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Histochemical and Immunohistochemical Investigation of Endocrine Tissue of Pancreas after Damage Caused by B-cytotoxic Chemicals and its Prevention by L-Hystidine

Authors investigated a possibility of prevention developing of experimental diabetes in animals caused by group diabetogenic Zn-binding substances by preliminary injection of amino acid L-Hystidine contains the sulfhydryl SH-groups in structure of molecule. It is established that intravenous administration of solution of 900-1000 mg/kg L-Hystidine to rabbits leads to the almost complete binding of ions of Zinc in B-cells of pancreas for 24 hours and prevent of formation in B-cells of a toxic complex Zinc-dithizon that is followed by prevention of developing of diabetes at 11 animals from 12. At the same time by comparative using of various histochemical research technics staining of tissue of pancreas it was shown that most sensitive for identification of insulin and Zinc in B-cells are fluorescent methods. The most qualitative results of assessment of features of gistotopography of insulin and Zinc in B-cells obtained by using of Aldehyde-fuchshine method.

Keywords: B-cells, L-Hystidin, insulin, zinc, experimental diabetes, aldehyde-fuchshine method, dithizon, pancreas, histochemical methods.

Background. Okamoto K. showed that Diphenylthiocarbazon (Dithizon) possess ability for selective destruction of B-cells that accompanied by developing of 1st type diabetes [1]. More later it was showed that injection of alloxan, of derivatives of 8-oxyquinolin and of Streptosotozin result selective destruction and death of B-cells too. Among methods for investigation result of action of these substances on islets histochemical and immunocytochemical methods have a few advantages as: 1) detail analysis of state of histostructure of islets; 2) analysis of insulin and Zn⁺²-ions content and disposition of hormone in cytoplasm of B-cells; 3) reveal the early histological and histochemical changes in islets. Pancreatic B-cells contains a large amount of Zn⁺²-ions [2–4]. In B-cells Zn⁺²-ions take part in processes of biosynthesis of insulin as of storage by forming of zinc-insulin complex [5, 6]. Zn⁺²-ions in B-cells formed with insulin a deposited form of hormone as Zn⁺²-insulin complex [5]. Proinsulin forms a zinc containing hexamer after synthesis. Zinc ions enhance proinsulin solubility and render insulin insoluble. Zinc ions also appear to play an important role in the microcrystalline character of the precipitated insulin granules [1]. Pancreas of rat, rabbit, dog, cat, some fish, human, birds, mice, hamster, porcine, hoerst, contains a large amount of Zn⁺²-ions [1]. By using

of electron histochemistry method it was showed that Zn^{+2} -ions concentrated in B-granules only contains deposited form of insulin [7]. Destruction of B-cells caused by Dithizon which formed in B-cells toxic complexes with Zn^{+2} -ions, started by destruction of B-granules [8, 9].

Aim of work: 1) to investigate influence of SH radical contains aminoacid L-Hystidine on activity of Diphenylthiocarbazon (Dithizon)[DZ], a diabetogenic zincbinding reagent; 3) to compare results of staining of islets for insulin and zinc in B-cells by using of various histochemical and immunocytochemical methods

Methods. Animals. 26 Rabbits 2240–2760g., 22 Rats 158–175g. 1. Experiences with Dithizon. 2. Experiences with L-Hystidine: injection of L-Hystidine, 900 mg/kg + injection of Dithizon, 48,5–50,2 mg/kg

Dithizon [DZ] as derivatives of 8-oxyquinolin possess a high chemical affinity for Zn^{+2} -ions and in vitro formed color complexes as Zn^{+2} -chelator [1, 2]. 8TSQ formed fluorescent green complexes with Zn^{+2} -ions visible using fluorescent microscopy and Dithizone formed red DZ- Zn^{+2} -ions complex visible using dark microscopy. Maximum of absorbance of Zn^{+2} -DZ complex on spectrum of absorbance correspond for 530 nm [3]. Diabetogenic properties of DZ as of other diabetogenic zunchbindng substances were investigated previously and determined by ability to form complex salt with Zn^{+2} -ions in cytoplasm of B-cells that result necrosis and death of cells within short time [8–10].

