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THE EFFECTS OF ACUTE HEAT STRESS ON PROLIFERATIVE AND APOPTOTIC PROCESSES IN THE RAT ADRENAL CORTEX

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Abstract - Hyperthermia can cause significant structural and functional reorganization of tissues and organs. The proliferative and apoptotic processes of rat adrenal cortex were analyzed by light and electron microscopy after an acute exposure to high ambient temperature. Animals were divided in two groups. The first group consisted of intact controls. The rats from the second group were exposed to a high ambient temperature of 38°C for 60 min. Mitotic chromosomes and the largest number of immunoreactive nuclei for the Ki-67 were observed in the *zona reticularis* (ZR) of the control animals. The relative number of mitoses after heat stress showed a significant decrease in the *zona glomerulosa* (ZG; 66.8%), *zona fasciculata* (ZF; 27.8%) and ZR (86.7%) (for all zones $p<0.05$), while in the whole adrenal cortex the after-treatment decrease was 61.9% ($p<0.05$) compared to the controls. Under heat stress numerous apoptotic nuclei were seen at the light and ultrastructural levels in all the zones of the adrenal cortex. Such dynamics of mitosis/apoptosis events seriously affect adrenal cortex morphology.

Key words: Acute heat stress, rat, adrenal cortex, proliferation, apoptosis

INTRODUCTION

The exposure of experimental animals to high ambient temperatures results in multi-organ disturbances when it comes to microcirculation, which determines functional organ failure and compromises the vitality of the tissues and the whole organism (Vlad et al., 2010). Hyperthermia increases apoptotic cell death, an event that is affected by the duration of hyperthermia (Pavlik and Aneja, 2007).

The adrenal gland is composed of two functionally distinct organs. The cortex synthesizes steroid hormones that mediate body homeostasis and chronic stress responses. It is organized into three concentric zones, *zona glomerulosa* (ZG), *zona fasciculata* (ZF), and *zona reticularis* (ZR). The adrenocorticotropic hormone (ACTH) acts on ZF to produce glucocorticoid hormones (e.g., corticosterone in rats and cortisol in humans), and can stimulate ZG to produce aldosterone in concert with the renin-angiotensin system, while inner ZR synthesizes androgens. The medulla produces catecholamines that facilitate acute mammalian stress or "fight-or-flight" response.

In 1998, Wolkersdorfer and Bornstein proposed three theories to explain the zonation of the adrenal cortex. The migration theory describes cell prolif-

eration in the cortex outer part, their migration and differentiation from ZG to ZF and from this to ZR where the cells degenerate and die. The transformation field theory proposes two transformation directions – one from ZG and the other from ZF, from which migration takes place in two opposite directions: on one side towards the medulla and on the other towards the capsule. Finally, the zonal theory proposes an equal proliferation and apoptosis in the three cortex zones, implying that each zone could be locally regulated without being affected by the other zones (Wolkersdorfer and Bornstein, 1998).

Taking all this into consideration, as well as the fact that there are no reports of semiquantitative analysis of the proliferation and apoptosis in the adrenal cortex after acute heat stress, we determined the effect of high ambient temperature (38°C) on these processes in the rat adrenal cortex.

MATERIALS AND METHODS

Experimental design

Male Wistar rats weighing 320 ± 30g were acclimated to 22±1°C and kept under a 12h light/dark cycle. Animals were fed with commercial rat food and drank tap water *ad lib*. The rats were divided in two groups, each consisting of ten rats. The animals from the first group were intact controls. The rats from the second group were exposed to a high ambient temperature of 38°C for 60 min in a hot chamber (Sutjeska, Beograd, R. Srbija), immediately before sacrificing.

After measuring the body mass and temperature, the animals were killed by decapitation with a guillotine (Harward-Apparatus, Holliston, MA, USA). The left adrenal gland from each animal was removed, freed of fat on ice, and weighed. Adrenal glands were fixed in 4% formalin solution (pH=7) and embedded in paraffin, according to the standard procedure, after which they were serially cut into 5 μm thick sections on a 'Reichert' rotation microtome.

The experiments were performed according as proposed by the Serbian Laboratory Animal Science Association (SLASA), a member of the Federation of European Laboratory Animal Science Association (FELASA).

Immunohistochemical and immunofluorescent studies

The Ki-67 antigen was used to detect cell proliferation in the adrenal cortex. After deparaffinization, antigen retrieval was performed in sodium citrate for 21 min in a microwave (700 W). Endogenous peroxidase was suppressed in 3% H_2O_2. Samples were incubated with primary antibody (1:40, Dako, Denmark) overnight (4°C) and with the secondary antibody (1:200, Santa Cruz biotechnology, Inc., Heidelberg, Germany) for one hour. After this, the sections were incubated with an avidin-biotin complex. Sections were rinsed in phosphate buffered saline (PBS) after every step. Visualization was performed with 3, 3'-diaminobenzidine (DAB, Serva, Heidelberg, Germany). Mayer hematoxylin was used for counterstaining. After dehydration, the sections were mounted in DPX.

