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THE INFLUENCE OF AGE FACTOR ON CHANGES IN MORPHOMETRIC INDICES OF RAT PANCREAS IN MODELING OF INSULIN RESISTANCE AND ITS CORRECTION WITH N-STEAROYLETHANOLAMINE

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Histological, histochemical and morphometric changes of the pancreas in young (4 months) and old (16 months) rats in the modeling of insulin resistance and its correction with N-Stearoylethanolamine were studied. In the experiment, insulin resistance was reproduced by a long-term, 6 months, keeping rats on a fat diet (fat content in the diet – 58 %), and its development was monitored by glycemic control. To correct insulin resistance, an aqueous suspension of NSE was used, which was administered to rats per os at a dose of 50 mg/kg for 2 weeks. Two-factors analysis of variance proved the leading role of age in changing the basic morphometric parameters that characterize the development of destructive changes in the pancreatic islets (number of apoptotic cells), inflammatory changes in pancreatic tissue (number of mononuclear cells in the stroma), and secretory dysfunction of ß-insulocytes (specific number of insulocytes with aldehyde-fuchsin -positive secretory granules). The contribution of the age factor to changes in these indices is 78.3 %, 56.1 % and 41.5 %, respectively. It was found that the number of apoptotic TUNEL-positive cells in the pancreatic islets is an informative integral index for the assessment of destructive changes in insulocytes, which should be recommended when screening for pharmacological correction of insulin resistance and diabetes.

Key words: pancreatic islets, rats, insulin resistance, apoptosis, pharmacological correction.

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ВПЛИВ ФАКТОРА ВІКУ НА ЗМІНИ МОРФОМЕТРИЧНИХ ПОКАЗНИКІВ ПІДШЛУНКОВОЇ ЗАЛОЗИ ЩУРА ПРИ МОДЕЛЮВАННІ ІНСУЛІНОРЕЗИСТЕНТНОСТІ ТА ЇЇ КОРЕКЦІЇ N-СТЕАРОЇЛЕТАНОЛАМІНОМ

Досліджено гістологічні, гістохімічні та морфометричні зміни підшлункової залози у молодих (4 місяці) та старих (16 місяців) щурів при моделюванні інсулінорезистентності та її корекції N-стеароілетаноламіном. В експерименті відтворювали інсулінорезистентність при тривалому 6-місячному утриманні щурів на жирній дієті (вміст жиру в раціоні – 58 %), а її розвиток контролювали за допомогою глікемічного контролю. Для корекції інсулінорезистентності використовували водну суспензію NSE, яку вводили щурам per os у дозі 50 мг/кг протягом 2 тижнів. Двофакторний дисперсійний аналіз довів провідну роль віку у зміні основних морфометричних параметрів, що характеризують розвиток: деструктивних змін в острівцях підшлункової залози (кількість апоптотичних клітин), запальних змін у тканині підшлункової залози (кількість мононуклеарів у стромі), а також секреторна дисфункція ß-інсулоцитів (специфічна кількість інсулоцитів з альдегід-фуцин-позитивними секреторними гранулами). Внесок вікового фактора у зміни цих індексів становить відповідно 78,3 %, 56,1 % та 41,5 %. Встановлено, що кількість апоптотичних TUNEL-позитивних клітин в острівцях підшлункової залози є інформативним інтегральним показником для оцінки деструктивних змін інсулоцитів, який слід рекомендувати при скринінгу для фармакологічної корекції інсулінорезистентності та цукрового діабету.

Ключові слова: острівці підшлункової залози, щури, інсулінорезистентність, апоптоз, фармакологічна корекція.

The study is a fragment of the research project "Study of age-related structural and ultrastructural changes of target cells in the modeling of insulin resistance and type 2 diabetes mellitus and their treatment", state registration No. 0120U100079.

Due to the progressive aging of the population, diabetes mellitus type 2 (DMT2) is defined as a multifactorial age-dependent disease, accompanied by the development of severe complications, attracting the attention of researchers in endocrinology, experimental pathology, biochemistry, molecular biology, biotechnology, pharmacology and others.