Isolation of pancreatic islets by Collagenase

Animals. Pancreas of 14 rats LEWIS 4–5 days old. Isolation procedures: dissected pancreas tissue were treated 3 times 3 min each by 2% solution of Collagenase (Boehringer Mannheim, Germany; FLUKA, Switzerland); rinse 3 times in cold Hanks solution and centrifugation; cultivation 12h at +37^o Celsius in medium RPMI 1640 (SERVA, Germany) with bovine serum+5.5 mM of Glucose, pH 7.32-7.41. Fixation in Bouin 15 min-1 h and filling in paraffin. Sections 4 mcm were used. Dithizon solution 0,4 ml was added in 10 ml of nutria media 199 contains islets for 20 min that correspond to concentration approximately about 40–45 mg/kg in experiences on animals; changing of media 199; pre-cultivation 5h.

Preparing of solutions

Preparing of Dithizon solution: 400 mg of Dithizon (Avocado chemical company, USA)+30 ml of bidistillate+0,2 ml of 25% ammonium solution; mixing 10 min on water bath at +70^oC, filtration. Frozen sections of Rabbit's pancreas 4 mcm were investigated 10 min after injection using of dark-condensor microscopy. Intensity of staining was measured by photometer. 2nd part of pancreas tissue was fixed in Ethanol 70% contains dissolved H₂S; staining of paraffin sections of tissue by 0,4% acetone solution of 8PTSQ [9] and investigation using of fluorescent microscopy. Pancreas tissue was fixed in Bouin 24h.

Staining technologies. Following methods were used for staining 4–5 mcm paraffin sections of pancreas.

Aldehyde-fuchshine method by Gomori G. Violet granules in cytoplasm of B-cells correspond to deposited form of insulin [10–11]. Intensity of color of cytoplasm of B-cells directly correspond to insulin content in cytoplasm [12–13]. Insulin content was calculated as parameter $K=AB1/AB2$ where: AB1-density of staining of intact B-cells; AB1-density of staining of B-cells past action of diabetogenic chemicals (calculated as 1,00).

Diethylpseudoisocyanine fluorescent method. Schiebler T. and Schiessler S. showed that A chair of oxidized insulin reacted with Diethylpseudoisocyanine chloride with formation of red fluorescent complex which fluoresces in UV light. We have used modernized by Coalson R.E. method [14–16].

Description of staining procedures. Preparing of staining solution: 0,04% water solution of Diethylpseudoisocyanine (SERVA, Germany). Staining procedures: 1) deparaffinization of sections in xylol; 2) alcohol 90^o,80^o,70^o 1 min in each; 3)washing in cold water; 4) oxidation 0,5–2 min; oxidation solution: 5 ml of 5% H₂SO₄+5 ml 2,5% solution of KMnO₄+30 ml bidistilled water at +28^o Celsius; 5)washing in cold water; 6) 5% solution of oxalic acid -5 sec; 6)washing in 2 portions of cold water; 7) 0,4% cold solution of Diethylpseudoisocyanine — 20 min in refrigerator at +4^oCelsius; 8) washing in cold water 5 min; 9) store in refrigerator 1,5–3h.

Insulin content was calculated using of relative units (r.u.) as parameter $K=IF1/IF2$ where: IF1-intensity of fluorescence of intact B-cells (B-cells/exocrine tissue); IF2-intensity of fluorescence of B-cells after action of diabetogenic chemicals (B-cells/exocrine tissue). Histofluorimetric complex was used [17].

Victoria Blue 4R method staining of insulin Diphenylnaphthylmetane (V4R), colour index 42563; MERCK, Germany; FERAК, West Berlin). It was showed [18] that V4R in aqueous solution interacted with oxidized A-chair of insulin that is accompanied by painting of cytoplasm of B-cells on a blue color proportionally to the amount of insulin [17]. V4R paints some peptides hormones but B-cells produce insulin only. This method is used not often, that is why we offer description of staining procedures. Staining procedures: 1) deparaffinization of sections; 2) washing in cold water a few min; 3) oxidation 3–5 min (oxidation solution: 0,3% $KMnO_4$ 50 ml+0,3% H_2SO_4 50 ml; wash sections; 4) place sections in 2–5 % water solution of sodium bisulphate — 1 min; wash sections; 5) 70° alcohol-1 min; 6) stain in staining solution (96° alcohol 100 ml+Victoria Blue 4R - 1g) 15 min — 2h; wash sections; 7) staining on 0,5% water solution of Phloxine 30–120 sec.; wash sections; 8) 5% water solution of phosphor wolframic acid 1–2 min; wash section in water; 9) stain in 0,5% water solution of Light Green 1–2 min; 10) dehydration in 96% alcohol. Method was adopted for using of sections of tissue culture of islets. Insulin content was calculated as parameter $K=AB1/AB2$ (17) where: AB1 — density of staining of B-cells, AB2 — density of staining of exocrine tissue.