For the detection of apoptotic cells in the adrenal cortex, propidium iodide staining was used (Telford et al., 1992). After deparaffinization, sections were stained with propidium iodide for 30 min. Upon dehydration, the sections were covered with glycerol and analyzed under a fluorescence "Leica" (Germany) microscope.

Ultrastructural study

The right adrenal glands from six animals were used for ultrastructural study. The glands were fixed in 4% glutaraldehyde in 0.1 M phosphate-buffer, postfixed in 1% osmium tetroxide, and then contrasted with a water solution of uranyl-acetate overnight at 4°C, dehydrated in alcohol and propylene oxide, and embedded in EPON. Semi-thin sections were cut on an LKB III Ultratome and stained with toluidine blue, and then used for identification of the adrenocortical zones. Ultra-thin sections were stained with lead-citrate, and examined under a CM 12 Philips electron microscope.

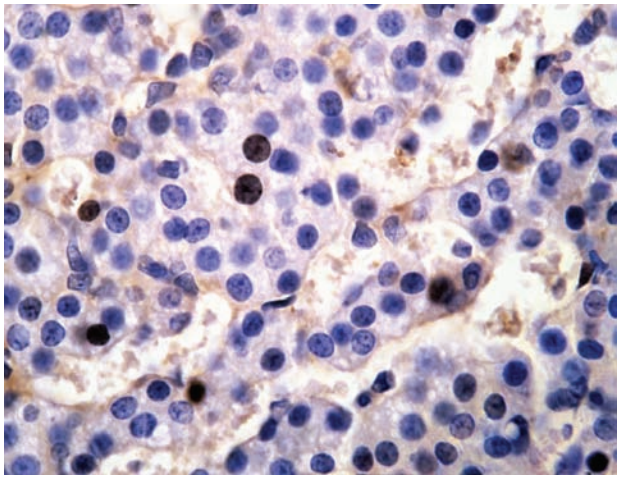

Fig. 1. Nuclei of *zona reticularis* (ZR) cells in the adrenal cortex of control animals labeled with anti-Ki-67 antibody (avidin-biotin method, magnification 1000x).

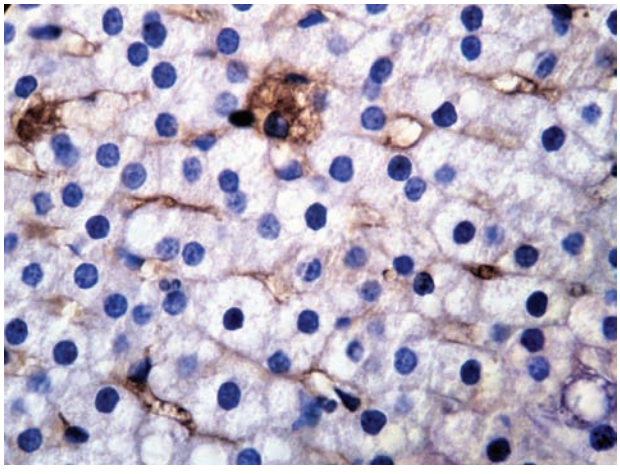

Fig. 2. Nuclei labeled with anti-Ki-67 antibody in *zona fasciculata* (ZF) cells of heat stressed animals (avidin-biotin method, magnification 1000x).

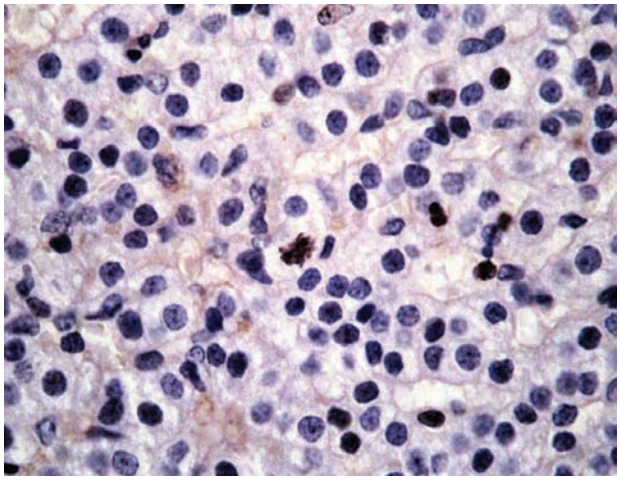

Fig. 3. Mitotic chromosomes in *zona reticularis* (ZR) cells of control animals labeled with anti-Ki-67 antibody (avidin-biotin method, magnification 1000x).

