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EFFECTS OF CHRONIC ETHANOL ADMINISTRATION ON THE GLUCAGON-PRODUCING A-CELLS IN RAT ENDOCRINE PANCREAS. A MORPHOMETRIC AND FINE STRUCTURAL STUDY

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The present study describes optical and ultrastructural features of glucagon-producing A-cells in the male Wistar strain rat after chronic (4 months) alcoholism where nutriition was well controlled. Male Wistar rats were given ethanol to provide 23% of the total energy (group E), while starch replaced ethanol isocalorically in controls (group C). The "point counting" technic was used to evaluate morphometric/stereological parameters on the islets and glucagon-producing A-cells. Immunohistochemically detected A-cells showed a ring like distribution in both groups of rats. At the ultrastructural level, most of the A-cells resembled those in the control, but a small number of A-cells were changed with deep invagination of the nuclear envelope and condenzed chromatin. The total number of endocrine cells, both per islet and per mm^2 of islet was increased in the ethanol-treated rats. Glucagon-producing A-, somatostatin-producing D-, and pancreatic polypeptide producing PP-cells were increased, while insulin-producing B-cells were decreased. Stereological investigation at the ultrastructural level showed that the profile area of the whole A-cell, cytoplasm, Golgi system and gER was diminished, and that of mitochondria increased. Volume density of the nuclei and Golgy system was decreased, while cytoplasm granules and mitochondria were more numerous the former because of a greater number of mature granules. The mean diametar of the granules was significantly reduced in ethanol treated rats. All the results obtained indicated hypoactivity of A-cells.

Key words: Ethanol; Langerhans islets; Glucagon-producing cells; Morphometry; Rat

INTRODUCTION

Chronic alcohol ingestion has been shown to produce different histological types of alcoholic chronic pancreatitis in man and various animal species (Sarles, 1975; Weesner et al., 1985; Singer et al., 1990; De Angelis et al., 1992). Mechanisms of functional disturbances of neuroendocrine pancreatic cells are

very complex due to the existence of interactions between endocrine and exocrine pancreatic components, as well as enteropancreatic interaction in chronic alcoholism (Klöppel et al., 1978; Owyang, 1986; Go, 1990). Studies have shown that exocrine deficiency had a significant effect on islet cell function (Sereny and Endrenyi, 1978), while functional disturbances of gastrointestinal neuroendocrine cells could also alter pancreatic function.

There are few morphometric data about the effects of chronic alcohol ingestion on gastrointestinal and pancreatic endocrine cells (Koko et al., 1995a, Koko et al 1995b, Todorović et al., 1993a, Todorović et al, 1993b). To the best of our knowledge, morphometric analysis of glucagon-producing cells of the pancreas and their ultrastructural properties has not yet been studied in chronic experimental alcoholism where nutrition was well controlled. Because very little is known about the effect of alcohol on islet A-cells, the aim of this work was to investigate the effects of chronic ethanol consumption on islet morphology and glucagon-producing A-cells.

MATERIALS AND METHODS

Thirty male Wistar rats 2 months old, and weighing approximately 250g at the beginning of the experiment, were randomly allocated into control (C) and experimental, ethanol fed (E) groups. Animals were maintained in single cages at a constant temperature (19 ± 1^0C) and humidity, on an alternate 12:12 h light-dark cycle (lights on at 7 a.m.).

Control rats were fed with a commercial stock cereal based pelleted diet (25% protein). The experimental rats were given free access to a hyperprotein cereal based pelleted diet (34% protein) and an aqueous solution of 25% sucrose - 32% ethanol as recommeded by Hartroft (1971). The amount of food fed to the control animals was adjusted to the energy intake of the animals receiving ethanol (i. e. the control rats were pair-fed).

Alcohol was replaced by starch in the control diet. The volume of tap water given to the ethanol-treated rats was 22 ml and mean consuption of the alcoholic solution per rat per day was 12 ml.

The amount of tap water given to the control group was adjusted so that the rats in both groups received the same amount (approximately 40 ml) of miquid per day. Both diets were balanced correctly in respect to consumption of vitamin-mineral mixture.

