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IMMUNO-HISTOMORPHOMETRIC AND –FLUORESCENT CHARACTERISTICS OF RAT GH CELLS AFTER CHRONIC EXPOSURE TO MODERATE HEAT

Popovska-Perčinić Florina[1], Ajdžanović Vladimir[2], Pendovski Lazo[1], Živanović Jasmina[2], Ilieski Vlatko[1], Dovenska Monika[1], Milošević Verica[2]

[1]*Faculty of Veterinary Medicine, "Ss Cyril and Methodius" University in Skopje, 1000 Skopje, R. Macedonia*
[2]*Institute for Biological Research "Siniša Stanković", University of Belgrade, 11060 Belgrade, Serbia*

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ABSTRACT

Growth hormone (GH) axis function appears to be changed in the warm milieu. The effect of chronic exposure to moderate heat on immuno-histomorphometric and –fluorescent characteristics of pituitary GH cells, in adult male rats, was examined. The experimental group was exposed to 35±1°C for 30 days, whereas the control group was kept at room temperature during the same period. GH cells were studied using the adequate immunostaining procedures. The body weight of animals in the experimental group was significantly decreased by 24.5% compared to the controls. Immuno-histochemically and –fluorescently identified GH cells in controls were intensely stained, oval in shape, with the centrally located spherical nucleus. In rats exposed to moderate heat the localisation of GH cells was not significantly changed, while their shape was slightly different. They were mostly organized in groups, with darker cytoplasmic regions/higher intensity of immunofluorescence signal throughout the whole cytoplasm. The cellular and nuclear volumes of GH cells in the experimental group were significantly decreased by 16.0% and 9.0% respectively, but the volume density was only slightly decreased in comparison with the controls. These findings suggest that 30 days of continuous exposure of adult male rats to moderately high ambient temperature has an inhibitory effect on the immuno-histomorphometric characteristics and increases the immuno-fluorescence signal of GH cells.

Key words: immuno-histomorphometric, fluorescence, GH cells, chronic exposure, moderate heat, rat

INTRODUCTION

Exposure to high ambient temperature represents stronger type of physical stress compared to the immobilization or cold stress (1). The impact of actual average temperature elevation, during the summer period in the southeastern parts of Europe, including Macedonia, represents an inevitable stress for all living organisms. Activation of the hypothalamic-pituitary-adrenal (HPA) axis during heat exposure is a well-known physiological concept, but the ambiguous data on the effects of chronic heat exposure on GH axis exist.

One of the metabolic processes during the exposure of an organism to heat, connected with the GH activity, is maintaining the body homeothermy by regulating the activity of the sweat glands (2). Pertinent to this, it was found that the patients with GH-deficiency have reduced sweat capacity and may be at risk for developing hyperthermia (2). Many studies have shown increased values of blood GH, within the time of several minutes to several hours after exposure to high ambient temperature, as a result of direct stimulation by increased body temperature (3, 4). In contrast, data concerning the prolonged exposure of domestic animals to elevated ambient temperature are rather contradictory.

Corresponding author: Ass. Prof. Florina Popovska-Percinic, PhD

E-mail address: florinap@fvm.ukim.edu.mk
Present address: Department of functional morphology,
Faculty of Veterinary medicine-Skopje,
"Ss. Cyril and Methodius" University in Skopje,
Lazar Pop- Trajkov 5-7, 1000 Skopje, R. Macedonia
tel: +389 2 3240 710; fax: +389 2 3114 619

Namely, some earlier studies have shown decreased (5), increased (6) or the absence of changes (7) of blood GH concentration, during exposure of cattle or swine to moderately high ambient temperature (32°C-35°C). Studies on chronic exposure of humans to moderate heat have also shown different results in serum GH concentration (8, 9). Also, the available analyses of GH cells during heat exposure show that thermal environment (32°C) did not affect GH secretion, cellular GH content, or plasma GH concentration in young barrows, during the first 3-5 weeks (10).

Bearing in mind all the above as well as considering the fact that high ambient temperature is one of the most common environmental factors that affects all living organisms, especially during prolonged exposure in summer period, the aim of this study was to investigate the immuno-histomorphometric and –fluorescent changes of the rat GH cells after 30 days of continuous exposure to 35±1°C.