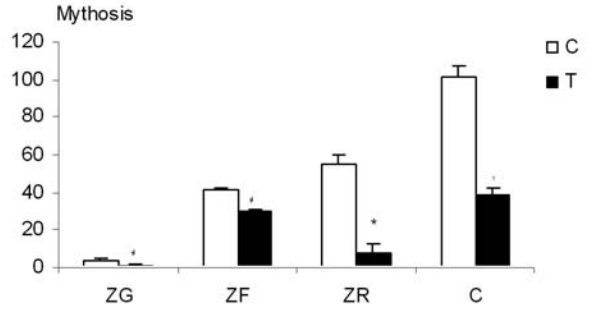

Fig. 4. Relative number of mitoses in the *zona glomerulosa* (ZG), *zona fasciculata* (ZF), *zona reticularis* (ZR), and in the whole adrenal cortex (C) after heat stress. Values are means ± SD. *p< 0.05.

Statistical analysis

For a comparison of the relative mitosis number between the two groups the Student t-test was used and the level of significance was set at $p<0.05$.

RESULTS AND DISCUSSION

The primary goal of the present study was to determine the levels of proliferating cells and the occurrence of apoptosis in zones of the rat adrenal cortex after acute heat stress.

In the adrenal cortex of the control animals, the largest number of immunoreactive nuclei for Ki-67 was observed in the ZR (Fig. 1), while the other two zones contained a small number of marked nuclei. The Ki-67 antigen is a nuclear protein that is primarily expressed in the G_1, S, G_2 and M phases of the cell cycle, while Ki-67 is not observed in the cells during interphase. Mitotic chromosomes were also observed in the ZR of the controls (Fig. 3). The relative number of mitoses after heat stress significantly decreased in the ZG (66.8%), ZF (27.8%) and ZR (86.7%) (for all zones $p<0.05$), whereas in the whole adrenal cortex,

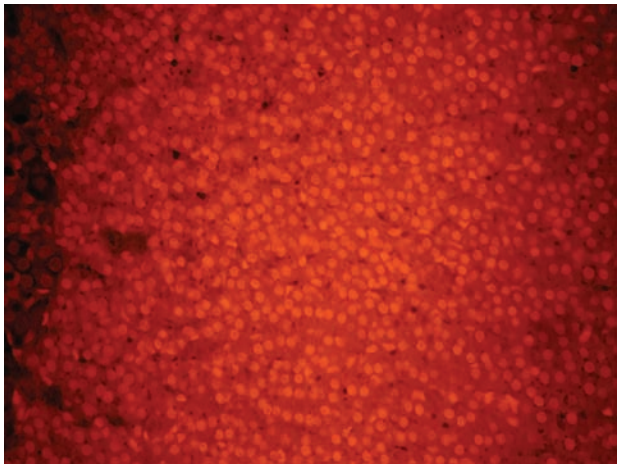

Fig. 5. Small number of fluorescent nuclei in the cells of the adrenal cortex of control rats (propidium iodide staining, magnification 400x).

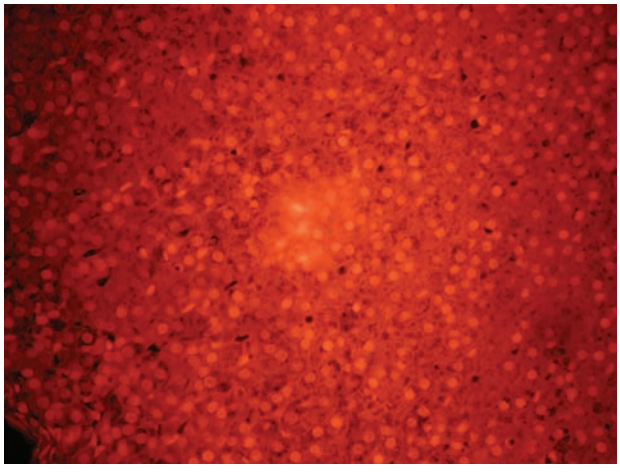

Fig. 6. Increased number of fluorescent nuclei in the cells of the adrenal cortex of heat stressed rats (propidium iodide staining, magnification 400x).