It is known that a significant role in the pathogenesis of insulin resistance (IR) and DMT2 is played by a progressive decrease in the pancreatic islets (PI) of the pancreas (PG), the total number of β-insulocytes and their volumetric density due to dystrophy and apoptosis of cells against the background of low-grade chronic systemic (low-grade) inflammation of PG and adipose tissue [1, 10, 13]. With aging in the pathogenesis of IR and DMT2, an important role plays dysfunction of β-insulocytes and reduces their ability to recover, due to inhibition of activator expression and increased expression of cell cycle regulators' inhibitors [5, 6]. Therefore, in the development of modern treatments for IR and DMT2, preference is given to pharmacological agents aimed not only at strict control of glycemia but also at the protection of β-insulocytes from damage and recovery of their population in PI [12].

Results of the studies performed on an experimental model of IR showed that N-Stearoylethanolamine (NSE) can have a membrane-stabilizing, anti-inflammatory, antioxidant effect, and helps restore the lipid composition of pancreatic tissue [2, 8]. This determines the prospects for the use of NSE for pharmacological correction of IR and DMT2.

The purpose of the study was to evaluate the influence of age factors on morpho-functional changes of the pancreas in young and old rats in modeling insulin resistance and its correction with NSE based on a two-factor analysis of variance.

Materials and methods. The studies were performed on Sprague-Dawley male rats of two age groups – young (4 months at the beginning of the experiment and 10 months after its completion) and old (16 months and 22 months, respectively), who were kept in the standard vivarium conditions. The study was performed in compliance with the basic principles of the "International Guiding Principles for Biomedical Research Involving Animals" (CIOMS-ICLAS, 2012). The IR model was reproduced by long-term (6 months) keeping rats on a fat diet (fat content in the daily diet of 58 %), as described previously [7]. Six months after the start of the experiment, we performed the oral glucose tolerance test. The rats of each age group (young and old) with impaired glucose tolerance (the blood glucose level (BGL) within 150 min after the oral glucose administration was higher than 5 mmol/L)) were selected and divided randomly into two groups: IR (young – n=16; old – n=12) and IR + NSE (young – n=15; old – n=12). The control group was rats kept on the standard diet (intact control "Contr." – 4 young and 3 old rats) and "NSE" (3 young and 3 old rats). NSE and IR+NSE rats received an aqueous suspension of NSE at the dose of 50 mg/kg per day per os for two weeks. Animals were sacrificed by decapitation after previous anesthesia with Nembutal (50 mg/kg).

For morphological studies, PG was fixed in Bouin's solution, dehydrated in ethanol, clarified in xylene, impregnated and poured into paraffin (Paraplast®, type 6). Microtomed sections (5 μm) were stained with hematoxylin and eosin, as well as Gomori's aldehyde-fuchsin (A-F). Apoptosis of cells was detected in paraffin sections using the TUNEL method, using the commercial ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon, USA).