Samples for blood ethanol determination were obtained from the tail vein, and blood ethanol concentration was determined using the Sigma diagnostic alcohol procedure (No, 322-UV, Sigma Chemical Co., St. Louis, MO). The mean blood ethanol concentration was determined for all ethanol-treated rats studied and based upon multiple blood ethanol determinations (at 10 a.m. on day 1, 15, 30, 45, 60, 75, 90, 105 and 120) throughout the 120-day exposure period.

Fasting blood glucose level was measured by the glucose oxidase method (Hyvarinen and Nikkila, 1962).

The experiment lasted for 4 monts. At the end of the feeding period the animals were fasted overnight and the abdomen opened under light ether anaesthesia.

Table 1. Daily nutrient and energy intake (mean± SEM)

PARAMETER	GROUP C	GROUP E
Food		
(g/day)	23.92 ± 0.01	16.10 ± 0.13
Ethanol		
(g/day)	–	3.06 ± 0.17
(g/100gBW/day)	–	0.72 ± 0.03
Protein		
(g/day)	5.87 ± 0.00	5.54 ± 0.05
(g/100gBW/day)	1.64 ± 0.03	1.34 ± 0.06
Fat		
(g/day)	1.15 ± 0.00	1.09 ± 0.01
(g/100gBW/day)	0.32 ± 0.00	0.26 ± 0.01
Carbohydrate		
(g/day)	13.68 ± 0.00	9.50 ± 0.16
(g/100gBW/day)	3.83 ± 0.06	2.27 ± 0.07
Vitamin-mineral mixture		
(g/day)	0.06 ± 0.00	0.06 ± 0.00
(g/100gBW/day)	0.19 ± 0.00	0.17 ± 0.00
Total energy intake		
(kJ/day)	375 ± 0.19	385 ± 7.45
% of total energy intake		
protein	27	24
fat	11	11
carbohydrate	62	42
alcohol	–	23

The pancreas was quicly removed, washed in cold 154 mM NaCl and weighed in air. The total pancreatic volume was determined using an immersion method (Scherle, 1970). Tissue specimens were taken from the splenic portion of the pancreas and immediately fixed in Bouin's solution for 8h and embedded in paraffin according to standard procedures, sectioned and stained with hematoxylin and eosin and Victoria trichrome stain.

Immunocytochemistry was performed on 5μm serial sections from Bouin-fixed paraffin-embedded tissue. For determination of insulin-, glucagon- and somatostatin-containing cells, sections were immunostained by the unlabelled peroxidase-antiperoxidase complex (PAP) tehnique (Sternberger, 1986).The sections were incubated with antisera against insulin (1:1000; A564, DAKO Corp., CA), glucagon (1:200; A565, DAKO Corp., CA), and somatostatin (1:500, by courtesy of Dr. J. Rehfeld, Univ. Aarhus, Denmark). An avidin biotin complex (ABC) technique was used to localise pancreatic polypeptide-producing PP cells (rabit anti-pancreatic polypeptide commercial prediluted; Histostain^{TM} SP (Peroxidase) Bulk kit 95-6143-B).

Immunoreactive sites were visualized by DAB/H_2O_2 (0.7 mM diaminobenzidine-HCl and 0.002% H_2O_2 in 0.05M Tris-HCl, pH 7.6), for 5min in the dark (result of reaction - brown deposits). The nuclei were counterstained with Mayer's haematoxylin.

For electron microscopy, the specimens were immediately placed in cold 3% glutaraldehyde in 0.1M Na-cacodylate buffer at pH 7,4, minced into very small pieces and fixed in the same fixative for 3h at 4^0C. After postfixation for 1h at room temperature in 1% osmium tetroxide, specimens were dehydrated in a graded series of ethanol and embedded in epoxy resin (Araldite). Ultrathin sections were stained with uranyl acetate and lead citrate before examination in an Philips CM 12 electron microscope.

Stereological analysis of islets and their neuroendocrine components (insulin-, glucagon-, somatostatin- and pancreatic polypeptide-producing cells) was performed using Weibel's multipurposetest grid(Weibel et al., 1966) by a "point counting" tehnique.

