

## EFFECT OF DIFFERENT TYPES OF STRESS ON ADRENAL GLAND PARAMETERS AND ADRENAL HORMONES IN THE BLOOD SERUM OF MALE WISTAR RATS

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**Abstract** — In the present study, we examined gross changes in the mass of whole adrenal glands and that of the adrenal cortex and medulla in mature male Wistar rats subjected to three different stress types: acute, chronic, and combined, i.e., chronic followed by acute stress. These parameters were correlated with adrenal activity as judged from serum levels of corticosterone and catecholamines, respectively, as well as with serum levels of ACTH and glucose. Under all three conditions, we observed bilaterally asymmetric and stress-type-independent hypertrophy of whole adrenals, as well as adrenal cortices and medullas. Under acute and combined stress, adrenal hypertrophy was followed by increase of adrenal hormones in the blood serum. However, under chronic stress, both cortical and medullar activities as judged from low or unaltered levels of the respective hormones and glucose were compromised and disconnected from the input signal of ACTH. Since all of the studied adrenal activities could be restored by subsequent acute stress, it is concluded that chronic isolation can be viewed as partly maladaptive stress with characteristics resembling stress resistance rather than the stress exhaustion stage of the general adaptation syndrome.

**Key words:** Wistar rats, immobilization, isolation, adrenal glands, adrenal hormones, stress

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### INTRODUCTION

The response to neuroendocrine stress begins with activation of the sympatho-adrenomedullary system (SAS) and hypothalamic-pituitary-adrenal (HPA) axis and through a cascade of coordinated neural and hormonal signals culminates in activation of the adrenal glands (McEwen, 2000, 2008). These paired organs through release of catecholamines (adrenalin, noradrenalin) from the medullar part of the gland and steroids (glucocorticoids, mineralocorticoids) from the adrenal cortex further orchestrate both fast and long-term adaptation of an organism to any stressful condition (Raber, 1998). For example, while medullar adrenalin maintains alertness, cortical glucocorticoids help replenish energy supplies of a stressed organism (McEwen, 2000). Alongside adaptation, the adrenal hormones (particularly glucocorticoids) also regulate termination of the stress response through a feed-back mechanism that operates at various levels of the brain. Acting both intrinsically, i.e., at the HPA axis itself, and extrinsically at

the limbic brain structures controlling the HPA axis, adrenal hormones help to regain stress-disturbed homeostasis (McQuade and Young, 2000). However, if stressful conditions persist, these systems sometimes fail to switch off when not needed or do not become active when needed, leading to a state that may result in a variety of maladaptive syndromes (Seley, 1998; Brown et al., 1999). At least in part, the maladaptation may emerge from exhaustion of adrenal gland secretion under the chronic stress conditions and loss of adrenal ability to recover from the stress. Such conditions are known as adrenal decompensation or fatigue (Brown et al., 1999).

In our previous study, we found that mature male Wistar rats subjected to chronic social isolation stress exerted marked hypocorticism 21 days after treatment (Adžić et al., 2008). Under these conditions, we also observed multiple molecular changes in the upper, extrinsic feed-back parts of the HPA axis: the hippocampus and the prefrontal cortex of the brain. In these structures, the composition of

corticosterone (glucocorticoid) receptor phosphoisoforms, their partitioning between the cytoplasmic and nuclear compartments, and the ratio of receptor chaperones Hsp90 and Hsp70 were significantly altered by chronic social isolation (Adžić et al., 2008; Djordjević et al., 2008). These changes may be caused by alterations in the cellular milieu of upper parts of the HPA axis, but they may also be a consequence of hypocorticism of the adrenal glands, i.e., adrenal fatigue. Consequently, in the present study, we tested the latter assumption and examined gross changes in mass of the adrenal glands as a whole and the adrenal cortex and medulla as separate gland structures in male Wistar rats subjected to three different stress types: acute, chronic, and combined, i.e., chronic followed by acute stress. Changes of cortical and medullar mass were correlated with adrenal activity as judged from serum levels of corticosterone and catecholamines, respectively, as well as with serum levels of ACTH and glucose. We found stress-type-independent changes in the mass of adrenal cortices and medullas, which did not correlate with their respective hormone levels or with other blood serum parameters and which were most pronounced under chronic stress.