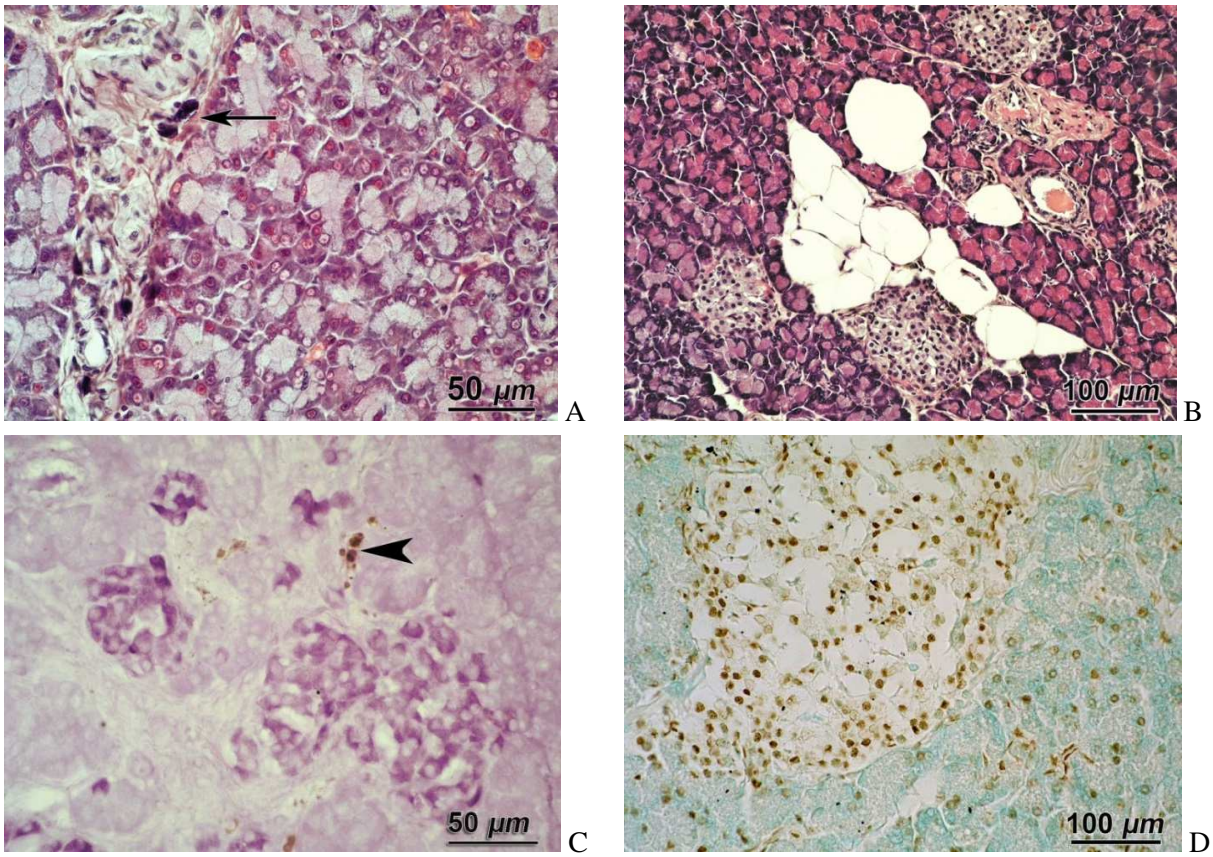

Fig. 1. Morphological changes in PG of old (a, d) and young rats (b, c) of the control group (a) and in the simulation of IR (b, c, d). Designation: mast cells with basophilic granules (←); l□□fu□□□gr□ul□□(◄). Hematoxylin and eosin (a, b); Gomori's aldehyde-fuchsin (c); TUNEL method (d).

Histological examinations were performed using an Olympus BX51 light microscope with Olympus DP-Soft 3.2 imaging system (Japan). For morphometric studies, the ImageJ software product 1.52a (National Institutes of Health, USA) was used, which was applied to determine: 1) the specific

volume of the connective tissue stroma PG (%); 2) the number of mononuclear cells (MNC) per 1 mm^2 of PG stroma ; 3) the number of PI per 1 mm^2 of the cross-sectional area of the section; 4) the mean PI section area per 1 mm^2 of PG cross-sectional area; 5) the specific proportion of insulocytes with A-F-positive secretory granules in the cytoplasm (%); 6) the number of TUNEL-positive cells per 1 mm ^{2} of the PI area.

Data processing. Statistical processing of the results was performed by non-parametric statistical methods using the program STATISTICA 13 (TIBCO Software Inc., SN AXA905I924220FAACD-N). The results represented the minimum and maximum values of indices, their median (Me) and inter-quarter intervals (25 %; 75 %). The ANOVA analysis package was used to assess changes. Statistical hypotheses were tested by Fisher, Mann-Whitney and Kolmogorov-Smirnov tests at α=0.05. Estimation of the age factor influence (AF in gradations "young" and "old") on changes in PG morphometric parameters of rats with IR and correction with NSE (factor "model" in grades "control", "IR", "IR+NSE") was performed using two-factors analysis of two-factors dispersion analysis (TFDA), for which linear models were built and analyzed, where one factor was fixed and the other was random, or both factors were random.