Ultrastructural stereological analysis of glucagon-producing A- cells was performed by micrographs at a final magnification of 9000. Morphometric analysis was done by the "point counting" technique using a transparent lattice grid (20x29 cm), with lattice ratio r=3, using the method of Weibel and Bolender as described by Aherne and Dunnill (1982). Test grid covering micrographs made in possible to determine the number of "hits" on chosen test organelles (coarse points for nucleus and cytoplasm and fine points fon the rest of the subcellular structures). The following morphometric/stereological parameters were obtained from this number of points by applying standard equations; the profile areas of the whole cell, nuclei, cytoplasm, mitochondria, Golgy complex and endoplasmatic reticulum; volume density of nuclei, cytoplasm, mitochondria, Golgi complex, endoplasmatic reticulum and cytoplasmic granules; number per cell profile of total cytoplasmic granules, mature and immature granules, and mitochondria; number per μm^2 of cytoplasm of total, mature and immature granules.

The mean granular radius was measured using, a Kotron MOP-AMO 3 semiautomatic analyzer.

The Student t-test was used to determine statistically significant differences between means.

RESULTS

The histological investigations showed that there were no differences in the general structure of the endocrine pancreas between controls and ethanol treated rats. Immunohistochemical detection of pancreatic glucagon-producing A-cells revealed their ring like distribution at the periphery of rach islet of Langerhans in both examined groups of rats.

The number of total and particular endocrine cells per islet and per μm^2 of islet is presented in Table 2.

Glucagon-producing A-cells, as well as somatoatatin-producing D-, and pancreatic polypeptide-producing PP-cells were increased in number, while

insulin-producing B-cells were decreased. In comparison with the control, the increase of A-cells ancounted to about 22% per islet and 20% per μm^2 of islet.

Table 2. Number of A, B. D, and PP-cells per islet and per μm^2 of islet (mean ± SEM)

	GROUP C (n = 10)	GROUP E (n = 12)	Statistics
	Number / islet profile		
Total	66.8 ± 3.55	72.3 ± 5.44	n.s.
A-cell	21.2 ± 1.95	27.1 ± 2.76	n.s.
B-cell	30.8 ± 1.49	25.5 ± 2.73	n.s.
D-cell	3.8 ± 0.38	4.2 ± 0.56	n.s.
PP-cell	11.0 ± 1.53	15.6 ± 2.04	n.s.
	Number / μm^2 of islet		
Total	0.0132 ± 0.002	0.0159 ± 0.0020	n.s.
A-cell	0.0048 ± 0.0009	0.0060 ± 0.0010	n.s.
B-cell	0.0070 ± 0.0008	0.0068 ± 0.0011	n.s.
D-cell	0.0008 ± 0.0001	0.0009 ± 0.0001	n.s.
PP-cell	0.0012 ± 0.0001	0.0022 ± 0/0002	$p < 0.01$

At the ultrastructural level, most of the A-cells in the e perimental group of rats were similar to those in the controls. However, smaller granules were observed in their cytoplasm (Figures 1 and 2).

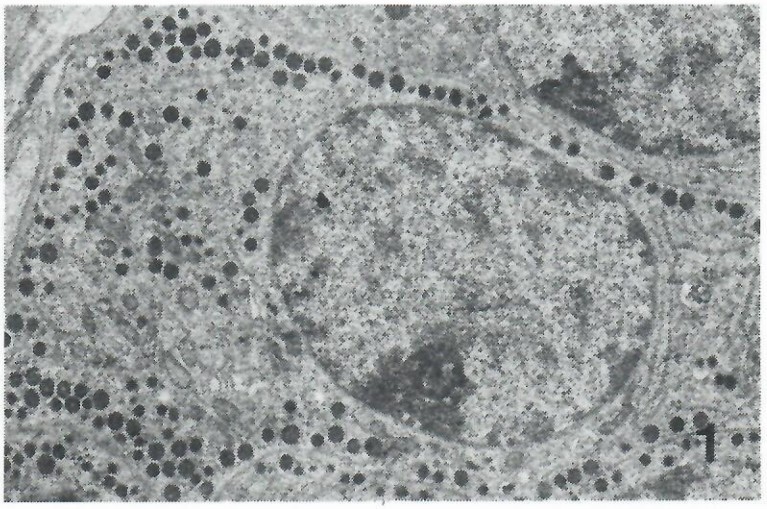

Figure 1. Glucagon-producing A-cell in the islet of Langerhans in the control rat. Uranyl acetate, lead citrate; 9000x.