## MATERIALS AND METHODS

### *Materials*

The OCTEIA Corticosterone EIA kit was purchased from Immunodiagnostic Systems Inc. (IDS Inc.), Fountain Hills, AZ, USA. Accutrend strips for determination of blood glucose were purchased from Roche Diagnostics GmbH, Mannheim, Germany.

### *Animal treatment and sacrifice*

All animal procedures were approved by the Ethical Committee for the Use of Laboratory Animals of the Vinča Institute of Nuclear Sciences in accordance with the guidelines of the EU FELASA-registered Serbian Laboratory Animal Science Association (SLASA). All experiments were performed with adult (3-month-old) male Wistar rats (body mass 330-400 g) housed four per standard size cage and offered food (commercial rat pellets) and water ad libitum. Light was kept on between 7:00 am and 7:00

pm, and room temperature (RT) was maintained at  $20 \pm 2^\circ\text{C}$ . For the stress experiments, animals were divided into four groups: group I consisted of unstressed animals (control group); group II animals were exposed to acute immobilization for 30 min according to the method of Kvetnansky and Mikulaj (Kvetnansky and Mikulaj, 1970); group III animals were subjected to chronic isolation stress by individual housing for 21 days as described by Sanchez et al. (Sanchez et al., 1998); and group IV was exposed to chronic (21-day) isolation followed by 30-min immobilization. To reduce variance in the physiological parameters due to daily rhythms, all animals were sacrificed at the same time point in the circadian cycle, between 9:00 and 11:00 am, i.e., immediately after stress treatment. Animals were sacrificed under no stress conditions by rapid decapitation.

### *Preparation of serum and determination of glucose and adrenal gland hormones*

Blood from each animal was collected at the time of sacrifice. Serum was prepared by 15-min centrifugation at 3000 rpm. The corticosterone (CORT) level was determined using the OCTEIA Corticosterone EIA kit according to the manufacturers' instructions (Immunodiagnostic Systems Inc.). Calibrators, controls, and diluted samples were loaded in duplicate on a 96-well plate coated with a polyclonal anti-CORT antibody, along with HRP-labelled CORT. The plate was incubated overnight at  $4^\circ\text{C}$  and washed, after which color was developed using a chromogenic substrate. The reaction was stopped by adding HCl and the absorbance at 450 nm (reference 650 nm) was determined with a microplate reader (Wallac, VICTOR2 1420, PerkinElmer). The concentration of CORT (ng/ml) was determined using a standard curve. Serum catecholamines were assayed by the modified radioenzymatic method of Peuler and Johnson (Peuler and Johnson, 1977), and ACTH was determined as previously described (Dronjak et al., 2004). For determination of glucose concentration, a drop of fresh blood from each animal was applied to an Accutrend strip and assayed colorimetrically with an Accutrend GCT reader (Roche, Mannheim, Germany).

*Preparation of adrenal tissues and determination of their mass*

After sacrifice, the adrenal glands were carefully excised *in situ* and placed in an ice bath. The mass of the right or left gland was determined by weighing with a Mettler AE 50 electronic analytical balance having a precision of 0.1 mg (Mettler, Toledo, OH, USA). The following procedures were all carried on ice-cold Petri dishes placed in the ice bath: the adrenal cortices were carefully peeled off and completely removed, after which the remaining medullas were weighed using the aforementioned Mettler electronic analytical balance. The mass of each adrenal cortex was calculated by subtracting mass of the respective medulla from total mass of the adrenal gland.

