Tkachuk Yurii. Morpho-functional changes of pituitary-adrenal system in the long-term course of experimental diabetes mellitus. Journal of Education, Health and Sport. 2016;6(4):375-384. eISSN 2391-8306. DOI http://dx.doi.org/10.5281/zenodo.50532
http://ojs.ukw.edu.pl/index.php/johs/article/view/3488

The journal has had 7 points in Ministry of Science and Higher Education parametric evaluation. Part B item 755 (23.12.2015).
755 Journal of Education, Health and Sport eISSN 2391-8306 7

The authors declare that there is no conflict of interests regarding the publication of this paper.
Received: 05.04.2016. Revised 25.04.2016. Accepted: 25.04.2016.

Morpho-functional changes of pituitary-adrenal system in the long-term course of experimental diabetes mellitus

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Summary

Different neurohumoral systems are involved in the development and advance of diabetes mellitus as well as its complications, including hypothalamic-pituitary-adrenal system. The increase of functional activity of the latter contributes to the worsening of the disease course and leads to the development of diabetic macroangiopathies (retinopathy, nephropathy). That's why our goal was to discover common regularities of morpho-functional changes in pituitary-adrenal system in the long-term course of experimental diabetes mellitus (EDM).

The investigation involved 20 mature male Wistar rats, which were equally divided into two groups: control group and experimental one. Diabetes mellitus was induced by single dose intraperitoneal administration of streptozotocin (diluted in 0.1 M of citrate buffer with the pH of 4.5) in a dose of 6 mg per 100 grams of body mass. The research work involved the use of histological (hematoxylin-eosin staining, Heidenhain's Azan staining technique) and electron-microscopic investigation methods. We have discovered that on the 56^{th} day of EDM hyperglycemia leads to the increase of functional activity of pituitary-adrenal system that is morphologically manifested in: corticotrophs – as increase of their number, hypertrophy and hyperplasia of structural components of Golgi complex and granular endoplasmic reticulum with the increase in volume ratio of secretory granules, including marginated ones, causing significant increase of ACTH in blood; adrenal cortex – as marked

thickening of zona fasciculata and zona reticularis, increase of endocrinocyte area in these zones and lipid droplets in them as well as the increase of blood cortisol level.

On the increase of experimental period (day 70) constant hyperfunctioning of pituitary-adrenal system results in its exhaustion. These changes in pituitary are morphologically characterized by ballooning degeneration of corticotrophs, decrease of volume density of secretory granules within them, including the marginated ones, decrease of ACTH in blood; while in adrenal cortex these changes are marked by hydropic degeneration of endocrinocytes of all zones, partial necrosis of endocrinocytes in zona fasciculata, decrease of blood cortisol level.

Key words: corticotrophs, adrenal glands, streptozotocin-induced diabetes mellitus.

Introduction. The incidence of diabetes mellitus (DM) has dramatically increased in the recent decades [1]. Different neurohumoral systems are involved in the development and advance of diabetes mellitus as well as its complications, including hypothalamic-pituitary-adrenal system [2, 4]. The researches put forward the hypothesis that glucocorticoid hormones of adrenal glands play a negative role in the development of the disease and its complications, the increased blood level of which leads to the stimulation of gluconeogenesis in target-tissues of the liver, kidneys, etc. [2, 3]. This hypothesis is confirmed by other scientists, whose experimental and clinical findings point out that the level of glucocorticoid hormones in blood increase in diabetes mellitus type I [4, 5], and enzyme strength of gluconeogenesis in the liver also increases [5]. However, there are other evidences concerning the functioning of hypothalamic-pituitary-adrenal system in diabetes mellitus. Thus, N.K. Mazurina [2] points to the normal cortisol level in blood plasma in patients without signs of acidosis, retino- and neuropathy. O.V. Korpachova-Zinych [6] testified to the fact that circadian rhythms of cortisol and dehydroepiandrosterone levels in patients with type II diabetes mellitus were reduced prior to treatment as compared to the age norm. Though, these findings lack data on morphological changes in adrenal cortex. Insufficient data is available about morpho-functional changes of pituitary-adrenal system in diabetes mellitus.

The **objective** of our investigation was to discover common regularities of morpho-functional changes in pituitary-adrenal system in the long-term course of experimental diabetes mellitus (EDM).