MATERIALS AND METHODS

Animals and the experimental protocol

The experiment was conducted on adult Wistar male rats. The experimental animals (n=7) were continually exposed for 30 days to moderately high ambient temperature (35±1°C), in a special heat chamber with controlled temperature and relative humidity of 30-40%. The control group (n=7) was kept at room temperature (20±2°C) during the same period. Animals were housed under standard light conditions (12h light\dark cycle), with food and water *ad libitum.*

The experimental protocols were approved by the Local Animal Care Committee in conformity with the recommendation provided in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123, Appendix A).

Immunohistochemistry and light microscopy

The pituitary glands were excised, fixed in Bouin's solution for 48 h and embedded in paraplast. Serial 5-μm thick tissue sections were deparaffinized in xylol and serial alcohol. GH in the granules was localized by the peroxidase-antiperoxidase complex (PAP) method (11). The endogenous peroxidase activity was blocked by incubation in a 9 mmol hydrogen peroxide solution in methanol, for 30 min, at ambient temperature. Before the application of specific primary antisera, nonspecific background staining was prevented by incubation of the sections with nonimmune, *i.e.* normal porcine serum, diluted with phosphate buffered saline (PBS) pH 7.4 for 60 min. Sections were then overlaid with the appropriate dilution of the specific primary antibodies (hGH antisera, Dako A/S, Glostrup, Denmark) for 48 h at 4°C. After washing in PBS for 5 min, sections were incubated for 60 min with a secondary antibody, swine anti-rabbit IgG (DAKO, Glostrup, Denmark; diluted 1:100 in PBS), rinsed again in PBS for 5 min and then incubated with rabbit PAP complex (DAKO A/S, Glostrup, Denmark; diluted 1:100 in PBS), for 45 min. Binding sites were visualised using 0.05% diaminobenzidine (DAB; Serva, Heidelberg, Germany) and 0.03% hydrogen peroxide in 0.2 mol/L TRIS-HCl buffer, pH 7.4. The sections were counterstained with hematoxylin and mounted in Canada balsam (Molar Chemicals KFT, Budapest, Hungary). For the control sections, the primary antibody was omitted and replaced by PBS, pH 7.4.

Digital images were made on a DM RB photomicroscope (Leica, Wetzlar, Germany) with a JVC TK 1280E video camera (Leica) for the acquisition and analysis of the images.

Immunofluorescent studies

Pituitary sections were washed in PBS and pretreated with blocking normal donkey serum (Dako, Denmark), diluted in PBS (1:10 v/v). After blocking, sections were incubated overnight at room temperature with polyclonal anti-rat GH (Dako, Denmark, 1:400 v/v). After rinsing in PBS, the sections were incubated for 2h at room temperature with secondary antibody-Alexa Fluor 488 donkey anti-rabbit IgG (Molecular Probes, Inc., USA, 1:200 v/v). Sections were mounted using Mowiol 4-88 (Sigma-Aldrich, Co., USA) and analyzed on Carl Zeiss AxioVision microscope (Zeiss, Germany).

Morphometry

Volume densities (Vv) of the nuclei and cytoplasm of GH-immunoreactive cells, as well as numerical density (Na) of their nuclei per mm^3 were measured using 50 test areas in the pituitary gland with a magnification of x1000, using the multipurpose test system M_{42} (12).

The number of nuclei of immunoreactive GH-cells per mm^3 was estimated using the appropriate formula (13). Since the rat ACTH cells are mononucleated, the numerical density of nuclei (Nv) corresponds to the number of cells per cubic millimeter.

$$Nv = (k/b) \times (Na^{3/2}/Vv^{1/2})$$

On the basis of earlier karyometric studies (12) the shape coefficient b was estimated to be 1.382, for the pituitary cells. It relates the Nv (number of cells counted per unit volume) to the Na (number of cells counted per square millimeter) and Vv (volume density), and depends on the axial ratio of the nuclei. The volume densities of GH-positive cells were expressed as percentages of total pituitary cells in mm^3.

Statistical analyses

Morphometric data obtained for each rat was averaged *per* experimental group and the standard deviation of the mean (SD) was calculated. The statistical analysis was performed by Student T-test. A confidence level of $p<0.05$ was considered statistically significant.