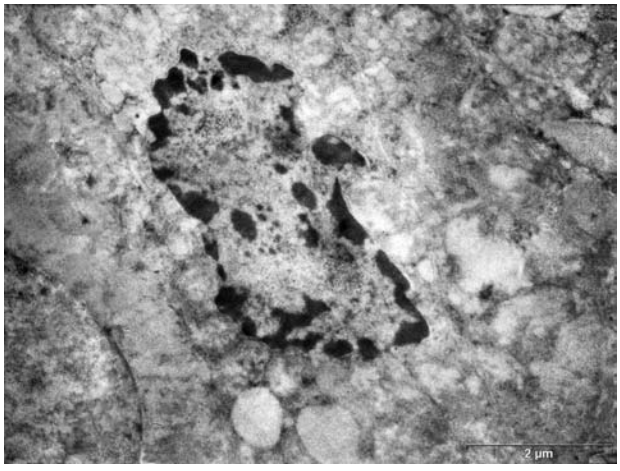

Fig. 7. Chromatin fragmentation of the apoptotic nuclei in cells of the rat adrenal cortex *zona reticularis* (ZR) after the heat stress (uranyl acetate, lead citrate). Scale bar, 2µm.

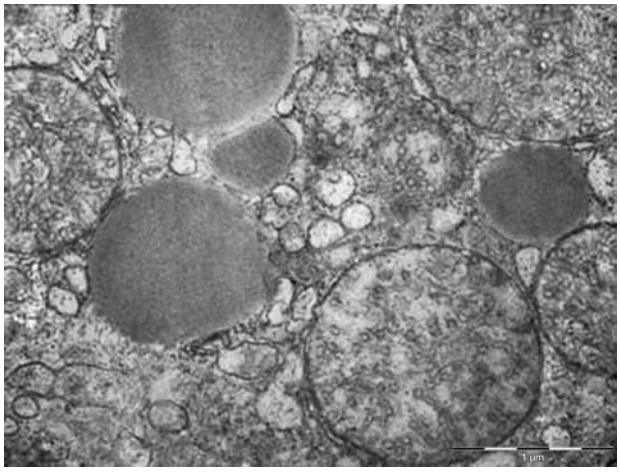

Fig. 8. Dilation of the endoplasmic reticulum in adrenal cortex *zona reticularis* (ZR) cells after heat stress (uranyl acetate, lead citrate). Scale bar, 1µm.

the after-treatment decrease was 61.9% ($p<0.05$) compared to the controls (Fig. 4). The use of Ki-67 antigen immunostaining to identify the proliferation revealed that cell proliferation after heat stress occurs primarily in the ZF (Fig. 2). Mitani et al. (1994) reported that the density of the bromodeoxyuridine (BrdU)-labeled cells at the border between the ZG and ZF was three or more times higher than in other cortical zones. The authors referred to this zone as an undifferentiated cell zone (ZU) of the adrenal cortex. Subsequently, these authors showed (2003) that ZU and its surroundings, i.e. the innermost portion of the ZG and the outermost portion of the ZF, are the sites for cell replication in the adult rat adrenal cortex, and that the cells raised there migrate to other regions. Our observations support the transformation theory which suggests that most of the adrenocortical cells proliferate at the border between the ZG and ZF, and then migrate bidirectionally, both towards the cortical surface and to the inner medullary surface.

The immunofluorescent study showed that apoptotic nuclei in the adrenal cortex differed from nonapoptotic ones in their strong fluorescence. Staining with propidium iodide demonstrated that in the control group fluorescence was present only in some adrenal cortex nuclei (Fig. 5), while the number of fluorescent nuclei increased after the heat stress in all zones (Fig. 6). Depending on the length and "severity" of heat stress, a halt in the cell cycle occurs as well as stagnation in the growth and proliferation (Zeuthen, 1971; Lindquist, 1986; Yost and Lindquist 1986), which can all result in cell death. Apoptotic cells were also observed in rats after exposure to heat stress for 30 min (Koldysheva and Lushnikova 2008). It is well known that high temperatures cause apoptosis in mice (Feng et al., 2009). Research on the U937 cell line has shown that hyperthermia leads to apoptosis and programmed cell death, which depends on the duration and degree of hyperthermia (Kameda et al., 2001).

Under the electron microscope, apoptotic signs such as chromatin fragmentation, nuclear morphology disruption and integrity loss (Fig. 7), cytoplasm condensation and numerous dilatations of the endoplasmic reticulum (ER) (Fig. 8) were observed in individual cells of the adrenal cortex after heat stress. In apoptotic cells, the ER produces unfolded proteins that accumulate, leading to the state of stress (Gupta et al., 2010) and dilatation of the ER. If ER stress is prolonged, or if the adaptive response fails, apoptotic cell death ensues (Gorman et al., 2012).

In conclusion, acute heat stress significantly reduces the number of mitoses and increases the occurrence of apoptosis in the adrenal cortex of rats. Such dynamics of the events seriously affect the morphology of the adrenal cortex.

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