Materials and methods of investigation. The investigation involved 20 mature male

Wistar rats, which were equally divided into two groups: control group and experimental (study) one. Diabetes mellitus was induced by single dose intraperitoneal administration of streptozotocin (diluted in 0.1 M of citrate buffer with the pH of 4.5) in a dose of 6 mg per 100 grams of body mass. The control group animals were administered 0.1 M of citrate buffer with the pH of 4.5 intraperitoneally in the equivalent dose. Euthanasia of animals was performed under thiopental anesthesia by means of decapitation with subsequent blood sampling for biochemical screening.

The level of glucose was determined from the blood sample taken from the tail vein by means of test strips on «Accu Chec» glucometer (Germany). The blood levels of glycosylated hemoglobin (HbA_{1c}), adrenocorticotropic hormone (ACTH) and cortisol were checked in approved testing laboratory «Diameb». The concentration of HbA_{1c} in blood was determined with the help of «ACCENT-200 HbA_{1c} DIRECT» diagnostic test kit (PZ Cormay S.A., Poland). The ACTH level in blood serum was identified with the help of enzyme-linked immunoassay using "EIA-3647 ACTH" kit (DRG, USA). The level of cortisol in blood serum was determined by means of enzyme-linked immunoassay using "EIA-1887 Cortisol ELISA" kit (DRG, Germany).

The test material involved pituitary and adrenal glands, which were collected at the 56^{th} - and 70^{th} day of experimental diabetes mellitus. In order to perform histological study the pituitary glands were fixed in Bouin's solution, paraffin blocks were made and the cuts were stained with azan by means of Heidenhain's Azan technique. The adrenal glands were fixed in neutral formalin solution, paraffin blocks were made and the cuts were stained with hematoxylin and eosin. With the purpose of electron microscopic study the pieces of material were fixed in 2% solution of osmium oxide, conducted and counterstained in a standard manner. Ultrathin sections were studied with the help of TEM-125 K electron microscope, at accelerating voltage of 75 kV, followed by photographic recording at magnifications variable between 1200 to12 000 X.

Histologic specimens were investigated under the MS 300 (TXP) optical microscope and photographed by means of CCD digital camera (Industrial digital camera UHCCD05100KPA-U-NA-N-C-SQ-NA). Morphometry was performed on the above-mentioned specimens in NIH USA "Image J" software as manual operation taking into account magnifications. We have also identified the indices of profile area of different endocrinocytes and their nuclei, as well as nucleocytoplasmic index (NCI) (The ratio of nuclear profile area to cytoplasmic area). The secretory process was estimated by the indices of volume density of secretory granules (SG) in endocrinocytes (Vi = Pi / Pt, where Vi –

stands for volume density, Pi – the number of points within the studied object, Pt – total number of points of the test-system). The thickness index of every zone of adrenal cortex was also defined.

Computer data processing was performed using Stat.Soft.Inc; Tulsa, OK, USA; Statistica 6 Package. Nonparametric research techniques were also used, namely Mann-Whitney test and Spearman rank correlation analysis (r_s).

Results of the investigation and their discussion. On the 56th day of experimental diabetes mellitus (EDM) development the blood glucose level increases to 20.01±2.37 mmol/L (control – 4.86±0.52 mmol/L, p=0.0011), and HbA_{1c} level runs up to 10.74±1.11% (control – 1.80±0.42%, p=0.0011). These biochemical indices point to the development of severe decompensated diabetes mellitus. Significant linear co-relation is noticed between the glucose and HbA_{1c} levels that makes up r_s=0.71 (p=0.0217).

