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VA Levytskyi
Galytska str. Ivano-Frankivsk National Medical University, Ukraine.

OYa Zhurakivska
MD, PhD, DSci, Professor of Human Anatomy Department of Ivano-Frankivsk National Medical University, Ukraine.

Yu L Tkachuk
MD, Ass Professor of Neurosurgery Department of Ivano-Frankivsk National University, Ukraine.

Corresponding Author
VA Levytskyi
Galytska str. Ivano-Frankivsk National Medical University, Ukraine.

Exenatide use for the correction of morphological changes of pituitary-adrenal system in experimental diabetes mellitus

VA Levytskyi, OYa Zhurakivska, Yu L Tkachuk

Abstract
The research work is devoted to issues of morpho-functional changes of pituitary-adrenal system in diabetes mellitus and its correction with exenatide and insulin. On the 28th day of streptozotocin-induced diabetes mellitus development we noticed the increase in number of corticotrophs of adenohypophysis and elevation of their functional activity (extension of nucleus area and increase of nucleocytoplasmic index, volume density of secretory granules due to their marginated form), leading to the increase of adrenocorticotropic hormone (ACTH) in blood. Qualitative and quantitative alterations of corticotrophs and hyperglycemia result in adrenal cortex restructuring, namely: thickening of fascicular and reticular zones of adrenal cortex, enlargement of the area of profile field of endocrinocytes and their nuclei, significant increase of volume density of lipid droplets in cells, increase of blood cortisol level. The use of exenatide in the treatment of morphological disorders of pituitary-adrenal system in streptozotocin-induced diabetes mellitus results in regeneration of quantitative cellular composition of adenohypophysis and adrenal cortex, ultrastructural composition and secretory activity of corticotrophs and endocrinocytes of adrenal cortex, decrease of adrenocorticotropic hormone and cortisol levels in blood, as well as normalization of glucose level and glycosylated hemoglobin.

Keywords: morphological changes, pituitary-adrenal system, experimental diabetes mellitus

Introduction
In recent decades a considerable increase in the incidence of diabetes mellitus has been observed [1]. Defining the threat posed to the humanity by diabetes, the General Assembly of the United Nations adopted a resolution (December 20, 2006) on diabetes, according to which the expenses related to medical treatment of diabetic patients came out of more than 2-3% of overall health care costs; almost 80% accounted for the treatment of complications, 20% - for the purchase of antihyperglycemic drugs and control means. In recent years the research work of many scientists has been focused on the discovery and development of anti-diabetic medications promoting regeneration of insulocytes in pancreatic islets. One of them is exenatide from the group of adrenoceptor agonists of incretin [2]. It lowers the secretion of glucagon by alpha-cells of pancreatic islets [3], reduces body weight [4, 5], decreases the level of triglycerides, low-density lipoproteins, as well as diastolic pressure and increases the level of high-density lipoproteins [6] in patients with type II diabetes mellitus. Experimental findings showed positive influence of exenatide on insulocytes, namely the increase of their mass due to stimulation of their proliferation [7, 8] and decrease in apoptosis [9]. However, we failed to find any records in the scientific literature concerning the influence of exenatide on the structure of pituitary-adrenal system in patients with diabetes mellitus, and as it is commonly known, namely diabetes causes hyperproduction of contrinsuline hormones (glucocorticoids, aldosterone) as a compensatory reaction responding to metabolic stress, which leads to the development of micro- and macroangiopathies [10].
Taking in consideration the above the objective of our research work was to study structural organization of corticotrophs of adenohypophysis and adrenal cortex in experimental diabetes mellitus and its correction with exenatide.