RESULTS

Data for the body weight, absolute and relative pituitary weights are summarized in Table 1. The body weight in rats chronically exposed to moderate heat was significantly decreased by 24.5%, compared to the controls. After the moderate heat exposure, the absolute and relative pituitary weight were significantly increased by 15.4% and 24.0 % respectively, in comparison with the corresponding control.

Table 1. Body weights, absolute and relative pituitary weights in control and rats exposed to 35±1 °C

Group	Body weight (g)	Absolute pituitary weight (mg)	Relative pituitary weight (mg%)
Control	337.5 ± 26.9	6.5±0.6	2.5±0.1
Moderate heat	254.9 ±16.8*	7.5±0.6*	3.1±0.2*

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

Immuno-histochemically and -fluorescently identified GH cells in control rat pituitaries were intensely stained, oval in shape, with a centrally located spherical nucleus (Fig. 1 A, C - arrows). In rats exposed to moderately high ambient temperature the localisation of GH was not significantly changed, while their shape was slightly different (Fig. 1 B, D - arrows). Precisely, most of them were oval in shape, but there were some stellate cells with prominent cytoplasmatical processuses (Fig. 1 D – arrow head). Generally, GH cells in rats exposed to moderately high temperature were smaller, mostly organized in groups, with darker cytoplasmic regions throughout the whole cytoplasm (immuno-histochemical finding, Fig. 1 B), or performing the higher intensity of immunofluorescence signal (Fig. 1 D).