On the 56th day of EDM the number of corticotroph cells per 0.01 mm^2 of adenohypophysis area increases to 3.7±0.82 (control – 2.9±0.73, p=0.0379). The area of corticotroph profiles and their nuclei increases correspondingly to 280.13±11.19 μm^2 (control – 233.93±15.71 μm^2, p=0.0001) and 51.72±2.91 μm^2 (control – 40.57±4.87 μm^2, p=0.0001), while the nucleocytoplasmic index does not significantly change and makes up 0.23±0.01 (control – 0.21±0.03, p=0.6501). Karyopyknosis, enlargement and destruction of cisterns in the granular endoplasmic reticulum is observed in corticotroph cell on the ultrastructural level. Perinuclear space is dilated and passes into the enlarged cisterns of the granular endoplasmic reticulum (Figure 1a). The latter reach huge proportions and force the organelles out to the cell periphery. Mitochondria with cleared matrix and partially destroyed cristae are seen in the cytoplasm. Secretory granules are scattered all over the cytoplasm and their matrix is of different density that points to the enhanced processes of both ACTH synthesis and its evacuation from the cell. Corticotrophs with hypertrophy of the Golgi complex are also present (Figure 1b), which is underdeveloped in animals from the control group. The volume density of secretory granules is significantly higher as compared to the control indices and makes up 5.54±0.28% (control – 4.78±0.19, p=0.0002), though in marginated secretory granules it decreases to1.47±0.19% (p=0.0010). These morphologic and morphometric changes in corticotrophs lead to a significant increase of ACTH in blood, up to 253.02±16.50 pg/ml (control –27.75±5.63 pg/ml). Thereby, a strong positive correlation is noticed between the glucose level and ACTH (r_s=0.94, p=0.0048), while such connection is unreliable in control group of animals (r_s=-0.57, p=0.1802).

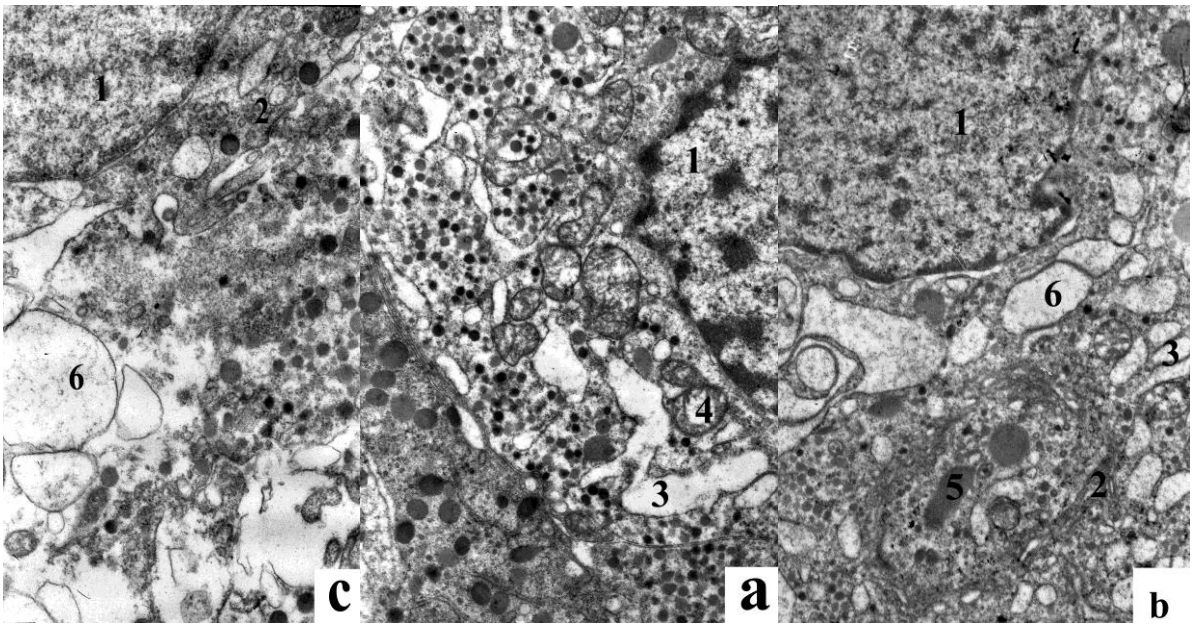

Figure 1. Ultrastructural restructuring of corticotrophs in experimental diabetes mellitus. Electron-diffraction photograph. Magnification: a, b) 8000, c) 9600.

1 – nucleus, 2 – Golgi complex, 3 – granular endoplasmic reticulum, 4 – mitochondria, 5 – lysosomes, 6 – vacuoles.

This restructuring of corticotrophs in pituitary gland and high ACTH level cause changes in adrenal cortex. Significant thickening of all zones of adrenal cortex is observed, namely: zona glomerulosa – to 124.79±13.93 μm (control – 60.47±7.99 μm, p=0.0001), zona fasciculata – to 922.06±86.41 μm (control – 491.49±67.51 μm, p=0.0001), zona reticularis – to 198.34±33.17 μm (control – 133.21±23.18 μm, p=0.0001). The endocrinocytes' profile area is significantly increased in all zones, and the area of their nuclei remains unchanged resulting in significant decrease of nucleocytoplasmic index, as compared to control indices (Table 1). Discomplexation of epithelial ducts of zona reticularis and especially of zona fasciculata is noticed on the light-optical level. It results from intercellular edema.