Staining by Dithizon. Preparing of Dithizon solution: 30 mg of Dithizon, (MERCK, Germany) +10 ml. bidistillate+0.2 ml 25% NH_4OH 10 min. mixing on temperature +70° at Celsius. Solution was injected intravenously to Rabbits and to Mice 46–48,6 mg/kg.

Frozen sections 4 mcm were investigated 5–10 min past injection by dark microscopy. Density of staining was measured using photometer. Insulin content was calculated as parameter $K=AB1/AB2$ where: AB1-density of staining of intact B-cells; AB1-density of staining of exocrine tissue.

Immunohistochemical method staining of insulin. Standart kits for insulin (DAKO, Demark) were used for staining sections of pancreas tissue.

Staining by 8PTSQ. Zn^{+2} -8PTSQ complex radiates intensive green fluorescence, and complex Cd-8PTSQ as yellow fluorescence under UV-light 360–370 nm length of wave that was confirmed by spectral analysis [19–21]. Cytoplasm of B-cells not contains Cadmium. Past long time prolonging testing in Institute of High Pure Chemicals (Moscow) 8PTSQ was proposed as fluorescent reagent for identification of very small amounts of Zn^{+2} in solutions and tissues. Later by Lasaris Y.A. and coll. [20] 8PTSQ was tested for revealing Zn^{+2} -ions. 8PTSQ is high specific reagent for staining of Zn^{+2} -ions in pancreatic B-cells. Frozen sections of rat's Pancreas 4 mcm were investigated on fluorescent microscope. Staining procedures: 1) staining by 0,4% acetone solution of 8PTSQ; 3–4 drop of solution placed on section; wash section by 3 portions of bidistillate. Intensity of fluorescence was measured [17]. Insulin content was calculated as parameter $K=AB1/AB2$ where: IF2-intensity of fluorescence of intact B-cells; IF1-intensity of fluorescence of B-cells past action of diabetogenic substances.

RESULTS

Isolated pancreatic islets. Intact islets

Aldehyde-fuchsine staining: histostructure and insulin content in B-cells (violet color) without changes, (Fig.1.1; Table 1); $K=1,80\pm 0,06$ (AB1/AB2: AB1-B-cells of isolated islets; AB2 — exocrine tissue of intact pancreas).

Victoria 4R staining: histostructure and insulin content in B-cells without changes (Table 1); $K = 1,62 \pm 0,05$.

Immunohistochemistry: histostructure and insulin content in B-cells without changes (Fig. 1.4; Table 1); $K = 1,74 \pm 0,04$.

Diethylpseudoisocyanine staining: histostructure and insulin content in B-cells without changes (Table 1); $K = 1,72 \pm 0,04$.

Fluorescent staining of Zn^{+2} -ions: a large amount of Zn-ions in B-cells: intensive green fluorescence of B-cells; $K=1,75\pm 0,03$.

Isolated pancreatic islets after action of Dithizon:

Aldehyde-fuchsine staining. Necrosis, destruction and death of B-cells; marked decreasing of insulin content in majority of B-cells (Fig. 1.2; Table 1); $K = 1,14 \pm 0,04$.

Victoria 4R staining. Destruction of islets, destruction and death of B-cells; decreasing of insulin content in majority of B-cells (Table 1); $K = 1,06 \pm 0,05$.

Immunohistochemistry. Deformation of islets; destruction and death of B-cells; decreasing of insulin content in majority of B-cells (Fig.1.5; Table 1); $K = 1,18 \pm 0,04$.

Diethylpseudoisocyanine staining. Destruction of islets; marked decreasing of insulin content in B-cells (Table 1); $K = 1,07 \pm 0,06$.