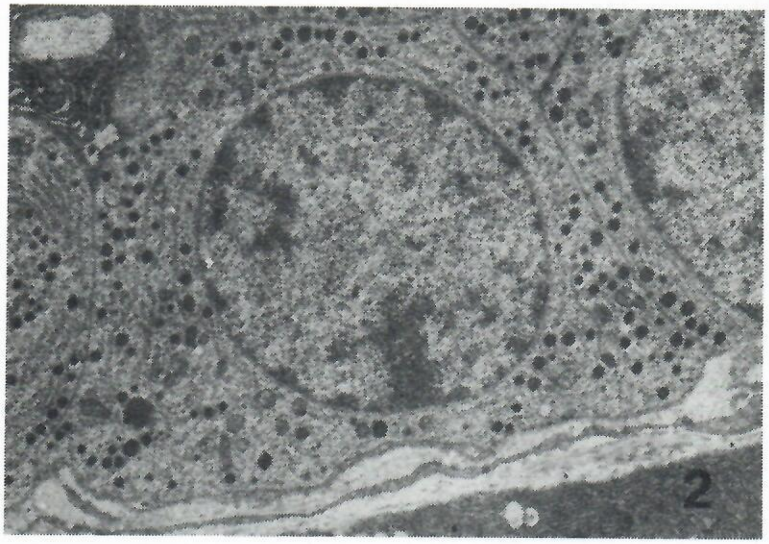

Figure 2. Glucagon producing A cell in the islet of Langerhans in the ethanol treated rat. Uranyl cetate, lead citrate; 9000x.

In addition, a small number of A-cells showed gross alteration (Figure 3).

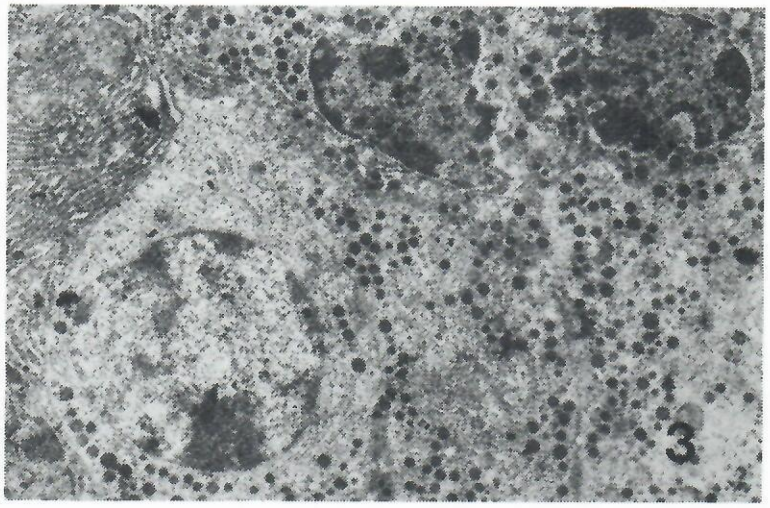

Figure 3. Altered glucagon producing A cell in ethanol treated rat. Uranyl acetate, lead citrate; 9600x.

The shape of their nuclei was irregular with deep invaginations. A heterochromatin condensation, close to the nuslear envelope, was observed indicating that apoptosis had taken place. Altered A-cells were in the vicinity of damaged B-cells.

Stereological investigation at the ultrastructural level is presented in Table 3.

Table 3. Ultrastructural morphometry of A-cells in the control (C) and experimental (E) rats: summary of quantitative analysis (mean ± SEM).