*Statistical analysis of data*

Data are presented as means  $\pm$  SEM from 17 to 22 animals per group. In order to establish significant differences between control and stressed animals, data were analyzed by one-way ANOVA, while comparison between the right and left adrenal glands was performed by the t-test. Values were considered statistically significant if the p value was less than 0.05.

RESULTS

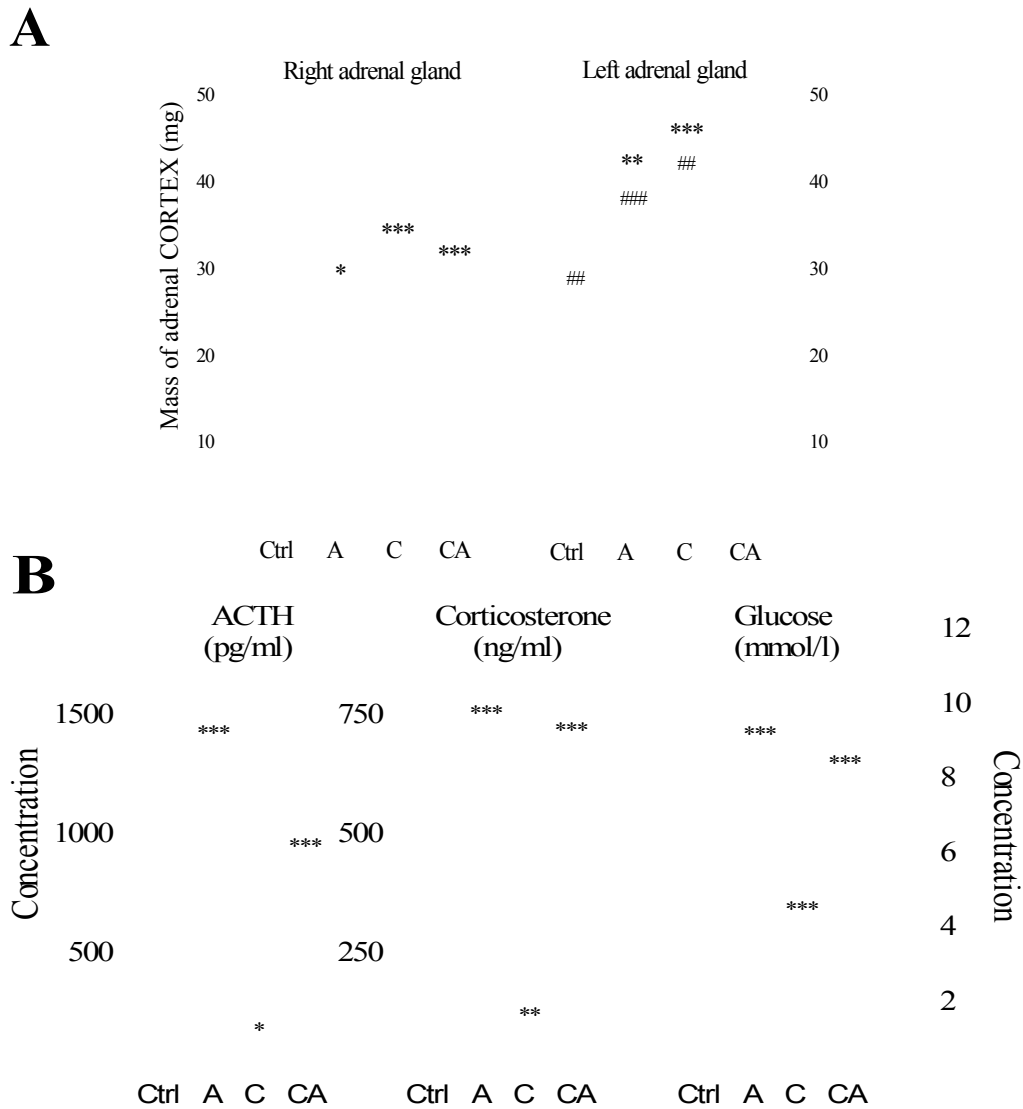
**Stress did not alter body mass but increased adrenal gland mass in male Wistar rats.** Body mass and adrenal gland mass were determined in 3-month-old Wistar males subjected to acute (30-min) immobilization, chronic social isolation