Fluorescent staining of Zn^{+2} -ions. Almost complete disappearing of Zn^{+2} -ions from B-cells (Table 1); $K = 1,02 \pm 0,01$.

Isolated pancreatic islets after action of L-Hystidine+Dithizon:

Aldehyde-fuchsine staining: necrobiosis of single B-cells; insulin content without changes in 80–85% of B-cells (Fig.1.3; Table 2); $K = 1,65 \pm 0,04$.

Victoria 4R staining: histostructure and insulin content without changes (Table 1); $K = 1,61 \pm 0,05$.

Immunohistochemistry: insulin content in B-cells without changes (Fig. 1.6; Table 1); $K = 1,68 \pm 0,06$.

Diethylpseudoisocyanine staining: insulin content in B-cells without changes (Table 1); $K = 1,72 \pm 0,05$.

Fluorescent staining of Zn^{+2} -ions: negative reaction for Zn^{+2} -ions in B-cells as result of binding with L-Hystidine for Zn^{+2} -ions in B-cells as result of binding with L-Hystidine (Table 1); $K = 1,12 \pm 0,05$.

Intact pancreas tissue

Aldehyde-fuchsine staining: oval form, histostructure without changes, a large amount of deposited insulin (violet color) in cytoplasm of B-cells (Fig. 1.7) which maximally are concentrated in B-cells located around blood capillaries; insulin content without changes (Table 1); $K = 1,86 \pm 0,05$

Victoria 4R staining: histostructure of islets without changes; a large amount of deposited insulin in cytoplasm of B-cells (Table 1); $K = 1,66 \pm 0,06$.

Immunohistochemistry: histostructure and insulin content in B-cells without changes (Fig. 1.9) (Table 1); $K = 1,90 \pm 0,04$.

Diethylpseudoisocyanine staining: insulin content in B-cells without changes (Table 1); $K = 1,92 \pm 0,06$.

Fluorescent staining of Zn^{+2} -ions: positive reaction for Zn-ions in B-cells (intensive green fluorescence of Zn^{+2} -ions); $K = 2,05 \pm 0,07$ (Table 1).

Pancreas tissue after action of Dithizon

Aldehyde-fuchsine staining: destruction and death of majority of B-cells, marked decreasing of insulin content in B-cells (Fig.1.8; Table 1); $K = 1,12 \pm 0,03$

Immunohistochemistry: destruction and death of B-cells; marked decreasing of insulin content (Fig.1.10; Table 1); $K = 1,03 \pm 0,02$.

Diethylpseudoisocyanine staining: marked decreasing of insulin content (Table 1); $K = 1,11 \pm 0,04$.

Victoria 4R staining: destruction and death of majority of B-cells, marked decreasing of insulin content in B-cells (Table 1); $K = 1,08 \pm 0,09$

Fluorescent staining of Zn^{+2} -ions, DZ: absence of Zn^{+2} -ions in cytoplasm of B-cells (Table 1); $K = 1,04 \pm 0,01$