Table 1

Morphometric indices of endocrinocytes of adrenal cortex in experimental diabetes mellitus (M±m, n=5)

Zones of adrenal cortex	Groups of animals	Cells' area (μm^2)	Area of the nucleus (μm^2)	NCI
56th day of experimental diabetes mellitus				
Zona glomerulosa	experiment	47.58±11.42*	18.63±2.79	0.77±0.48*
	control	39.29±11.94	18.43±2.26	1.051±0.40
Zona fasciculata	experiment	167.96±29.51*	25.90±2.94	0.19±0.04*
	control	130.61±22.53	27.72±4.09	0.28±0.06
Zona reticularis	experiment	95.41±20.55*	24.56±3.51	0.36±0.08*
	control	61.04±13.40	23.22±2.67	0.68±0.15
70th day of experimental diabetes mellitus				
Zona glomerulosa	experiment	59.90±9.22*, #	19.95±2.58	0.60±0.17*
	control	44.79±10.34	19.44±4.29	0.89±0.42
Zona fasciculata	experiment	142.62±33.15*, #	27.15±3.51	0.25±0.06*, #
	control	120.91±11.57	26.97±3.69	0.29±0.07
Zona reticularis	experiment	95.41±20.55*	24.56±3.51	0.36±0.08*
	control	61.04±13.40	23.22±2.67	0.68±0.15

Notes:
1 - * - the difference between the control and experiment indices, $p<0.05$.
2 - # - the difference of the indices as compared to the previous observation period $p<0.05$.

Perinuclear space widening is observed at the ultrastructural level in endocrinocytes of all zones, as well as the breakdown of mitochondrial crustae and occasionally even their complete destruction with the formation of vacuoles. The most significant ultrastructural changes are observed in endocrinocytes of zona fasciculata. They are characterized by the widening of cisterns of granular endoplasmic reticulum, irregular outlines of lipid droplets (Figure 2a). The latter acquire huge size in certain endocrinocytes and fill the entire cell cytoplasm (Figure 2b), indicating the delay of secretion evacuation from the cell. Together with the modified endocrinocytes, one may notice the ones with the preserved ultrastructure. Blood cortisol level is significantly higher as compared to the control indices and makes up 18.93±1.42ng/ml (control – 10.05±0.98 ng/ml, $p=0.0027$). The correlation analysis showed no significant co-relations between the ACTH and cortisol levels, while in control group of animals they are positive and strong ($r_s=0.96$, $p=0.0008$).