Materials and methods of investigation: The investigation involved 23 mature male Wistar rats, which were divided into three groups: 1 – control group (5 animals), 2 - 6 animals with streptozotocin-induced diabetes mellitus, 3 - 12 animals with streptozotocin-induced diabetes mellitus, which were under antidiabetic treatment. Experimental diabetes mellitus (EDM) in animals from groups 2 and 3 was induced by a single intraperitoneal administration of

streptozotocin (diluted in 0.1 M of citrate buffer with the pH of 4.5) at 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. Animals from group 3 were administered antidiabetic treatment beginning from the 14th day of diabetes development: namely, animals from subgroup 3a (6 animals) were injected exenatide («Byetta» medication) at a dose of 0.04 mcg/100g of body mass once a day under the skin, while animals from subgroup 3b received subcutaneous injections of insulin glargine (3 units/day). Euthanasia of animals was performed under thiopental narcosis by means of decapitation followed by blood sampling for biochemical screening.

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

The test material involved pituitary and adrenal glands, which were collected at the 28th 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). Computer data processing was performed using Stat.Soft.Inc; Tulsa, OK, USA; Statistica 6 Package.

Results of the investigation and their discussion: After two weeks of treatment the blood glucose levels significantly decreased to 9.35±1.07 mmol/L (p=0.0039) in animals from subgroup 3a, and to 8.71±0.96 mmol/L (p=0.0039) in subgroup 3b animals as compared with animals from group 2 (blood glucose level was 15.35±1.36 mmol/L); though these levels are significantly higher that control ones (4.71±0.44 mmol/L, p=0.0062 in all the cases). The level of HbA_{1c} also decreases in animals from group 3, as compared with those from group 2 (9.07±0.58%), and in subgroup 3a it decreases to 5.91±0.56% p=0.0039, and to 5.61±0.74% p=0.0039 in subgroup 3b animals. Such levels of HbA_{1c} in animals from group 3are higher than control indices (control – 2.27±0.76%), but still are within acceptable standards.

On the 28th day of streptozotocin-induced diabetes mellitus corticotrophs in group 2 animals are characterized by low level of hydropic degeneration that shows up as marginal enlightenment of cytoplasm and presence of scattered small vacuoles, therefore vacuoles are not revealed in these cells in animals from group 3 and they are well stained with varied intensity. The total count of corticotrophs over the area of $0.01mm^2$ of adenohypophysis remains higher than control indices and makes up 4.4±0.17 (p=0.0461) in subgroup 3a, and 3.9± 0.09 (p=0.0219) in 3b subgroup of animals, and does not significantly differ from indices in group 2. The area of nuclei and nucleocytoplasmic index (NCI) of corticotrophs increase in animals from groups 2 and 3, as compared to the control ones, though NCI in group 3 animals is significantly lower than in group 2 (table 1).

Table 1: Morphometric indices of corticotrophs of adenohypophysis in streptozotocin-induced diabetes mellitus and its correction (M±m)

Groups of animals	Area of nucleus (μm²)	Nuclear density index	Cell's area (μm²)	NCI
DM	49.89±1.41*	0.77±0.02	226.54±6.45	0.28±0.02*
3a	45.82±1.21*	0.76±0.02	241.23±11.21*,#	0.24±0.01*,#
3b	46.96±1.34*	0.78±0.02	236.32±9.28*	0.25±0.02*,#
control	42.34±1.81	0.76±0.01	239.74±6.85	0.21±0.02

Note:

1. * - significant difference, as compared to the control group animals, p <0.05.
2. # - significant difference, as compared to animals with experimental diabetes mellitus (group 2), p<0.05.

Polymorphism of corticotroph changes is noticed in group 2 animals at the ultrastructural level. All nuclei are irregular in shape through the invagination of the nuclear membrane therewith karyoplasm is clear, and heterochromatin forms small aggregations near the karyolemma. Mitochondria have cleared matrix, some of them are represented by vacuoles arising from the destruction of their inner membrane (Figure 1). The cytoplasm of some of them is overcrowded with a large number of secretory granules, which also fill the cell processes (Figure 1a). They are small, with the matrix of moderate and high electron density, however coated granules are hardly observed that points to the intensification of ACTH synthesis. The cytoplasm of other corticotrophs is of low electron density and contains many enlarged cisterns of the granular endoplasmic reticulum, small and large vacuoles and a small number of secretory granules (Figure 1b), that indicates the hydropic degeneration pattern of destructive changes in these cells. Volume density of secretory granules

increases to 8.94±0.37% (control – 4.39±0.48, p=0.0002) and coated ones – to 2.97±0.39% (control – 1.05±0.21, p=0.0002) as compared with the control indices. The level of ACTH increases to 334.31±49.26 pg/ml (control – 25.45±2.43 pg/ml, p=0.0062).