Results of the study and their discussion. The results of BGL studies were revealed in young and old rats of the IR group, the development of hyperglycemia was revealed, which was characterized by Me values (25 %; 75 %) of BGL: in young rats – 6.10 mmol/L (5.60; 6.70) (in control – 4.6 mmol/L (4.25; 5.1), p=0.0001), and in older animals – 5.80 mmol/L (5.50; 6.40) (control 4.9 mmol/L (4.3; 5.1), p=0.0064), which determined significant age differences (p=0.005) in the development of hyperglycemia in rats with IR.

The results of histological examinations of the old rats' PG of the IR group revealed moderate edema of the organ's connective tissue stroma and its MNCs infiltration, among which were lymphocytes, macrophages and mast cells with signs of their partial degranulation (Fig. 1) (a, b, c, d).

Such histological changes in the pancreas in old rats were accompanied by a significant (p=0.006) compared to young rats increase in the number of MNCs in the connective tissue stroma of the organ, the specific volume of which did not change significantly (fig. 2 a, b).

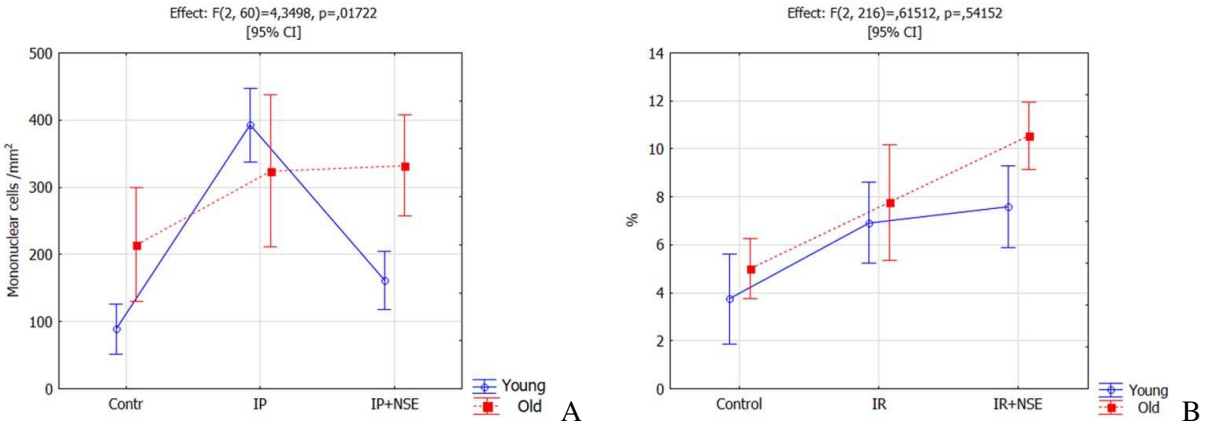

Fig. 2. Influence of AF on changes in morphometric parameters of rat PG: a – the number of MNCs in 1 mm ^{2} of the organ's stroma, b – specific volume of connective tissue stroma (%).

Microscopically, changes in PI were observed with aging, which acquired an oval and irregular shape were surrounded by thickened layers of connective tissue, in which accumulations of lipofuscin granules were detected. During the morphometry of PG in the PI of old rats, there was a decrease in the specific proportion of insulocytes with A-F-positive secretory granules (p=0.001; fig. 3 a) and an increase in the number of TUNEL-positive cells (p=0.001; fig. 3 b), indicating significant impairment of the secretory function of ß-insulocytes and development of destructive changes and apoptosis of cells.

In the simulation of IR in PG of both old and young rats edema of the connective tissue stroma and its infiltration of MNCs were observed. However, the pancreas of young and old rats showed dilatation of the lumens and plethora of capillaries, dilation of the excretory ducts' lumen, as well as dystrophic changes in cells of exocrine and endocrine tissue of the pancreas. Focuses of periductal and intralobular sclerosis, and intra- and perilobular lipomatosis were observed in the pancreas of young rats. In the layers of connective and adipose tissue observed immured small PI formed by insulocytes with A-F-positive secretory granules in the cytoplasm and clusters of polymorphic granules of lipofuscin.

In the analysis of morphometric studies in PG of young rats with IR, in contrast to the old, a significant, compared to the control the increase was found in the specific volume of connective tissue stroma of the organ (p=0.017) and an increase in MNC (p<0.001) which indicates the development of inflammation in PG.