Pancreas tissue after action of L-Hystidine +Dithizon

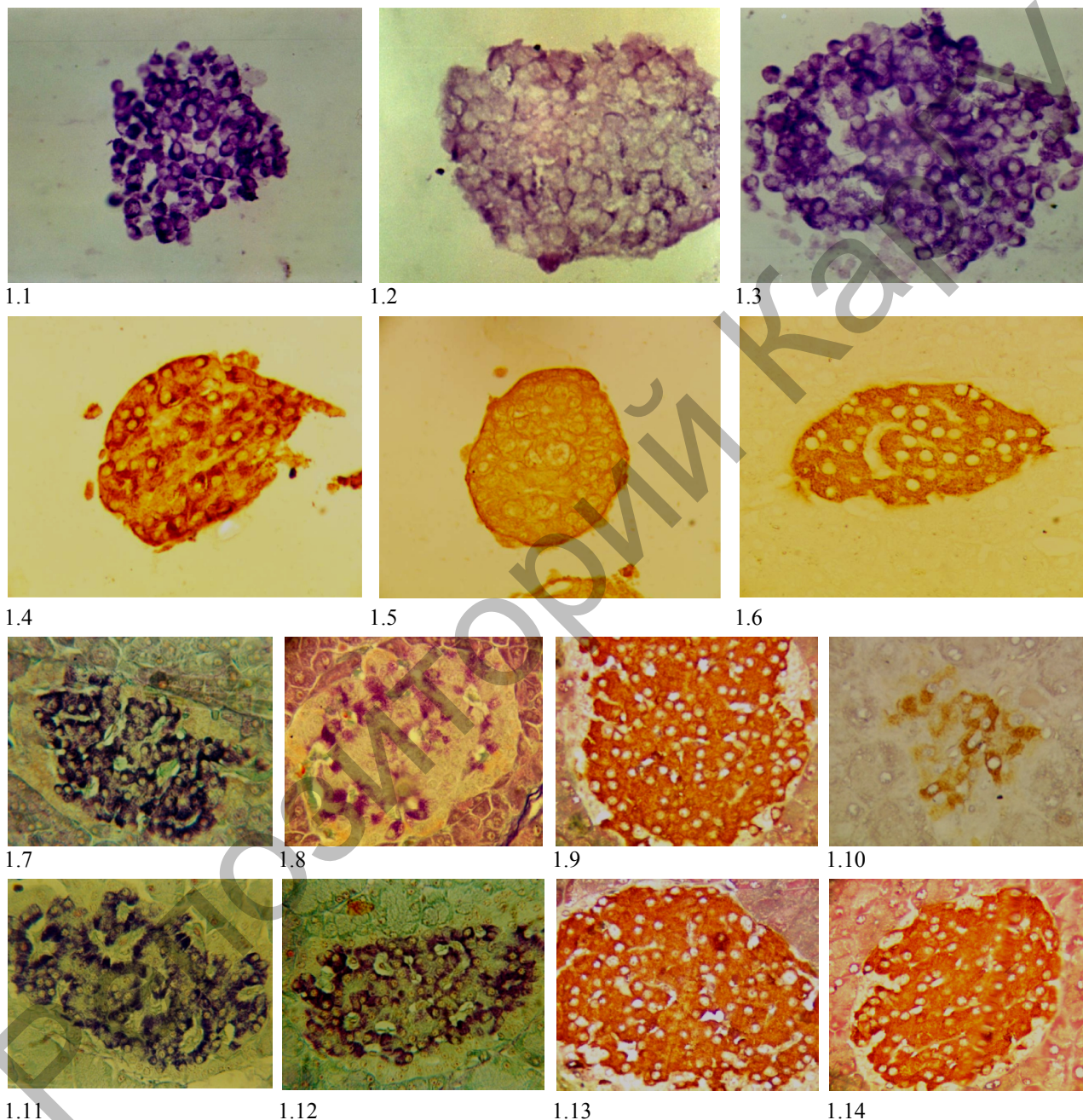
Aldehyde-fuchsine staining: histostructure and insulin content without changes (Fig. 1.11;1.12; Table 2); $K = 1,80 \pm 0,03$.

Victoria 4R staining: histostructure and insulin content without changes (Table 2); $K = 1,66 \pm 0,09$.

Immunohistochemistry: histostructure and insulin content without changes (Fig. 1.13;1.14; Table 2); $K = 1,82 \pm 0,04$.

Diethylpseudoisocyanine staining: histostructure and insulin content without changes, (Table 2); $K = 1,94 \pm 0,04$.

Fluorescent staining of Zn^{+2} -ions: negative reaction for Zn^{+2} -ions in B-cells as result of binding with L-Hystidine (Table 2); $K = 1,14 \pm 0,06$.



- 1.1 Isolated intact islet. Aldehyde-fuchshine. Histostructure and insulin content without changes; x280;
 1.2 Isolated islet. Dithizon. Aldehyde-fuchshine. Destruction of B-cells, negative reaction for insulin; x280;
 1.3 Isolated islet. L-Hystidine+Dithizon. Aldehyde-fuchshine. Histostructure and insulin content without changes; x280;
 1.4 Isolated intact islet. Immunohistochemistry. Histostructure and insulin content without changes; x280;
 1.5 Isolated intact islet. Dithizon. Immunohistochemistry. Destruction of B-cells. Negative reaction for insulin, x280;

