detail [9,22,23,24]. Briefly, seven immunohistochemically labelled pituitary sections were analyzed (two from the superior and inferior part and three sections from the middle part of the gland). Stereological analysis was conducted with a point-counting method, using a M_{42} multipurpose test grid [25]. 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 analyzed *per* group. A 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 the 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 according to the formula:

$$N_V = (k/\beta) (N_A^{3/2}/ V_{Vn}^{½})$$

According to the previous reports [26], 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 analyses

The blood plasma and serum samples were collected from the rat trunks and stored at –70 °C until assayed. Plasma levels of ACTH were determined without diluting the plasma, by the IMMULITE method (DPC, Los Angeles, USA), in duplicate samples within a single assay, with an intra-assay CV of 9.6%. Serum corticosterone concentrations were determined without diluting the sera, by immunoassay (R&D Systems Inc., Minneapolis, USA), in duplicate samples within a single assay, with an intra-assay CV of 8.0%.

Statistical analysis

STATISTICA® version 7.0 (StatSoft, Inc) was used for the statistical analysis. All stereological and hormonal parameters were averaged and the standard deviation of the mean value was calculated. One-way analysis of variance (ANOVA) followed by a Duncan test was used for comparison of differences between the groups. A probability value of 5% or less was considered statistically significant.

RESULTS

Body mass and pituitary weights

The body mass values are presented in Table 1, while the body mass fluctuation during long-term exposure to moderately high temperature is presented in Figure 1. After 7 and 21 days of exposure to moderately high temperature, body mass decreased by 22.6% ($p<0.05$) compared to the control group. In rats exposed to moderately high temperature for 14 and 30 days, the body mass reduction was 16.4% and 24.5 % ($p<0.05$) respectively, in comparison with the control values. The most prominent body mass reduction was noted in the experimental animals exposed to elevated temperature for 60 days, which measured 37.6% ($p<0.05$) when compared to the control group.

Table 1. Body mass in control rats and rats exposed to 35±1°C during different periods of time (7, 14, 21, 30 and 60 days).

Group	Body mass (g)
Control	337.5 ± 26.9
7 days	261.2 ± 21.8* (-22.6%)↓
14 days	282.1 ± 20.4* (-16.4%)↓
21 days	261.3 ± 24.6* (-22.6%)↓
30 days	254.9 ±16.8* (-24.5%)↓
60 days	210.5 ± 6.5* (-37.6%)↓

All values are the means ± SD, n=7 animals *per* group; *p<0.05 *vs.* control.

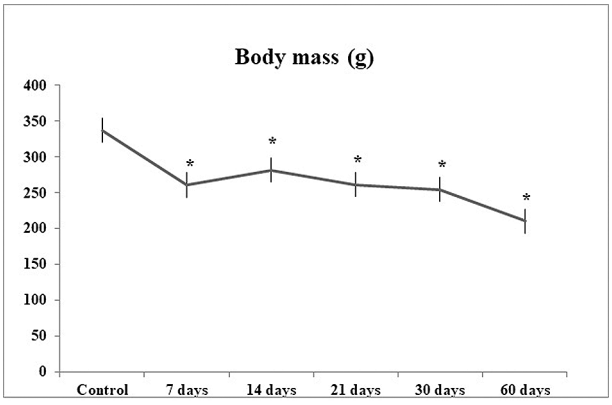

Figure 1. Body mass fluctuation during long-term exposure (7, 14, 21, 30 and 60 days) to moderately high temperature (35 ± 1°C).

Absolute pituitary weight was increased by 27.7% ($p<0.05$) after 14 days of exposure to moderately high temperature, while relative pituitary weight was increased by 24.0% ($p<0.05$) upon 30 days of the same treatment, all in comparison with adequate control values (Fig 2A,B). In other groups of rats, subjected to elevated ambient temperature, absolute and relative pituitary weights were insignificantly changed ($p>0.05$) compared to corresponding controls (Fig 2A,B).