Regeneration of the ultrastructural organization of corticotrophs (Figures 1 c, d) is noticed in group 3 animals, which received treatment: nuclei become rounded and euchromatin predominates; young mitochondria with electron-dense matrix and well-differentiated cristae appear in the cytoplasm, hypertrophy of granular endoplasmic reticulum is also observed. In common with the control group of animals, we observed moderately-, hyper- and degranulated corticotrophs indicating the re-establishment of the process of synthesis, accumulation and evacuation of ACTH from these cells. It is important to note that the volume density of secretory granules in these cells decreases to 5.40±0.53% (p=0.0002) in animals from subgroup 3a and to 5.43±0.81% (p=0.0002) in subgroup 3b as compared to group 2 animals, though it remains significantly higher than the control indices (p=0.0017 and p=0.0046 respectively). The same applies to the marginated secretory granules, namely: their volume density makes up 1.92±0.43% and 1.89±0.24 in animals from subgroups 3a and 3b that is significantly lower than the indices in group 2animals (p=0.0002 and p=0.0002), though it is significantly higher than the control (p=0.0003 and p=0.0002). This restructuring of corticotrophs caused the ACTH blood level to become three-times lower in animals from subgroups 3a (96.85±14.3 pg/ml, p=0.0062) and 3b (32.27±4.8 pg/ml, p=0.0062), than in group 2 animals, however these indices remain higher than the control ones in subgroup 3a (p=0.0062).

Such metric and ultrastructural changes in corticotrophs in group 3 animals are associated with the compensation of diabetes mellitus and considerable daily glucose fluctuations, while in group 2 animals they are connected with the decompensation of DM, and on the one hand, with high functional activity of these cells in response to metabolic disorders in the body and with depletion and destructive changes as a result of their continuous hyperfunction, on the other hand.

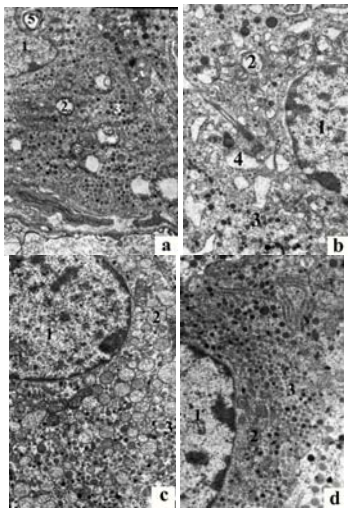

Fig 1: Ultrastructure of corticotrophs in animals from group 2 (a, b) and 3a subgroup (c, d) in experimental DM and its correction. Magnification: a, b, c, d) 9600. 1 – nucleus of corticotroph, 2 – mitochondria, 3 – secretory granules, 4 – granular endoplasmic reticulum, 5 – vacuoles.

On the 14th day of treatment we noticed significant decrease in thickness index of various zones of adrenal glands (Figure 2). At the same time the area of glomerular and reticular zones does not significantly differ from that in control group of animals, while the area of fascicular zone remains higher than the control one. The cells' profile field area and areas of glomerular and reticular zones as well as their nucleocytoplasmic index (NCI) are not significantly different from the control indices (Table 3). Significant reduction of profile field area of endocrinocytes is observed in fascicular zone as compared to group 2 animals, while the area of nucleus is significantly higher than the control index that causes NCI increase (see table 3).