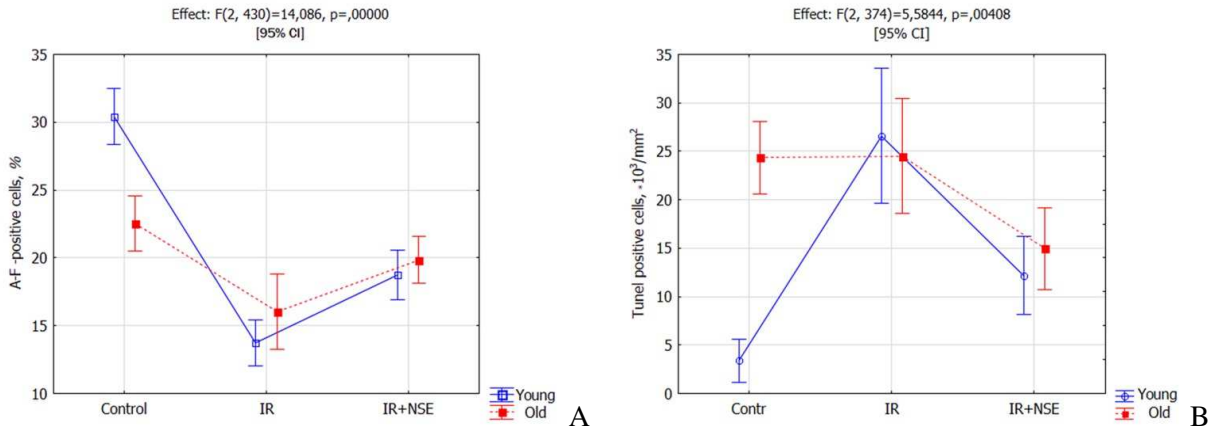

Fig. 3. Influence of AF on changes in morphometric parameters of rat PG, a – specific proportion of PI insulocytes with A-F-positive secretory granules (%); b – the number of TUNEL-positive cells in 1 mm^2 PI.

It should be noted that in both age groups of rats with IR, morphometric changes in PI were significant (p<0.001), compared to the control, a decrease in the specific proportion of insulocytes with A-F-positive secretory granules, indicating the impaired secretory function of ß-insulocytes, in both young and old rats. At the same time, in young animals, in contrast to the old ones, a significant increase in the number of TUNEL-positive cells (p=0.002) was observed in PG, which indicates a high intensity of cell destruction processes and apoptosis.

The use of NSE in young rats with IR led to a significant increase in the specific proportion of insulocytes with A-F-positive secretory granules (p<0.001) and a decrease in the number of TUNEL-positive cells (p=0.002), which was associated with the restoration of secretory function of ß-insulocytes and reduction of destructive changes and apoptosis of cells. At the same time, in the connective tissue stroma of young rats' pancreas due to NSE, there was a significant decrease in the number of MNC (p<0.001), which may indicate suppression in the pancreas of inflammatory processes observed in rats with IR.

It should be noted that in older rats with IR, in contrast to young, the effect of NSE was manifested in a wider range of PG changes. In the body, there was a significant increase in the number of PI (p=0.041; fig. 4 a) and their specific area (p<0.001; fig. 4 b).