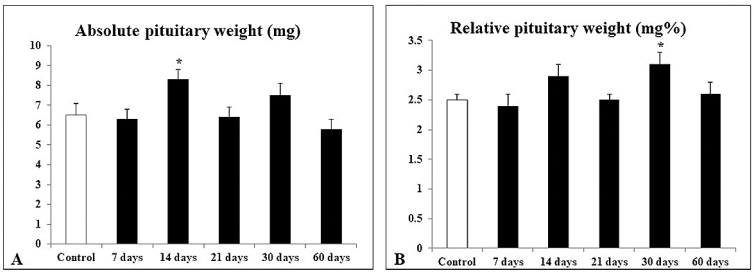

Figure 2. A) Absolute and **B)** relative weight of pituitary glands in control and rats exposed to moderately high temperature (35 ± 1°C) for 7, 14, 21, 30 and 60 days. All values are the means ± SD, n=7 animals *per* group; *$p<0.05$ *vs.* control.

Immunohistochemical findings

The pituitary ACTH cells in control rats are dispersed as individual or remain in groups and have an irregular or star-like shape. The cytoplasmic protrusions of control ACTH cells have a tendency to envelop adjacent cells or to extend between them. Oval nucleus with visible nucleolus as well as peripheral, dark secretory granules characterize these ACTH cells as well (Fig. 3A). In experimental groups of rats, subjected to moderately high temperature from 7 – 60 days, the shape of ACTH cells was not markedly changed when compared to the controls. On the other hand, their abundance appears to be variable (Fig. 3B-F). Pronounced dark granules are noticeable in the cytoplasm of ACTH cells exposed to high ambient temperature for 30 days (Fig. 3E). To some extent dilated capillaries are also visible in the pituitary gland tissue of rats exposed to high temperature for 60 days (Fig. 3E, 3F).

Stereological results

The volume of pituitary ACTH cells of rats exposed to moderately high temperature for 7, 14, 21, 30 and 60 days decreased ($p<0.05$) by 18.1%, 14.5%, 13.5%, 8.6% and 14.2% respectively, compared to the value of the same parameter in the controls (Fig. 4A). The volume of ACTH cell nuclei decreased ($p<0.05$) only in the animals exposed to elevated temperature for 7 and 14 days, by 14.8% and 20.0% respectively, in comparison with the same parameter in the control group (Fig. 4B). Volume density of pituitary ACTH cells of experimental groups exposed to moderately high temperature for 7, 14, 21, 30 and 60 days decreased ($p<0.05$) by 40.0%, 33.3%, 26.7%, 13.3% and 26.7% respectively, compared to the ACTH cells volume density in control rats (Fig. 4C).