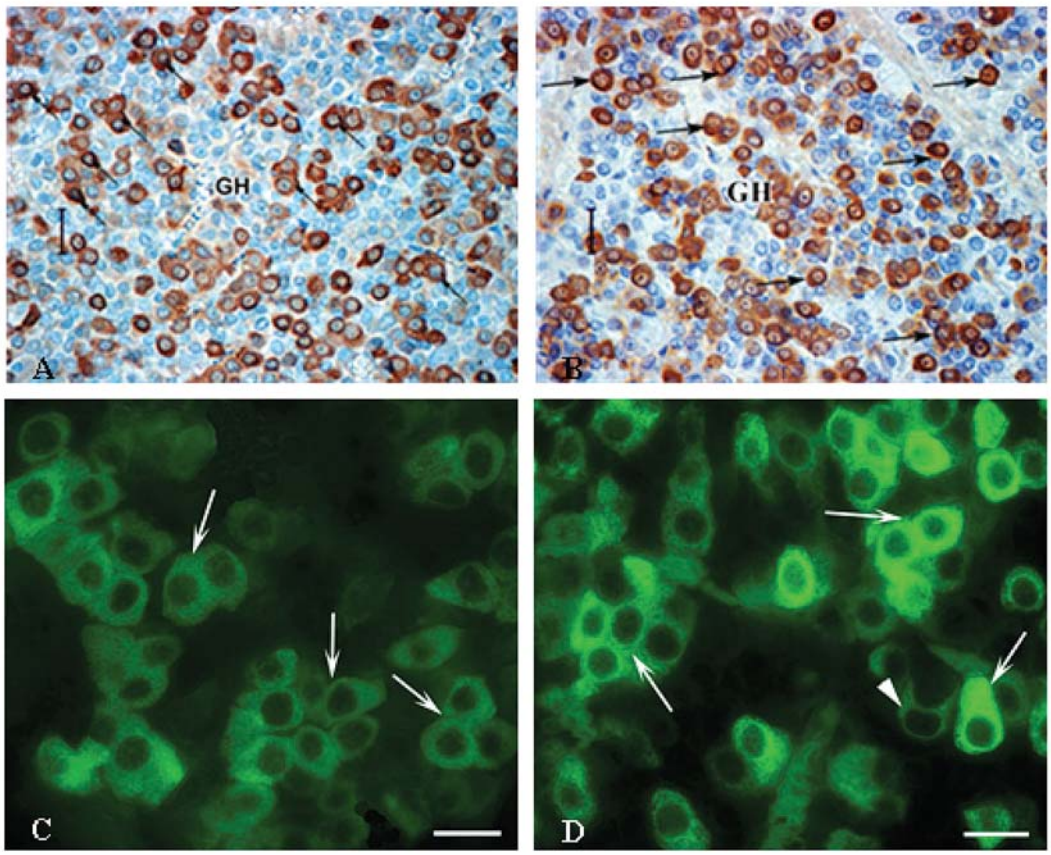
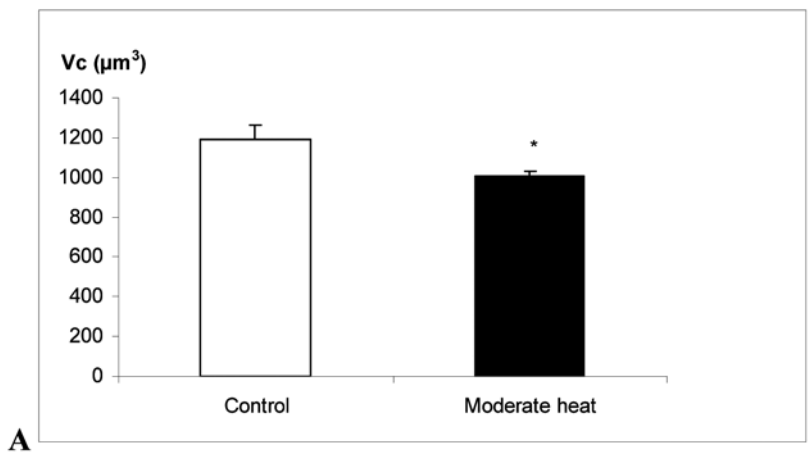
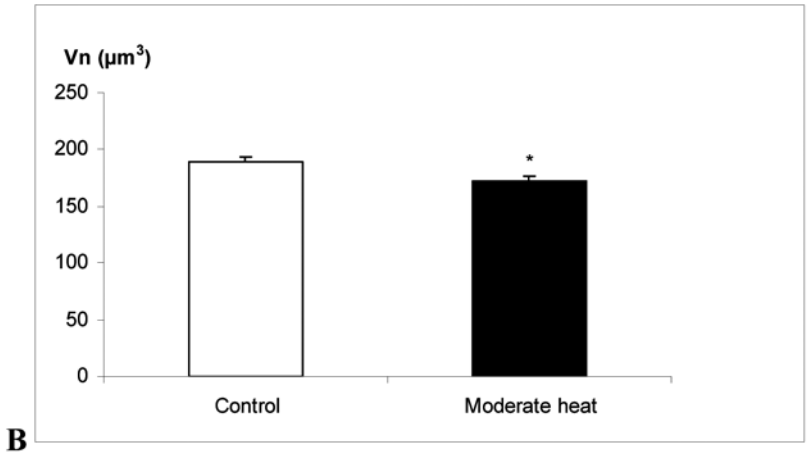


Figure 1. Immunopositive GH cells in: A) control rats, B) rats exposed 30 days to 35±1°C (PAP, bar= 16μm); and immunofluorescent labelling of GH cells in: C) control rats, D) rats exposed 30 days to 35±1°C (bar=20μm).

Morphometrical analysis of the pituitary GH cells have shown that the cellular and nuclear volumes of GH cells in rats exposed to moderate heat were significantly ($p<0.05$) decreased by 16.0% and 9.0% respectively (Fig. 2A, B), in comparison to the control rats. The volume density was slightly (un-significantly) decreased in comparison with the controls (Fig. 2C).



A



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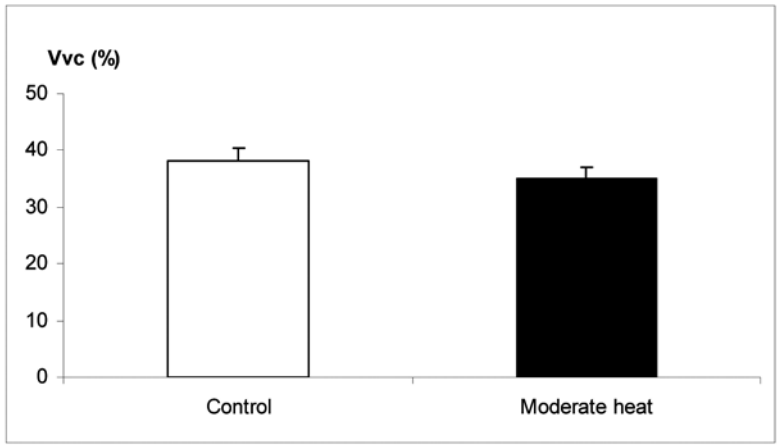