	GROUP C	GROUP E	Statistics
Cell			
Profile area (μm^2)	104.30 ± 4.75	79.40 ± 3.65	$p < 0.01$
Nucleus			
Profile area (μm^2)	31.10 ± 1.84	26.40 ± 1.05	$p < 0.05$
Volume density (μm^0)	0.370 ± 0.019	0.350 ± 0.027	n.s.
Cytoplasm			
Profile area (μm^2)	73.20 ± 3.83	52.90 ± 3.86	$p < 0.01$
Volume density (μm^0)	0.630 ± 0.019	0.650 ± 0.027	n.s.
Cytoplasmic granules			
Volume density (μm^0)	0.198 ± 0.016	0.240 ± 0.026	n.s.
No. per cell profile	129.4 ± 13.00	141.0 ± 16.14	n.s.
No. per μm^2 of cytoplasm	1.77 ± 0.163	2.66 ± 0.940	$p < 0.01$
Mature to immature granule ratio	2.24 ± 0.310	4.20 ± 0.940	n.s.
Mature granules			
No. per cell profile	89.3 ± 10.98	104.0 ± 16.06	n.s.
No. per μm^2 of cytoplasm	1.62 ± 0.143	2.81 ± 0.270	$p < 0.01$
Mean diameter (nm)	352 ± 7.50	323 ± 4.20	$p < 0.001$
Immature granules			
No. per cell profile	39.90 ± 4.66	37.00 ± 5.39	n.s.
No. per μm^2 of cytoplasm	0.77 ± 0.082	0.68 ± 0.081	n.s.
Mitpchondria			
Profile area (μm^2)	2.87 ± 0.346	3.20 ± 0.630	n.s.
Volume density (μm^0)	0.054 ± 0.0054	0.070 ± 0.0020	$p < 0.01$
No. per cell profile	15.0 ± 1.66	16.5 ± 2.99	n.s.
Golgy system			
Profile area (μm^2)	0.999 ± 0.329	0.560 ± 0.270	n.s.
Volume density (μm^0)	0.017 ± 0.006	0.009 ± 0.270	n.s.
GER			
Profile area (μm^2)	4.15 ± 0.43	4.08 ± 0.67	n.s.
Volume density (μm^0)	0.080 ± 0.009	0.100 ± 0.020	n.s.

The profile area of the whole A-cell, as well as of the cytoplasm was significantly reduced. Reduction of the profile area of the Golgi system and gER, was also found, while that of the mitochondria significantly increased.

The volume density of the nuclei and Golgy system was diminished while the cytoplasm, granules and mitochondria showed increased volume density.

The number of cytoplasmic granules was greater due to an increase in number of mature granules, both per cell profile and per unit of cytoplasmic compartment (μm^2), compared with controls.

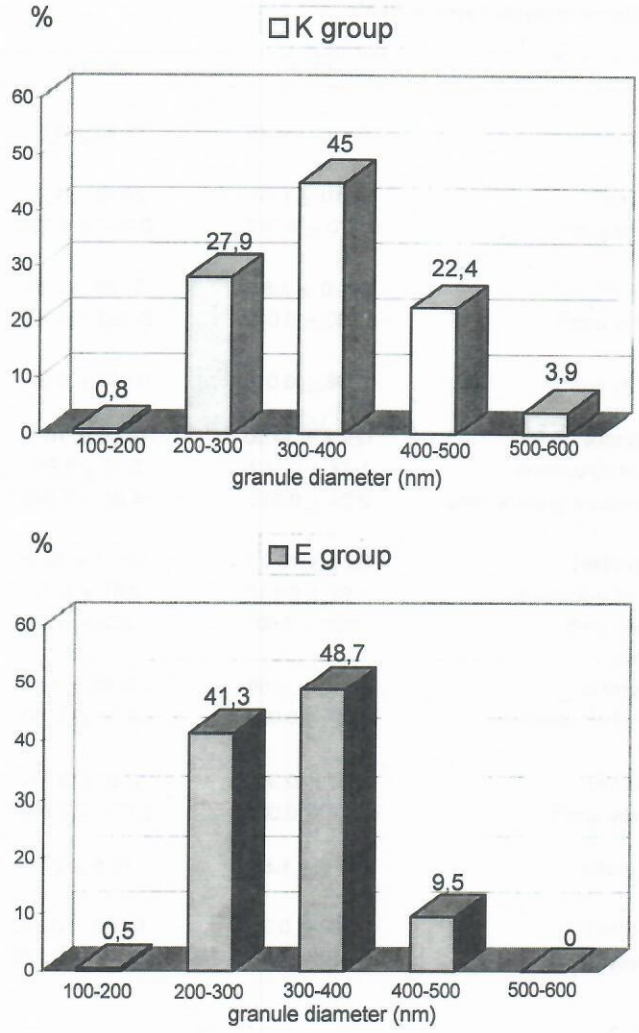

Figure 4. Frequency distribution of granule diametar in control (C) and experimental (E) rats.