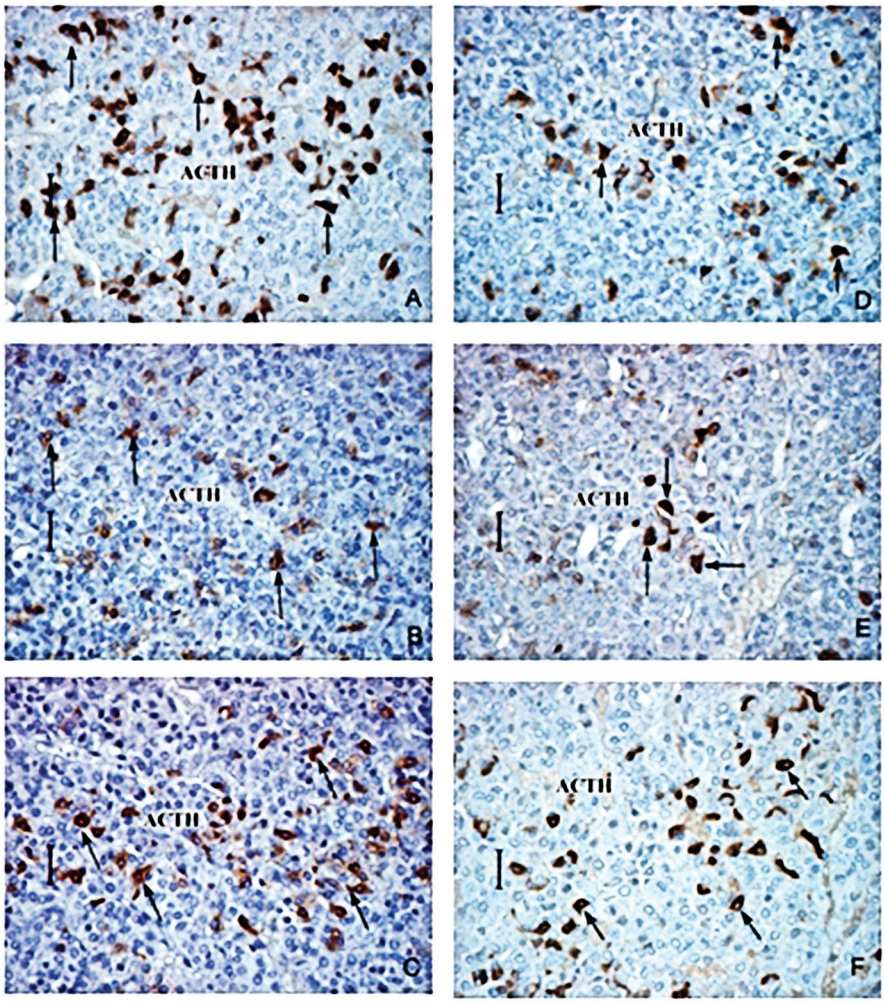

Figure 3. Immunopositive ACTH cells (black arrows) in *pars distalis* of the pituitary gland from: **A)** control and rats exposed to moderately high temperature (35 ± 1°C) for **B)** 7 days, **C)** 14 days, **D)** 21 day, **E)** 30 days and **F)** 60 days (objective magnification 40x, bar=25 μm).

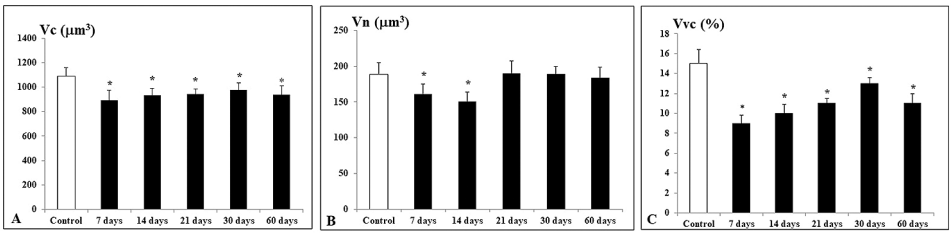

Figure 4. A) Cellular volume (Vc, μm^3), **B)** nuclear volume (Vn, μm^3) and **C)** relative volume density (Vvc, %) measured in ACTH cells from control and rats exposed to moderately high temperature (35 ± 1°C) for 7, 14, 21, 30 and 60 days. All values are the means ± SD, n=7 animals *per* group; *p<0.05 *vs.* control.

Hormonal levels

Plasma concentration of ACTH decreased ($p<0.05$) by 27.3% and 36.2% in rats exposed to elevated ambient temperature for 7 and 30 days respectively, all compared to the control values. On the contrary, plasma concentration of ACTH in animals exposed to moderately high temperature for 14 and 60 days increased ($p<0.05$) by 27.2% and 15.8% respectively, in comparison with the same parameter in control rats (Fig. 5A). Serum concentration of corticosterone decreased ($p<0.05$) by 54.9%, 24.4%, 29.9%, 21.1% and 24.4% in groups subjected to moderately high temperature for 7, 14, 21, 30 and 60 days respectively, all compared to the control corticosterone value (Fig. 5B).

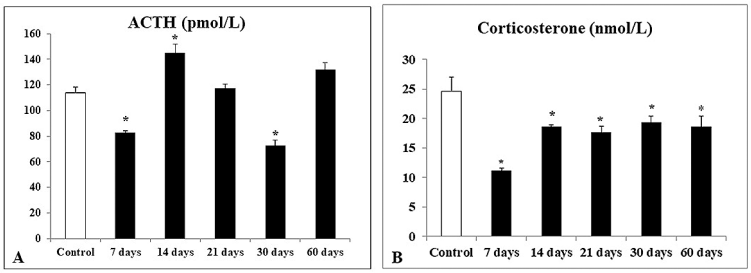

Figure 5. A) Plasma concentration of ACTH and **B)** serum concentration of corticosterone in control and rats exposed to moderately high temperature (35 ± 1°C) for 7, 14, 21, 30 and 60 days. All values are the means ± SD, n=7 animals *per* group; *$p<0.05$ *vs.* control.