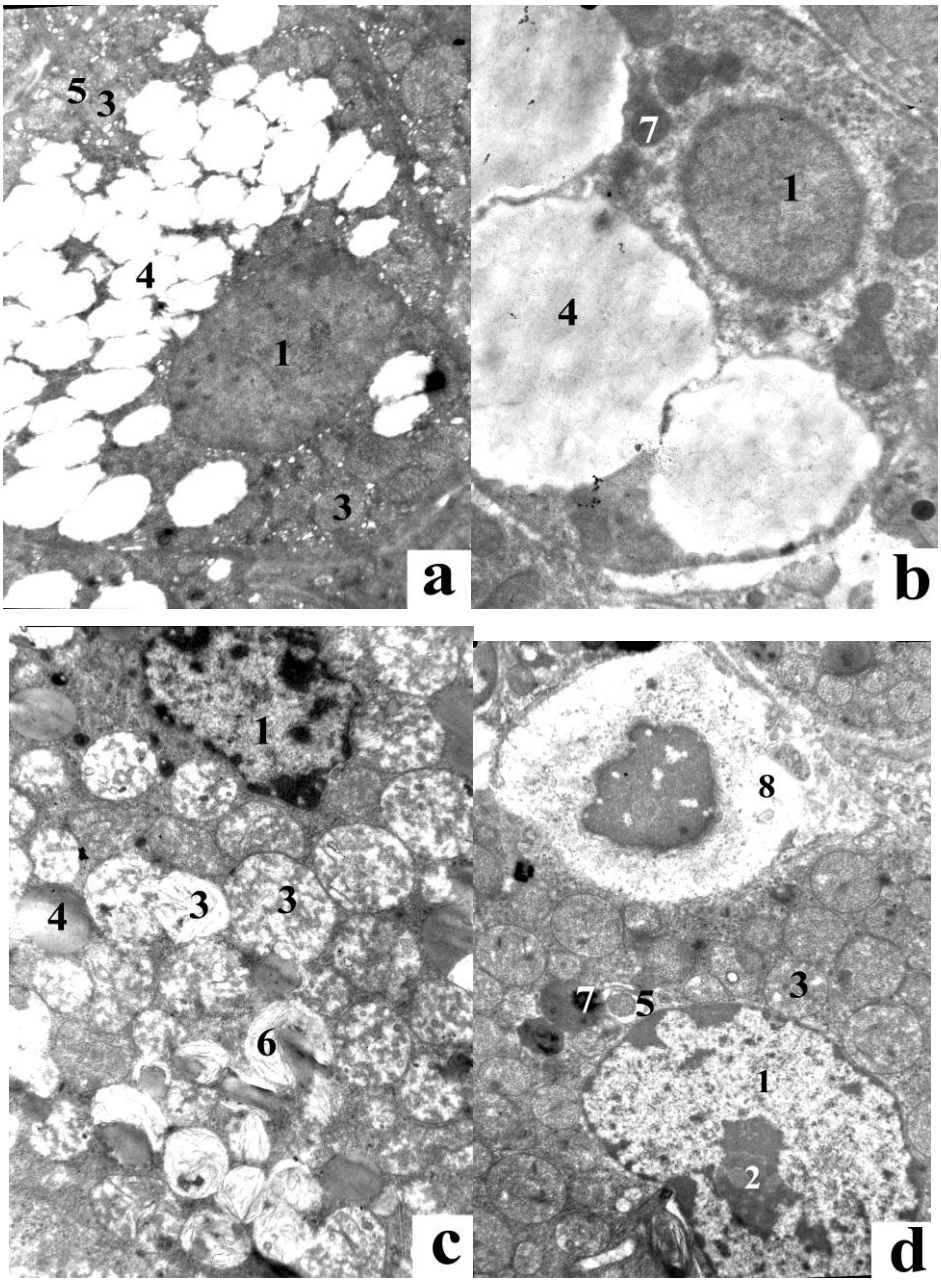

Figure 2. Ultrastructural changes in zona fasciculata endocrinocytes of adrenal cortex on the 56th (a, b) and 70th (c, d) days of experimental diabetes mellitus. Electron-diffraction photograph. Magnification: a, c, d) 6400, b) 8000.

1 – nucleus, 2 – nucleolus, 3 – mitochondria, 4 – lipid droplets, 5 – granular endoplasmic reticulum, 6 – mitochondrion with myelin-like inclusion structures, 7 – lysosomes, 8 – cell with partial necrosis.

On the 70th day of experimental diabetes mellitus the blood glucose level is statistically non-significantly higher as compared to the previous period of experiment, namely it increases to 21.64±3.52 mmol/L, ($p=0.4712$), while the HbA_{1c} level significantly

increases to 12.23±0.62% (p=0.0249). The indices of glucose and HbA_{1c} levels still show significant direct co-relation r_s= 0.84 (p=0.0022).

On the 70th day of EDM the number of corticotrophs per 0.01 mm^2 of adenohypophysis area decreases to 3.1±0.57 (p=0.1405) and does not differ from the control indices (p=0.5453). The corticotroph profile area significantly increases to 294.05±11.64 μm^2 (p=0.0191) as compared to the previous period of experiment, while the area of nuclei does not significantly change (51.61±2.83 μm^2, p=0.9696), leading to significant decrease of NCI to 0.21±0.01 (p=0.0046). Increase of the destructive changes in corticotrophs is observed on ultrastructural level. They show prominent features of ballooning degeneration (Figure 1c), thus the cytoplasm becomes comb-like by means of large vacuoles. Among the latter there occur certain ribosomes, polysomes and secretory granules. Marginated secretory granules are rare, and their volume density decreases to1.15±0.29% (p=0.0113, p=0.0008) as compared to the indices of previous period of experiment and control ones. Generally, the volume density of SG decreases to 4.30±0.89% (p=0.0004), as compared to the previous period of experiment, and does not significantly differ from control indices (p=0.0963). The cisterns of granular endoplasmic reticulum are partially destroyed, widened, and occasionally expand into the perinuclear space. There still remain some deeply swollen mitochondria with destroyed inner and outer membranes. Corticotrophs with apoptotic cells are also present. These corticotrophic changes cause significant decrease of ACTH levels in blood to 232.37±12.60 pg/ml (p=0.0249), as compared to the previous period of experiment, though its level still remains significantly higher than control indices (p=0.0027). The glucose and ACTH levels show significantly direct and strong co-relation (r_s=0.83, p=0.0416).