- 1.6 Isolated intact islet. Immunohistochemistry. Histostructure and insulin content without changes; x280;
- 1.7 Intact rabbit. Pancreas. Aldehyde-fuchshine staining. Histostructure and insulin content in B-cells without changes; x280;
- 1.8 Diabetes caused by Pancreas. Dithizon. Aldehyde-fuchshine staining. Destruction of B-cells and marked decreasing of insulin content in B-cells; x280;
- 1.9 Intact rabbit. Pancreas. Immunohistochemical staining of insulin. Histostructure and insulin content in B-cells without changes; x280;
- 1.10 Diabetes caused by Dithizon Pancreas. Immunohistochemical staining of insulin. Marked decreasing of insulin content in B-cells; x280;
- 1.11 Intact rabbit. Pancreas. Aldehyde-fuchshine staining. Histostructure and insulin content in B-cells without changes; x280;
- 1.12 L-Hystidine+Dithizon. Pancreas. Aldehyde-fuchshine staining. Histostructure of B-cells without changes; minimal decreasing of insulin content; x280;
- 1.13 Intact rabbit. Pancreas. Immunohistochemical staining of insulin. Insulin content in B-cells without changes; x280;
- 1.14 L-Hystidine+Dithizon. Pancreas. Immunohistochemical staining of insulin. Insulin content in B-cells without changes; x280;

Figure 1. State of histostructure and insulin content in B-cells in animals with diabetes caused by Dithizon and its prevention by L-Hystidine

Table 1

Comparative analysis results of measuring of insulin content in B-cells using of various methods (r.u., parameter K)

№	Method	Intact animals	Diabetes induced by Dithizon	Difference of Indexes: intact/dithizon
Isolated islets				
1	Aldehyde-fuchshine	1,80±0,06	1,14 ±0,04	0,79
2	Victoria 4R	1,62±0,05	1,06±0,05	0,59
3	Immunohistochemistry	1,74±0,04	1,18±0,04	0,63
4	Diethylpseudoisocyanine	1,72±0,04	1,07±0,06	0,95
5	8PTSQ (zinc reaction)	1,75±0,03	1,02±0,01	
Pancreas tissue				
1	Aldehyde-fuchshine	1,86±0,05	1,12±0,03	0,89
2	Victoria 4R	1,66±0,06	1,08±0,09	0,60
3	Immunohistochemistry	1,90±0,04	1,03±0,02	0,84
4	Diethylpseudoisocyanine	1,92±0,06	1,11±0,04	0,93
5	8PTSQ (zinc reaction)	2,05±0,07	1,04±0,01	

Table 2

Influence of L-Hystidine on insulin and zinc content in B-cells (r.u., parameter K)

№	Method	Intact animals	L-Hystidine + Dithizon	Difference of indexes Intacts/ L-Hystidine (max./min/)
1	2	3	4	5
Isolated islets				
1	Aldehyde-fuchshine	1,72±0,05	1,65±0,04	0,79
2	Victoria 4R	1,64±0,04.	1,61±0,05	0,59
3	Immunohistochemistry	1,71±0,03	1,68±0,06	0,63
4	Diethylpseudoisocyanine	1,73±0,04	1,72±0,05	0,95
5	8PTSQ (zinc reaction)	1,79±0,05•	1,12±0,05•	

1	2	3	4	5
1	Pancreas tissue			
2	Aldehyde-fuchshine	1,89±0,05*	1,80±0,03*	0,89
3	Victoria 4R	1,68±0,04	1,66±0,09	0,60
4	Immunohistochemistry	1,98±0,03	1,82±0,04	0,84
5	Diethylpseudoisocyanine	2,04±0,06 ⁺⁺	1,94±0,04 ⁺⁺	
	8PTSQ (zinc reaction)	2,02±0,06 ⁺	1,14±0,06 ⁺	

Note. *p< 0,05; ⁺ p< 0,005; ⁺⁺ p< 0,005; • p< 0,005.