DISCUSSION

Apart from the natural cycles, the anthropogenic factor has also a significant role in the process of global warming. According to the state report of the global climate in 2020, issued by the WMO, the atmospheric concentrations of greenhouse gases have continued to rise despite the COVID-19 lockdown [1]. An increase in the average annual temperature represents the most significant consequence of global warming. Consequently, the Mediterranean, South-Eastern Europe and Western Balkans regions are characterized by prolonged periods of high ambient temperature during the summer months (heatwaves), being a persistent stressogenic factor [4,6,7,9]. The function of the HPA axis follows the seasonal and daily temperature rhythm, whereby circulating ACTH and glucocorticoids have an important role in successful acclimatization [27]. Therefore, the main objective of this study was to evaluate the histological parameters and secretory ability of pituitary ACTH cells (along with the corticosterone output), following a long-term (7-60 days) exposure of adult rats to moderately increased ambient temperature (35 ± 1°C).

The observed decrease in the body mass of rats in all groups continuously exposed to elevated ambient temperature (7-60 days) was the most prominent in the 60-day group. This is in line with data from other studies, which showed that continuous exposure

to the temperatures between 34°C and 37°C, for up to 60 days, leads to a decrease of body mass in guinea pigs [28], mice [29,30] and rats [31]. A body mass decrease upon exposure of animals to high ambient temperature probably results from the decreased food intake and increased water consumption [32,33], as well as decreased energy or basal metabolism in hyperthermia [32,34,35]. An increase in the absolute (after 14 days of exposure) and relative (after 30 days of exposure) pituitary weight was observed in the current research. Although Koko and co-workers [14] found higher pituitary weight in rats subjected to acute heat stress (38°C), presumably due to dilation of small blood vessels and thickened hypothalamic axons in the posterior pituitary lobe, in our study such an observation cannot be stated.

During the long-lasting period (7-60 days) of acclimation of rats to elevated ambient temperature, a decrease in ACTH cell volume and relative volume density (indicating signs of exhaustion) was observed in all groups compared to the controls. Overall, the most pronounced decrease of the quantitative histology parameters, with a significant fall in blood ACTH and corticosterone concentrations, was noticed after 7 days of exposure to the treatment. From this point, until the end of the treatment, the mentioned parameters (except ACTH concentrations in the blood) had a gradual trend of rise/recover, but remained significantly lower than the control ones. Plasma concentration of ACTH varied differently during exposure to elevated temperature. Namely, it significantly decreased after 7 and 30 days of exposure to high ambient temperature (in line with the corresponding corticosterone fall), but it was markedly increased after 14 and 60 days of the same treatment (opposite to the corresponding corticosterone fall). These changes were likely coinciding with the negative feedback activation on the 14^{th} and 60^{th} day of exposure to elevated temperature. However, there was no subsequent rise in the blood levels of corticosterone. Gjuladin observed a decreasing trend in blood ACTH concentration in rats after 14 days of exposure to high ambient temperature, reaching the control values on the 30^{th} day of treatment [36]. Similarly, we have observed a decrease in circulating ACTH in the period between the 14^{th} and 30^{th} day of rat exposure to elevated ambient temperature. It was shown that long-lasting exposure of female rats to elevated temperature (30 days on 35°C) causes a multiple reduction in blood ACTH concentration [37]. This is in accordance with the findings in our study, demonstrating the lowest values of blood ACTH on the 30^{th} day of treatment. The decrease in stereological parameters and hormonal output of ACTH cells, observed on the 7^{th} and 30^{th} day of exposure, may result from decreased corticotropin-releasing hormone (CRH)−induced ACTH secretion upon chronic stress [38]. It is well known that arginine-vasopressin, with the mediation of protein kinase C, intensifies CRH-stimulated ACTH secretion by cAMP increase [39]. Zeisberger and colleagues [40] revealed a decrease in AVP release in guinea pigs exposed to long-term heat, due to excessive water intake and excretion of urine with low osmolality. Thus, probably decreased AVP input on the CRH-ACTH sub-axis additionally contributes to the morpho-functional status of rat ACTH cells after long-term exposure (7 and 30 days) to elevated ambient temperature. Several authors suggest

that exposure to elevated ambient temperature suppresses thyroid function as a sort of "heat thyroidectomy" [36,37,41]. Thyroidectomy is well known to decrease both the expression of CRH genes in the hypothalamic paraventricular nucleus and the secretion of ACTH [42,43], which may additionally explain our findings. As indicated above, the 14th and 60th day of heat exposure in our experiment may represent the approximate time points of temporary functional recovery of ACTH cells and the negative feedback activation.