(21-day), or the combination of both stressors, as well as in the corresponding age-matched controls (see Methods). As can be seen in Table 1, the body mass of Wistar males was not affected by any of the stress conditions applied. However, adrenal mass was significantly increased under all three types of stress (Table 1). In addition to this, under chronic and combined stress we observed bilateral asymmetry of adrenal gland mass gain, i.e., mass of the left gland was consistently higher than that of the right one (Table 1).

**Stress-induced adrenal cortex mass gain not correlated with serum parameters.** In the same animal groups, we determined stress-induced changes in mass of the right and left adrenal cortex (Fig. 1, upper panel). We also followed serum levels of three parameters: pituitary ACTH, which is the major activator of adrenal cortex activity; corticosterone (CORT), as an indicator of cortical secretion; and glucose, as the end point of CORT activity in maintenance of the carbohydrate balance (Fig. 1B). The results indicated that mass of the adrenal cortex was significantly increased under all stress conditions (Fig. 1A). Also, bilateral asymmetry of cortical mass gain was observed in control as well as in stressed animals, with size of the left cortex being consistently higher than that of the right one (Fig. 1A, compare right and left panels). Increase of cortical mass in acute stress was in correlation with increased ACTH, CORT, and glucose in the blood serum (Fig. 1B). However, chronic stress led to a much smaller increase in the level of serum ACTH compared to acute stress, and it also caused significant decrease

**Table 1.** Stress-induced changes of whole body and adrenal gland mass in Wistar male rats. The total number of animals in each experimental group [control (Ctrl), acute (A), chronic (C), or combined (C+A) stress] is indicated above, while means  $\pm$  SEM values for whole body mass and mass of the right and left adrenal glands are given below. Differences are statistically significant at \*p<0.05, \*\*p<0.01, and \*\*\*,###p<0.001 (\*stress vs. control, #right vs. left adrenal gland).

Treatment → Parameter ↓	Control (Ctrl)	Acute (A) immobilization	Chronic (C) isolation	Combination C+A	
Number of animals	22	17	17	20	
Body mass (BM) (g)	371.6 $\pm$ 44.1	387.4 $\pm$ 40.9	386.3 $\pm$ 28.0	377.9 $\pm$ 52.8	
Adrenal gland mass (mg)	Right	18.6 $\pm$ 6.6	32.2 $\pm$ 1.9***	31.5 $\pm$ 5.3***	29.4 $\pm$ 3.5***
	Left	23.5 $\pm$ 6.5	34.7 $\pm$ 4.8***	41.8 $\pm$ 5.2***###	37.8 $\pm$ 4.1***###