Table 2: Thickness of various zones of adrenal cortex in rats on the 28th day of EDM development and its correction (M±m, μm)

Group of animals		Glomerular zone	Fascicular zone	Reticular zone
2		80.14±15.93*	947.30±77.81*	163.49±32.66*
3	3a	60.34±7.12#	543.15±65.57#,*	123.52±21.34#
	3b	61.22±6.32#	562.31±45.62#,*	115.63±26.31#
control		58.41±7.48	468.37±33.48	122.49±22.73

Note:
1. * significant difference, as compared to the control group animals, p <0.05.
2. # significant difference, as compared to the animals with EDM (group 2), p<0.05.

Table 3: Morphometric indices of endocrinocytes of adrenal cortexin experimental diabetes mellitus and its correction (M±m)

Zones of adrenal cortex	Groups of animals	Area of cells (μm 2)	Area of nucleus (μm 2)	NCI
Glomerular zone	DM	82.71±2.78 *	31.19±0.63 *	0.65±0.03 *
	3a	45.02±3.03 #	20.96±0.45 #	0.87±0.05 #
	3b	49.56±2.12 #	22.23±0.62 #	0.81±0.06 #
	control	47.01±2.02	20.13±0.85	0.85±0.07
Fascicular zone	DM	260.81±9.11 *, #	46.98±0.69 *, #	0.23±0.01 #
	3a	155.23±8.63 #	33.24±0.65 #,*	0.27±0.06 #,*
	3b	156.24±6.17 #	31.56±2.12 #,*	0.25±0.07 *
	control	153.18±42.28	26.64±3.94	0.22±0.06
Reticular zone	DM	102.87±3.71 *, #	32.96±1.42 *, #	0.50±0.04
	3a	66.45±3.56 #	24.96±0.85 #	0.60±0.07 #
	3b	68.46±4.21 #	26.31±1.12 #	0.62±0.05 #
	control	67.44±3.02	25.01±0.79	0.62±0.04

Note:
1. * significant difference, as compared to the control group animals, p <0.05.
2. # significant difference, as compared to the animals with EDM (group 2), p<0.05.

Morphological characteristics of increased functional activity are observed on the ultrastructural level in endocrinocytes of all zones of adrenal cortex in group 2 animals (Figure 2a). These cells contain oval-shaped nucleus with micro-invaginations of the nuclear membrane. The nucleoplasm is of moderate electron density with heterochromatin lumps among

fine euchromatin. Structural components of granular endoplasmic reticulum are visually smaller in the cytoplasm. A large number of lipid droplets is observed in the cytoplasm of endocrinocytes in glomerular and fascicular. Their matrix is non-homogenous that points to intense processes of synthesis and evacuation of secretion from the cells. Most mitochondria have enlightened matrix, and some of them have partially destroyed cristae. Single vacuoles, lysosomes and autophagosomes are observed in cytoplasm of endocrinocytes. Lipid droplets fill the cytoplasm of endocrinocytes of fascicular zone, and their volume density significantly increases to 46.39±0.27% (control – 9.16±0.36%, p=0.0001), that indicates marked release of glucocorticoid hormones in blood and is confirmed by biochemical findings, namely, the cortisol level increases to 23.05±2.95 ng/ml (control – 10.14±1.03, p=0.0039).

The use of exenatide leads to the re-establishment of ultrastructural organization of endocrinocytes of various zones of adrenal cortex (Figures 2 b, c). Group 3 animals have mitochondria with moderate electron-optical density and clearly differentiated vesicular cristae, as opposed to animals from group 2 (Figure 2 c). Both degranulated and lipid-filled endocrinocytes are noticed in fascicular zone. Hyperplasia of granular endoplasmic reticulum and lipid droplets are visualized in the cytoplasm of endocrinocytes (see figure 2 b). The latter have matrix of varied electron-optical density, and their middle part is light that points to the process of glucocorticoids secretion. This process is also confirmed by the biochemical findings, which show that the blood cortisol level in group 3 animals is significantly lower than in animals from group 2and makes up 13.11± 2.02 ng/ml (p=0.0062) in subgroup 3a and 11.51±1.41 ng/ml (p=0.0062) in 3b one. At the same time, blood cortisol level of 3a subgroup animals is considerably higher than the control indices (p=0.0176), though it does not significantly differ from control indices in subgroup 3b (p=0.1441).