In conclusion, continuous long-term (7-60 days) exposure of rats to moderately elevated ambient temperature decreased the quantitative histology parameters of pituitary ACTH cells in a manner that indicates either decreased synthesis (7 and 30 days of exposure) or forced emptying (14 and 60 days of exposure) of their hormonal content, followed by the corresponding fluctuations of blood ACTH concentration. The serum corticosterone concentration was uniformly decreased in all treated groups (7-60 days of exposure). Although there were some signs of a functional recovery of ACTH cells at the 14th and 60th day of exposure to moderately elevated temperature, a general impression is that the long-term character of this stressor overcomes the capacity of the HPA axis for active resistance.

Abbreviations: adrenocorticotropic hormone, ACTH; Community Earth System Model, CESM; corticotropin-releasing hormone, CRH; diaminobenzidine, DAB; hypothalamic-pituitary adrenal, HPA; one-way analysis of variance, ANOVA; peroxidase-antiperoxidase, PAP; phosphate-buffered saline, PBS; World Meteorological Organization, WMO.

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Authors' contributions

All authors named have participated in the work in a substantive way. JČK, VA and FPP the manuscript concept creators, have processed the experimental material, performed the stereological measurements, written majority of the manuscript, designed the figures, analyzed and interpreted results of the research group in broader context of literature. LP and NP have performed the hormonal analyses, histological stainings and light microscopy analysis/photography, as well as discussed some stereological aspects

of the results. LP and MD have organized and conducted the experiment (work in the animal unit, daily care and treatment, sacrifice, pituitary gland extraction, etc.). NR and VM 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 DUGOTRAJNOG IZLAGANJA UMERENOJ TOPLOTI NA HIPOFIZNE ACTH ĆELIJE PACOVA: HISTOLOŠKA I HORMONALNA STUDIJA

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Globalno zagrevanje podrazumeva povećanje prosečne temperature vazduha i dugotrajne toplotne talase tokom letnjeg perioda, što čini stresogene faktore koji utiču na hipotalamo-hipofizno-adrenokortikalnu (HPA) osu kod sisara. Cilj ove studije je podrazumevao ispitivanje efekata dugotrajnog (7-60 dana) izlaganja umereno povišenoj ambijentalnoj temperaturi (35 ± 1 °C) na histološke parametre i sekretornu aktivnost hipofiznih adrenokortikotropnih (ACTH) ćelija, kao i na sekreciju kortikosterona, kod odraslih pacova. Stereološki parametri ACTH ćelija su mereni nakon imunohistohemijskog bojenja. Koncentracije ACTH i kortikosterona u krvi su određivane imunoesejima. Volumen ACTH ćelija pacova izloženih umereno povišenoj temperaturi 7, 14, 21, 30 i 60 dana je smanjen ($p<0,05$) za 18,1%, 14,5%, 13,5%, 8,6% i 14,2% redom, u poređenju sa vrednošću istog parametra kod kontrola. Volumenska gustina ACTH ćelija grupa izloženih povišenoj temperaturi 7, 14, 21, 30 i 60 dana je smanjena ($p<0,05$) za 40,0%, 33,3%, 26,7%, 13,3% i 26,7% redom, poredeći sa volumenskom gustinom kod kontrolnih pacova. Koncentracija ACTH u plazmi je varirala u smislu povišenja i sniženja vrednosti ($p<0,05$) sa trajanjem izlaganja povišenoj temperaturi. Koncentracija kortikosterona u serumu je bila snižena ($p<0,05$) za 54,9%, 24,4%, 29,9%, 21,1% i 24,4% u grupama izloženim umereno povišenoj temperaturi 7, 14, 21, 30 i 60 dana redom, sve u poređenju sa kontrolnom vrednošću. Uprkos izvesnim znacima funkcionalnog oporavka ACTH ćelija tokom tretmana, utisak je da dugotrajno izlaganje umerenoj toploti prevazilazi kapacitet HPA ose za aktivan otpor navedenom stresoru.