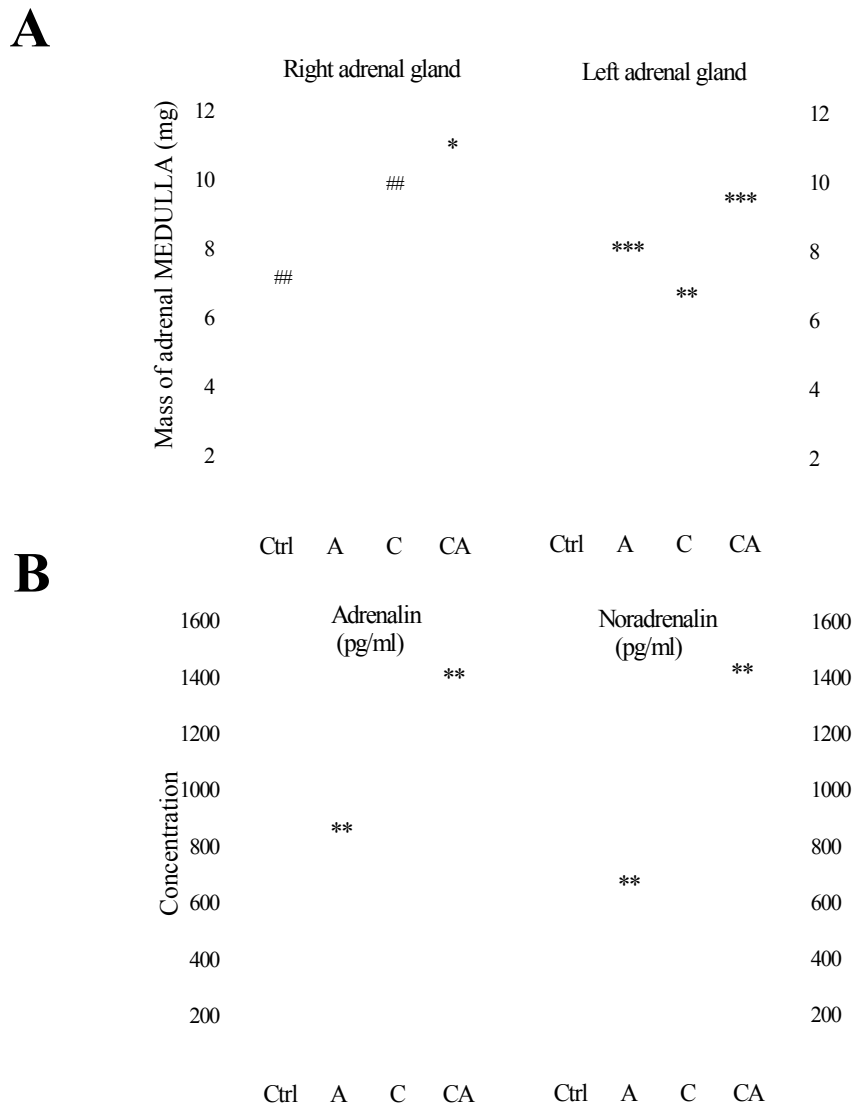


**Fig. 1.** Changes in parameters of the adrenal cortex in stressed Wistar rats. The cortical mass of the right and left adrenal glands (A) and serum levels of ACTH, corticosterone, and glucose (B) are presented as means ± SEM. The number of animals in each experimental group [control (Ctrl), acute immobilization (A), chronic isolation (C), and the combination of both stressors (C+A)] is given in Table 1. Differences are statistically significant at \* $p < 0.05$ , \*\*,# $p < 0.01$ , and \*\*\*,### $p < 0.001$  (\*stress vs. control, #right vs. left adrenal gland).

in CORT and glucose levels (Fig. 1B). When chronically stressed animals were subjected to subsequent acute immobilization (i.e., combined stress), the serum levels of ACTH, CORT, and glucose were again all increased (Fig. 1B), but the ACTH increase was less pronounced than in acute stress. Taken together, these results suggest that stress-induced gain of adrenal cortex mass was bilaterally asymmetric and mostly stress-type-independent, while

cortical activity (to judge from serum parameters) seemed to depend on the type of stress.

**Stress-induced adrenal medulla mass gain not correlated with serum catecholamines.** Analysis of stress-induced changes in mass of the adrenal medulla (Fig. 2A) also indicated the presence of bilateral asymmetry. To be specific, while mass of the right medulla was almost unaffected by stress, that



**Fig. 2.** Stress-induced changes in parameters of the adrenal medulla in Wistar rats. The medullar mass of the right and left adrenal glands (A) and serum levels of adrenalin and noradrenalin (B) are presented as means  $\pm$  SEM. The number of animals in each experimental group [control (Ctrl), acute immobilization (A), chronic isolation (C), and combined stress (C+A)] is given in Table 1. Differences are statistically significant at \* $p < 0.05$ , \*\*, $\#p < 0.01$ , and \*\*\*, $\#\#\#p < 0.001$  (\*stress vs. control, #right vs. left adrenal gland).