The mean diameter of the granules was significantly reduced in the ethanoltreated rats. Although,the largest number of mature granules was found in the same diameter range in both groups of rats (300-400 nm), in the ethanol-treated group the number of granules belonging to smaller class intervals was greater (Figure 4).

DISCUSSION

This study represents an attempt to determine the effects of chronic ethanol intake on morpho-functional characteristics of pancreatic glucagon-producing A-cells, under strictly controlled nutritional conditions.

Previous studies revealed that poor composition of the diet could mask or over emphasize the actual effects of ethanol per se (Tsukamoto et al., 1988; Roger et al., 1987; Goslin et al., 1965; Rämö 1987)

Der et al. (1988) reported a diet with 26% daily energy intake from alcohol which provided adequate intake of other nutrients. The amount of alcohol consumed (expressed per kg of body weight per animal) was identical to the amount consumed with Lieber's 36% alcoholic liquid diets. Similar results were found by Gomez-Dumm et al. (1968), Donald et al. (1981) and Todorović et al.(1988). Therefore we concluded that the rats of our experimental group were chronically consuming high doses of ethanol.

The existence of entero-insular, entero-acinar and insulo-acinar axes,as well as mechanisms of functional disturbances of neuroendocrine pancreatic cells in chronic alcoholism are very complex and the morphological substratum of these disturbances is almost completely unexplored.

Consumption of large amounts of alcohol often produces chronic pancreatitis in human and experimental animals (Sarles, 1975; De Angelis et al., 1992). This is characterized with many changes in each of the three compartments of the exocrine pancreas (acini, ducts and neurovascular elements). It was found that exocrine pancreatic deficiency had a significant influence on the function of islet endocrine cells (Owyang, 1986; Klöppel et al., 1978) and vice versa. The demonstration of the insulo-acinar portal system (Bonner- eir and Orci, 1982; eawer and Sorenson, 1989) lead to the conclusion that islet hormones have regulatory roles in the function of the exocrine pancreas. Also, different features of exocrine cells elose to islets in relation to more distant cells ("halo" phenomenon) suggest the existence of insulo-acinar interactions.

Our earlier examination showed a singificant increase of volume, as well as absolute and relative weight of the pancreas in alcoholic rats (Koko et al., 1995a). At the same time, stereomorphometric analysis of the islets of Langerhans revealed significant reduction of their volume. These results indicated that chronic ingestion of ethanol during 4-months caused a decrease of islet volume, probably due to decreased number and size of islets in alcoholic rats. There are few studies about the morphological apperance of islets of Langerhans in chronic alcoholism. Buchelr et al. (1992) reported regrission of pancreatic islets in humans suffering from alcohol-induced pancreatitis. Dissociation between whole pancreatic blood flow and islet blood flow, reported by Jansson and Petersson (1988), was

responsible for "smaller damage" to the islets of Langerhans in comparison with tissue of the exocrine pancreas. Although, the frequency distribution of islet diameter was similar in both investigated groups of rats, a different distributionof total islet volume among islets of different diameter classes was found between control and experimental animals (Koko et al, 1995a).

Our earlier examination of the blood vessels of islets of Langerhans showed that alcohol caused a significant increase of both volume density and total length of the vessels (Koko et al, 1995b). This results could be explained by the direct effect of ethanol on small blood arterioles which were predominant in the islets of Langerhans (eawer and Sorenson, 1989). This is in accordance with the finding of Oates and Hakkinen (1988) who showed that ethanol caused vasodilation of small gastric arteriole vessels and vasoconstriction of venules. Jansson and Petersson (1988) also showed that lower ethanol concentration increased islet blood flow and total vascular resistance, probably due to stimulation of the central nervous system. Higher ethanol concentrations decreased blood flow in the whole pancreas and increased islet blood flow.