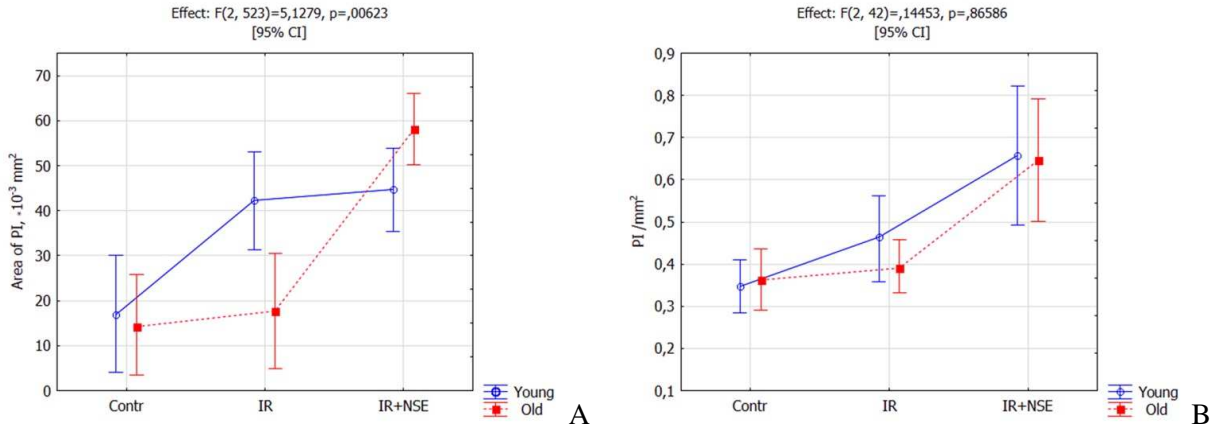

Fig. 4. Influence of AF on changes of morphometric indices of PG in rats: a – specific area of PI in 1 mm^2 of PG; b – the area of PI in 1 mm^2 of PG.

The proportion of insulocytes with A-F-positive secretory granules (p=0.015) increased and the number of TUNEL-positive cells (p=0.009) decreased, which was associated with the development of structural changes in the PI aimed at compensating for impaired IR functions of ß -insulocytes.

Based on our data, the TFDA of linear models showed that AF is gaining leading importance in the development of morphological changes in PG in IR modeling and its correction with NSE. This is indicated by changes in morphometric parameters of PG, which characterize the development of inflammatory processes in PG (number of MNCs in 1 mm^2 of stroma; p=0.017), destructive changes and compensatory-adaptive changes in the organ (mean PI area in 1 mm^2 of PG; p=0.006; the specific proportion of PI insulocytes with A-F-positive secretory granules, p<0.0001, the number of TUNEL-positive cells in 1 mm^2 of PI, p=0.004).

Among all the factors taken into account in the experiment, AF has the most significant effect on changes in the number of MNCs in PG stroma and the number of TUNEL-positive cells in the PI: the share

of AF contribution is 56.1 % (F=4.621; p=0.035) and 78.3 % (F=7.577; p=0.006), respectively Table 1). Instead, in combination with other factors taken into account in the experiment the share of AF's contribution to changes in these indices is 73.3 % and 85.5 %, respectively.

Table 1

Statistical indices of linear TFDA models

Factors	*SS*	*df*	*MS*	*F*	*p*	*P, %*
The specific volume of the connective tissue stroma of PG, %						
Free member	8482.371	1	8482.371	202.902	0.00000	100.0
“Model”	572.431	2	286.215	6.846	0.00130	91.1
“Age”	127.774	1	127.774	3.056	0.08183	4.1
“Model+Age”	51.431	2	25.715	0.615	0.00566	15.2
Error	9029.916	216	41.805	–	–	–
The number of MNCs in 1 mm2 of PG stroma area/mm2						
Free member	3505290	1	3505290	203.165	0.00000	100.0
“Model”	321003	2	160501	9.3026	0.00030	97.2
“Age”	79743	1	79743	4.6219	0.03560	56.1
“Model+Age”	150097	2	75048	4.3498	0.01721	73.3
Error	1035201	60	17253	–	–	–
The number of PI in 1 mm2 of PG area,/mm2						
Free member	10.889	1	10.889	184.599	0.00000	100.0
“Model”	0.760	2	0.380	6.442	0.00362	88.2
“Age”	0.005	1	0.005	0.101	0.75215	6.1
“Model+Age”	0.017	2	0.008	0.144	0.86585	7.1
Error	2.477	42	0.058	–	–	–
The mean PI area in 1 mm2 of PG area, mm2						
Free member	366814	1	366813.6	126.020	0.00000	100.0
“Model”	100806	2	50403.1	17.316	0.00000	99.8
“Age”	1400	1	1399.8	0.480	0.48832	10.6
“Model+Age”	29852	2	14926.0	5.127	0.01923	82.3
Error	1522316	523	2910.7	–	–	–
Specific proportion of insulocytes with A-F positive secretory granules, %						
Free member	158672.6	1	158672.6	2235.027	0.00000	100.0
“Model”	8090.4	2	4045.2	56.980	0.00000	100.0
“Age”	217.3	1	217.3	3.061	0.081	41.5
“Model+Age”	2000.1	2	1000.0	14.086	0.00000	99.8
Error	30527.2	430	71.0	–	–	–
The number of TUNEL-positive cells in 1 mm2 PI area,/mm2						
Free member	91766.3	1	91766.32	181.3660	0.00000	100.0
“Model”	11033.2	2	5516.59	10.9029	0.00002	99.0
“Age”	3833.9	1	3833.9	7.5773	0.00619	78.3
“Model+Age”	5651.1	2	2825.5	5.5844	0.02899	85.5
Error	189233.9	374	505.97	–	–	–