of the left medulla was significantly increased under all stress conditions (Fig. 2A). This increase in the left medulla mass was most prominent in acute and combined stress and was correlated with increase in the serum levels of adrenalin and noradrenalin (Fig. 2B). However, although the medullar mass was increased under chronic stress, both serum catecholamines were at the control level (Figs. 2A and

2B). Taken together, these data indicate that stress caused bilaterally asymmetric gain of medullar mass which was mostly stress-type-independent, while medullar activity, i.e., the respective levels of serum catecholamines, were stress-type-dependent.

#### DISCUSSION

According to Hans Selye's concept of the general

adaptation syndrome (GAS), in the stress-resistant stage of the GAS, the adrenal glands (as terminal effectors of the stress-activated HPA axis) begin to adapt and re-build themselves, increasing in size and function to the state known as hyperadrenia. However, if stress persists, this adrenal reaction may progress further into the exhaustion stage of the GAS, i.e., to the point of total loss of adrenal ability to adapt, known as hypoadrenia.

Our previous observation of marked hypocorticism in chronically isolated Wistar rats prompted us to evaluate this animal model, i.e., to determine if these animals are in the stress-resistant or exhaustion stage of the GAS. To that end, we followed changes in gross structure of the adrenal glands in relation to adrenal activity as defined by the level of adrenal hormones, the input signal of ACTH, and the resulting glucose level in the blood serum. These parameters were also followed in Wistar males subjected to acute immobilization, since the response to this high-intensity physical-emotional-psychosocial stress is defined in the literature as the 'normal' response to stress (McEwan, 1998; Garcia et al., 2000). Finally, the ability to retain 'normal' stress signalling after chronic stress was traced by imposing the combination of both stressors.

Our experimental data indicate that animals subjected to any of the three stress types exhibited marked bilateral increase in mass of the whole adrenal gland, as well as in mass of the adrenal cortex (Table 1, Fig. 1A). This stress-related adrenal hypertrophy was not an unexpected finding, since hypertrophy has been previously reported to be a ubiquitous phenomenon in response to any form of noxious treatment (Hornsby, 1985). Also, the adrenals have been shown to possess one of the highest rates of blood flow per gram of tissue during stress (Hornsby, 1985). Thus, the adrenal hypertrophy found in our experiments is interpreted as a consequence of stress conditions. However, in our experiments the increase of adrenal gland and cortical mass was not always accompanied by a corresponding increase of blood serum corticosterone (CORT). To be specific, while the increases of these parameters were found to be nicely correlated in acute and combined stress, CORT was significantly

below the control level in chronically isolated animals. A low CORT level may be a consequence of HPA axis hypoactivity under isolation (as reported by other authors) and it may lead to attenuated secretion of ACTH (Sanchez et al., 1998; Malkesman et al., 2006). However, it may also be a consequence of compromised adrenal secretion. This assumption is based on our observation that both ACTH and CORT levels in isolated animals could be increased by the subsequent immobilization. This increase of ACTH and CORT also correlated with less pronounced hypertrophy of the adrenal cortex in combined stress compared to isolation (Fig. 1). The discrepancy between elevated ACTH and decreased CORT in chronic isolation may result from partial modulation of the ACTH signal by other cellular agents, as reported by Salas et al. (2008). Nevertheless, the control of energy pools as judged from blood glucose remained more closely related to the CORT level than to the ACTH level (Tabata et al., 1991) under all conditions. Thus, when CORT was below control levels under chronic stress, we also observed hypoglycaemia, which according to Tabata et al. could itself be a significant stress on the entire body, and especially on the adrenals (Tabata et al., 1991). However, the hypoglycemia of chronically isolated Wistar rats seemed to be a transient state, since it could be normalized and even increased by subsequent acute stress, i.e., by CORT increase. In summary, our results on stress effects in the adrenal cortex indicate that Wistar rats subjected to chronic isolation experienced the stress-resistant rather than the exhaustion stage of the GAS.