The total number of endocrine cells per islet and per μm^2 of islet, as well as the number of A-cells per islet and per μm^2 of islet was increased. Moreover, in ethanol-treated rats changes in the frequency distribution of single islet cell types were noted. Similar results were obtained by analysis of human endocrine pancreas with primary or secondary chronic pancreatitis (Klöppel et al., 1978). Insufficiency of the exorcine pancreas may influence the cell composition of islets by reduction of the local circulation and diffusion of glucose with a consequent reduction in the number of glucose-sensitive B-cells. A changed numerical ratio of A- and B-cells, in the sense of an increased frequency distribution of A-cells, which had lost contact with adjecent B-cells, was noticed in chronic pancreatitis (Owyang, 1986). On the other hand, A-cells together with B-cells form a bihormonal unit. The discovery of tight and gap junctions between islet cells in man (Orci et al., 1975a) and in the rat (Orci et al., 1975b) suggests the existence of internal islet interactions.

The results of our morphometric and stereological studies of pancreatic glucagon-producing A-cells indicated a significant decrease in mean diameter, surface area and volume density of those cells in ethanol-treated rats. Ultrastructural study showed decrease of the cytoplasmic profile area and increase of the total number of cytoplasmic granules (expressed per μm^2 of cytoplasm), with reduction of their mean diameter. Amindzanov (1986) reported that blood glucagon level decreased in chronic alcoholics, and Simanovski et al. (1989) showed that blood glucagon level increased in chronic ethanol-treated animals after stimulation with an acute intraperitoneal dose of ethanol. The described morphological changes of glucagon-producing A-cells could be a secondary alteration of decreased insulin secretion in respect to the known antagonistic effects of these two hormones in homeostasis of glucose metabolism. The increased volume density and profile area of mitochondria could be explained as a direct effect of ethanol and this is in accordance with the previous results of Tandler et al (1996).

In conclusion, this study offers a description of the optical and ultrastructural features of glucagon-producing A-cells in rat pancreas after 4-months of ethanol intake. All investigated parameters indicated that ethanol caused hypoactivity of A-cells probably as a consequence of losing local paracrine influences of the B-cells which were damaged in close vicinity to the A-cells.

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EFEKAT HRONIČNOG UNOSA ETANOLA NA GLUKAGON-PRODUKUJUĆE A-ĆELIJE U ENDOKRINOM PANKREASU PACOVA. MORFOMETRIJSKA I ULTRASTRUKTURNA ISPITIVANJA

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SADRŽAJ

Ova studija predstavlja optičku i ultrastrukturnu sliku glukagon-produkujućih A-ćelija u endokrinom pankreasu pacova, u eksperimentalnim uslovima hroničnog alkoholizma i strogo kontrolisane ishrane. Mužjaci pacova soja Wistar konzumirali su etanol, što im je obezbedilo 23% od ukupnih kalorija (grupa E). Nakon 4 meseca eksperimentalnog perioda, pankreas je izvađen i pripremljen za svetlosna i ultrastrukturna ispitivanja. Weibel-ova mnogo namenska testna mrežica (M_{42}) je korišćena za procenu morfometrijskih/stereoloških parametara Langerhansovih ostrvaca i glukagon produkujućih A-ćelija. Ukupan broj endokrinih ćelija po ostrvcu i po mm^2 ostrvca bio je uvećan u grupi životinja tretiranoj etanolom. Broj glukagon-produkujućih A-, somatostatin-produkujućih D-, i ćelija koje produkuju pankreasni polipeptid -PP, bio je uvećan, dok je broj insulin-produkujućih B-ćelija bio smanjen. Imunohistohemijski detektovane A-ćelije imale su prstenast raspored u obe grupe životinja. Na ultrastrukturnom nivou, većina A-ćelija u životinja eksperimentalne grupe nalikovala je A-ćelijama u kontroli. Mali broj A-ćelija bio je izmenjen, sa dubokim uvratima nukleusnog ovoja i kondenzovanim hromatinom. Stereološka istraživanja na ultrastrukturnom nivou pokazuju da je površina obrisa celih A-ćelija, citoplazme, Goldžijevog aparata i gER bila smanjena a mitohondrija uvećana. Zapreminska gustina jedara i Goldijevog aparata je bila smanjena, dok je zapreminska gustina citoplazmatičnih granula i mitohondrija uvećana. Broj citoplazmatičnih granula je uvećan u smislu uvećanja broja zrelih granula. Srednji dijametar granula je značajno smanjen u životinja tretiranih etanolom. Svi dobijeni rezultati ukazuju na hipoaktivnost A-ćelija.