Notes: 1) SS – the total amount of squares; 2) df – degree of freedom; 3) MS – the mean square of the effect; 4) F – intragroup variance; 5) p – statistical significance at $\alpha = 0.05$; 6) P – power of factors influence at $\alpha=0.05$.

Analysis of the trend of the morphometric parameters changes of PG in rats of different age groups in modeling IR and its correction with NSE revealed an inverse relationship with changes in the number of TUNEL-positive cells in PI and the proportion of insulocytes with AF-positive secretory granules in the cytoplasm.

At the same time, according to the TFDA, AF itself in IR and its correction with NSE did not significantly affect the changes in the secretory function of PI insulocytes (F=3.061; p=0.081). Instead, the combination of AF with other factors considered in the experiment increases the share of its influence on changes in this index to 99.8 % (F=14.086; p<0.0001), which proves the leading role of AF in the mechanisms of IR and DMT2.

Thus, the results of PG morpho-functional studies in young and old rats showed that AF is gaining leadership in the development of experimental IR. Structural changes that occur in PG during aging characterize the effect of low-grade inflammation in the body (edema of the stroma and its infiltration with MNCs), destructive changes and apoptosis of insulocytes (increase in TUNEL-positive cells in PG), as well as violation of the secretory function of ß-insulocytes (reduction in the specific proportion of insulocytes with A-F-positive secretory granules in PI).

The obtained results correspond to the previously obtained data, which convincingly testify to the crucial role of low-grade inflammation of the pancreas and adipose tissue, as well as the destruction and apoptosis of ß-insulocytes in the pathogenesis of IR and DMT2[1, 3, 11].

It should also be noted that the results proved the leading role of AF in the formation of PG responses to the action of NSE in the correction of IR, which should be considered in preclinical studies and screening of pharmacological correction in IR and DMT2.

Conclusions

1. The use of NSE in PG of young and old rats in the IR+NSE group reduces the morphological manifestations of inflammatory and dystrophic processes, against which compensatory changes develop in the form of moderate hypertrophy and increase in the number of insulocytes with A-F-positive secretory granules. This indicates the restoration of impaired IR secretory function of β-insulocytes, which is more pronounced in young rats.

2. Studies of the AF effect on morpho-functional changes in the pancreas of rats with IR and its correction with NSE showed that the PI insulocytes in old rats undergo the most significant changes. Destructive changes of these cells against the background of the pancreas chronic inflammation cause more pronounced disorders of the secretory function of β-insulocytes than in young rats.

3. TFDA linear models revealed a significant effect of AF ($p<0.05$) on changes in PG morphometric parameters, which characterize the development of destructive changes in the pancreas (number of TUNEL-positive cells), inflammatory processes in PG (number of MNCs in connective tissue stroma), as well as disorders of the secretory function of ß-insulocytes (relative number of insulocytes with A-F-positive secretory granules). The value of AF contribution to changes in these indices is 78.3 %, 56.1 % and 41.5 %, respectively.

4. To assess the age features of morpho-functional changes that develop in the endocrine tissue of the pancreas in modeling of IR and its correction with NSE index of the number of apoptotic TUNEL-positive cells (78.3%) is more important, which changes permit an integrated assessment of destructive changes in insulocytes in screening means of pharmacological correction of IR and DMT2.

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