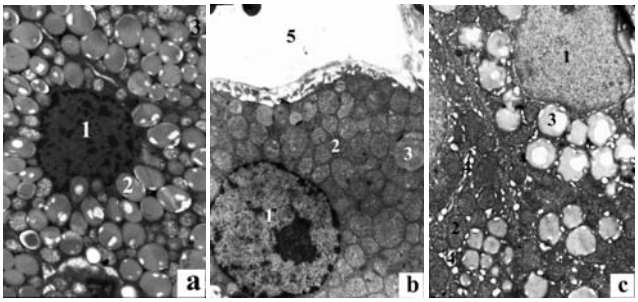

Fig 2: Ultrastructure of endocrinocytes of fascicular zone of adrenal cortex in group 2(a) animals and 3a (b, c) subgroup of rats in streptozotocin-induced diabetes mellitus and its correction. Magnification: a, b, c) x 6400. 1 – nucleus, 2 – mitochondria, 3 – lipid droplets, 4 – agranular endoplasmic reticulum, 5 – capillary lumen.

Discussion: The findings of our research work indicate activation of pituitary-adrenal axis in streptozotocin-induced DM that is expressed by significant increase in blood levels of ACTH (13 times) and cortisol (2.2times). Other authors [11] also point to the increase of glucocorticoids secretion in blood of rats with streptozotocin-induced DM and associate this fact with changes in hippocampus, namely: with the increase of glial fibrillar acid protein (GFAP) in astrocytes, which is considered to be the marker. While some others [12] link the high level of corticosteron in mice with streptozotocin-induced DM to the hyper sensibility of cell culture of adrenal cortex to ACTH, the level of which considerably increases in paraventricular nucleui of hypothalamus and hippocampus. A. Gohshi and others [13] indicate high levels of ACTH at early stages of streptozotocin-induced DM development and associate this fact with high corticoliberin levels.

We marked increased morphofunctional activity of pituitary-adrenal system during the course of treatment of streptozotocin-induced DM. It should be pointed out that ACTH and cortisol levels reduced significantly in animals treated with exenatide and insulin as compared to animals with diabetes mellitus. However, the ACTH and cortisol levels were higher in animals treated with exenatide injections, as compared with animals receiving insulin. High ACTH levels were also observed by other authors in animals treated with exenatide [14, 15]. They showed that high blood levels of ACTH, aldosterone and cortisol in rats were observed for 1-2 hours after exenatide administration in both intact and diabetic animals. We think that high levels of ACTH in the course of exenatide therapy have positive influence on the secretion of insulin by β-cells. Many authors [16, 17] confirmed that ACTH stimulates glucose-dependant production of insulin by β-cells due to endogenous influence on Langerhans islets. P.M. Jones *et al*. [16] investigated the mechanisms of paracrine effect of ACTH on Langerhans islets, particularly expression and function of ACTH receptors (melanocortin-2 receptor (MC2-R)) on the human and mouse islet tissue *in vitro*. They proved the presence of a large number of MC2-R in β-cells, whereas ACTH, due to its effect on these receptors, triggers insulin secretion by means of cAMP activation in combination with the influx of cytosolic Ca^{2+}. Other authors [17] point to complex endocrine and paracrine mechanisms that regulate the function of pancreatic islets in diabetes mellitus, particularly glucose-like peptide-1 has positive effect on the synthesis and secretion of insulin, while somatoliberin, corticoliberin and ACTH have paracrine effects on the secretion of the latter.

Conclusions: The use of exenatide in the treatment of morphological disorders of pituitary-adrenal system in EDM results in re-establishment of quantitative cell composition of adenohypophysis and adrenal cortex, ultrastructural composition and secretory activity of corticotrophs and endocrinocytes of adrenal cortex, reduction of the blood levels of adenocorticotropic hormone and cortisol, and normalization of glucose and glycosylated hemoglobin levels.

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