C

Figure 2. A) Cellular volume (Vc; μm^3), **B)** Nuclear volume (Vn; μm^3) and **C)** Volume density (Vvc; %) of GH cells in control and rats exposed 30 days to 35±1°C. The values are the means±SD (n =7), *p<0.05 *vs.* control.

DISCUSSION

Keeping in mind the ambiguous literature data regarding the effects of chronic heat exposure on GH axis, our ambition was to supplement the data 'pool' and clarify the immuno -histomorphometric and –fluorescent aspect of GH cells changes in rats, after their exposure to moderately warm milieu.

We have found that the body weight in rats continuously exposed to moderate heat was significantly decreased by 24.5%, compared to the controls. Our data for decreased body weight are in agreement with the results of some other authors (14, 15), according to which the body weight decreases regardless of the duration of heat exposure. Reduction of food-intake and increase of water consumption was registered during prolonged exposure of rats to moderate heat (16). Lower heat production and body growth were also observed in pigs chronically exposed to high temperatures (17). Supposedly, that the increase of the relative pituitary weight, found in our study, is primarily due to the evidently decreased body weight.

Obtained data from the immuno-histomorphometric and –fluorescence analysis in our study, showed that continous exposure of rats to moderate heat has an inhibitory effect on the morphometrical characteristics of GH cells and changes their immunostaining properties, which may suggest some low functional/secretory level of GH cells. This is in accordance with data indicating that stress has an inhibitory effect upon GH release (18, 19). According to reduced secretion of GH, found in cattle during prolonged thermal exposure (5), this mechanism seems to be necessary for the survival in warm ambients, maintaining the heat balance. Therefore, it is possible that decreased immuno-histomorphometric characteristics and

a more intense immunofluorescence signal of GH cells, found in this study, represent the consequence of acclimatization of these cells to chronic heat exposure.

It is well-known that the activity of GH cells is somatostatin dependent, bearing in mind its' GH suppressive effects (20). Studies with sheeps showed that their long term exposure to mildly elevated ambient temperature (30^{o}C; 30% humidity) increases the blood somatostatin levels (21). Therefore, one of the reasons for decreased immuno-histomorphometric parameters of GH cells in this study could be the inhibitory effect of somatostatin.

Some studies have established a positive correlation between thyroid hormones levels and GH function *via* increased GH gene expression (22), as well as between testosterone levels and GH secretion through promoted GHRH release (23). Consequently, thyroid hormones deficiency was associated with the impairment in GH secretion (24, 25), while castration provokes decrease of GH mRNA level in pituitary GH cells (26). Bearing in mind the reports of decreased serum concentration of TSH and T4 (16, 17, 27), as well as of testosterone (27, 28) during prolonged exposure of rats and pigs to moderate heat, it can be postulated that one of the reasons for having decreased immuno-histomorphometric characteristics and some higher immunofluorescent signal of GH cells in our study might be the result of decreased activity of the thyroid gland and testicles.

It should be emphasized that glucocorticoids also regulate GH secretion. They increase GH and growth hormone releasing hormone receptor (GHRH-R) gene expression on the pituitary level (29), while adrenalectomy provokes decreased pituitary GHRH-R, growth hormone secretagogue receptor (GHS-R) and GH mRNA levels (30). Bearing in mind the significant decrease of the serum corticosterone after 30 days of continuous exposure of rats to moderate heat in our previous study (31), it may be suggested that one of the reasons for the observed qualitative and quantitative parameters of GH cells in this study might be the decreased concentration of corticosterone.

From the above, it can be concluded that the continuous exposure of rats to moderate heat acts toward the decrease of GH cells immuno-histomorphometric characteristics and the increase of their immuno-fluorescence signal, which may be a way of acclimatization of these cells to chronic heat exposure.

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