Discussion

Obtained results showed that administration of L-Hystidine result binding of almost all amount of Zn-ions in B-cells reversibly as least for 24 hours. Injection of dithizon after L-Hystidine not accompanied by forming in B-cells of chelat complexes Zn-DZ and by prevention of damage and of death of majority B-cells and by prevention developing of diabetes in 11 animals from 12. It is known that amino acids as Cystein and oxidized form of Gluthatione [22, 23] possess same property: injection of both acids result prevention of destruction of B-cells after following injection of dithizon and dia- betogenic derivatives of 8-oxyquinolin as of developing of diabetes in animals [1]. Both acids con tains in structure of molecule SH-radicals. Meanwhile molecule of reduced form of Gluthatione has only one difference: molecule not contains of SH-radical. As result, reduced form of Gluthatione not protect B-cells from binding of Zn-ions by zincbinding chelat active chemicals and from developing of diabetes [23]. Binding of Zn-ions in B-cells by oxidized form of Gluthatione is apparently confirmed by existence of negative reaction for Zn for 24 hours. After that the complex gradually dissociated up and 48–72 hours later dithizon is able to form in B-cells toxic complex that accompanied by developing of experimental diabetes in animals.

Comparison of results of histochemical estimation of insulin and zinc content in B-cells showed following: most sensible are fluorescent Diethylpseudoisocyanine, 8PTSQ zinc reaction and immunohistochemical methods. Decreasing of index in sections painted by Victoria 4 method determined, as we suppose, by more dark color of exocrine tissue cells that result distortion of results of photometry.

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О.Л. Коваленко, Л.В. Хлыстова, М.К. Бутумбаева

Ұйқы безінің эндокринді ұлпасын В-цитотоксикалық заттарымен бұзылуын және L-гистидинмен алдын алуын гистохимиялық және иммуногистохимиялық зерттеу

Мақалада мырышбайланыстырушы диабетогенді заттар тобын тудыратын сульфгидрильді SH тобы молекула құрылымында бар амин-қышқылды L-гистидинді алдын ала енгізу арқылы жануарларда диабетті болдырмау жолдары зерттелген. L-гистидин ерітіндісін қояндарға 900–1000 мг/кг мөлшерде көктамырға ендіру нәтижесінде мырыштың ұйқы безінде В-жасушаларында 24 сағат ішінде ионның толық байланысуы және В-жасушаларында аса қауіпті мырыш-дитизонды кешендердің қалыптасуын болдырмау, осы уақытта дитизон енгізу арқылы 12 жануардың ішінде 11-де диабет туындауының алдын алуымен байқалды. Бір уақытта ұйқы безі ұлпасының кесінділерін зерттеу нәтижесінде әр түрлі гистохимиялық әдістер ішінде ең сезімтал болып люминесцентті әдіс есептеледі. В-жасушаларда инсулин және мырыштың гистотопографиялық ерекшеліктері ең сапалы көрсеткіштерге ие альдегидфуксинді әдіс арқылы алынды.

Кілт сөздер: В-жасушалар, L-гистидин, инсулин, мырыш, эксперименталды диабет, альдегидфуксинді әдіс, дитизон, ұйқы безі, гистохимиялық әдіс.

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Гистохимическое и иммуногистохимическое исследование эндокринной ткани поджелудочной железы при ее повреждении В-цитотоксическими веществами и в условиях предупреждающего действия L-гистидина

Авторами исследована возможность предотвращения развития диабета у животных, вызываемого группой цинк-связывающих диабетогенных веществ, путем предварительного введения аминокислоты L-гистидина, содержащего в структуре молекулы сульфгидрильные группы SH. Установлено, что внутривенное введение раствора L-гистидина кроликам в дозе 900–1000 мг/кг ведет к практически полному связыванию ионов цинка в В-клетках поджелудочной железы минимально на 24 часа и предупреждению формирования в В-клетках токсичного комплекса цинк-дитизон, что сопровождается предупреждением возникновения диабета у 11 животных из 12, вызванного введением дитизона после L-гистидина. Одновременно при сравнительном использовании различных гистохимических методов исследования срезов ткани поджелудочной железы показано, что наиболее чувствительными для выявления инсулина и цинка в В-клетках являются люминесцентные методы. Наиболее качественные результаты оценки особенностей гистотопографии инсулина и цинка в В-клетках получены при использовании альдегидфуксинового метода.

Ключевые слова: В-клетки, L-гистидин, инсулин, цинк, экспериментальный диабет, альдегидфуксиновый метод, дитизон, поджелудочная железа, гистохимические методы.

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