Furthermore, in animals subjected to any of the three types of stress, we also observed increase in mass of the adrenal medulla, which was an expected finding, since it is in accordance with previous observations of medullar changes in stress conditions (Ulrich-Lai et al., 2006). As in the case of the adrenal cortex, the observed medullar hypertrophy did not always correlate with the serum level of medullar catecholamines, adrenalin and noradrenalin (Fig. 2). Thus, increase of medullar mass under acute and combined stress correlated well with the significantly elevated levels of adrenalin and noradrenalin, but this correlation was not

observed under chronic isolation, since the levels of both hormones under this type of stress were indistinguishable from the control. Based solely on the catecholamine level, it might therefore be concluded that the animals were successfully adapting to prolonged isolation stress. However, hypertrophy of the adrenal medulla remained present in the left gland of chronically isolated animals, suggesting a possible attempt of the gland to meet the increased demand for these hormones under prolonged stress, as well as the possibility of their compromised secretion. Moreover, these medullar conditions seemed to result in medullar sensitization to the next stress signal, since the levels of blood catecholamines were two times higher in response to subsequent acute immobilization. Thus, even though stress-related changes in the adrenal medulla were somewhat different from those of the adrenal cortex, they further strengthen the previous assumption that Wistar rats subjected to chronic isolation most likely experienced the stress-resistant stage of the GAS.

The similarity of cortical and medullar responses to the three types of stress used in the experiments may also be a consequence of crosstalk between cortical and medullar signals. This assumption is based on data indicating that catecholamines secreted within the first minute of the stress response may modulate subsequent ACTH and CORT release (Sapolsky et al., 2000). Conversely, glucocorticoids may influence expression of catecholamine-synthesizing enzymes (Evinger et al., 2007). Further insight into these interactions gained in future research would help us to understand the molecular basis for compromised secretion of adrenal hormones and the partly maladaptive response of experimental Wistar males to chronic isolation.

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## ЕФЕКТИ РАЗЛИЧИТИХ ВРСТА СТРЕСА НА ПАРАМЕТРЕ АДРЕНАЛНИХ ЖЛЕЗДА И НИВО АДРЕНАЛНИХ ХОРМОНА У КРВИ МУЖЈАКА WISTAR ПАЦОВА

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У приказаној студији пратили смо промене масе адrenalних жлезда, адrenalног кортекса и медуле код полно зрелих мужјака Wistar пацова који су излагани различитим стресовима: акутном, хроничном и комбинованом стресу, односно хроничном стресу након кога је следио акутни стрес. Наведене промене корелисане су са активношћу адrenalног кортекса и медуле, која је праћена преко нивоа кортикостерона и катехоламина у крвном серуму, и такође су корелисане са нивоом АСТН и глукозе. Под деловањем сва три третмана запазили смо билатерално асиметричну хипертрофију адrenalних жлезда, кортекса и медуле и ове промене су биле независне од типа стреса. У акутном и хронич-

ном стресу хипертрофија адrenalних жлезда била је праћена порастом адrenalних хормона у крвном серуму. Међутим, у хроничном стресу, активност адrenalног кортекса и медуле била је или снижена или неизмењена, судећи по ниском нивоу хормона и глукозе у крвном серуму, и била је неповезана са улазним сигналом АСТН. Будући да су сви посматрани параметри активности адrenalних жлезда могли да се ревертирају накнадним акутним стресом, заључили смо, да хронична изолација доводи до делимично маладаптивног одговора на стрес, који по карактеристикама наликује фази резистенције на стрес, пре него фази исцрпљености по класификацији генералног адаптационог синдрома.