Significant widening of all zones of adrenal cortex is still observed in this period: zona glomerulosa widens to 92.68±18.31 μm (control – 64.56±8.55 μm, p=0.0001), zona fasciculata – to 1179.41±259.12 μm (control – 477.04±67.03 μm, p=0.0001), zona reticularis – to 200.42±59.83 μm (control – 120.68±29.19 μm, p=0.0001). The endocrinocyte profile area of all zones is significantly higher than control indices, as it was observed in the previous period of experiment (see Table 1), causing significant decrease of NCI. At the same time, the fasciculata cell area decreases significantly, as compared to its indices in the previous period of experiment (Table 1). The signs of hydropic degeneration are observed in endocrinocytes of different zones of adrenal cortex. Mitochondria undergo the most evident polymorphic changes. Mitochondria with cleared matrix and ruptured cristae prevailed in endocrinocytes of zona fasciculata. Some mitochondria in endocrinocytes of zona fasciculata had high cristae density; others had cleared matrix with partial destruction of vesicular cristae; some had

widely spaced vesicular cristae; still others showed the presence of myelin-like intramitochondrial inclusion structures, as well as fusion of lipid droplets and mitochondria (Figure 2c). Apart from endocrinocytes with hydropic degeneration, there are also cells with preserved ultrastructure, and those with partial necrosis (Figure 2d). These changes in adrenal cortex lead to significant decrease of blood cortisol level to 13.36±4.15 ($p=0.0163$), as compared to the previous period of experiment, though its level does not significantly differ from the control indices ($p=0.4750$).

We are of the opinion that changes in pituitary-adrenal system on the 56-70th days of experimental diabetes mellitus are associated with hyperfunctioning of cells in response to hyperglycemia, resulting in their destruction. The discovered morphological changes of secretory activity of corticotrophs in adenohypophysis correlate with the findings of other researchers [5, 6], who noticed the increase of corticoliberin level in blood at early stages of experimental streptozotocin-induced diabetes mellitus development, while its level decreased at later stages of diabetes course. The revealed changes in adrenal cortex were also observed by other researchers in various diseases and acute stressful situations [7, 8] and may point to their increased functional activity associated with metabolic changes in the body in case of diabetes mellitus [2, 3]. However, the findings of other researchers show that on the 70th day of EDM the destructive changes in endocrinocytes and the development of diabetic microangiopathy in the gland lead to the failure of glucocorticoids production and the delay of their evacuation from endocrinocytes of zona fasciculata that is confirmed by unreliable increase of cortisol level in blood. Our findings contradict the data provided by other scientists who point to the increase of cortisol level in blood plasma in cases of decompensated diabetes mellitus type I, ketoacidosis and diabetic coma [11]. Other researchers indicate normal cortisol level in blood plasma in diabetic patients [10] and in rats with decompensated experimental diabetes mellitus [9, 12, 13].

Conclusions. On the 56th day of EDM hyperglycemia leads to the increase of functional activity of pituitary-adrenal system that is morphologically manifested in: corticotrophs – as increase of their number, hypertrophy and hyperplasia of structural components of Golgi complex and granular endoplasmic reticulum with the increase in volume ratio of secretory granules, including marginated ones, causing significant increase of ACTH in blood; adrenal cortex – as marked thickening of zona fasciculata and zona reticularis, increase of endocrinocyte area in these zones and lipid droplets in them as well as the increase of blood cortisol level.

On the increase of experimental period (day 70) constant hyperfunctioning of

pituitary-adrenal system results in its exhaustion. These changes in pituitary are morphologically characterized by ballooning degeneration of corticotrophs, decrease of volume density of secretory granules within them, including the marginated ones, decrease of ACTH in blood; while in adrenal cortex these changes are marked by hydropic degeneration of endocrinocytes of all zones, partial necrosis of endocrinocytes in zona fasciculata, decrease of blood cortisol level.

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