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Victoria-4 histochemical method staining of insulin

Results using of histochemical Victoria-4 method staining of insulin in pancreatic B-cells are presented by authors comparatively with results of using other histochemical methods: immunofluorescent, immunohistochemical, pseudoisocyanine and aldehyde fucshine. By authors it is shown that this method being specific concerning insulin revealing in B-cells, is not high precise for quantitative measuring of insulin content in cells because not only B-cells but blood vessels and connecting tissue of islets are painted in dark colour registered by a photometer as well as blue colour of B-cells. Using of Victoria-4 method is possible for stain of various histostructures of islets that gives possibility to estimate the state of histostructure of islets not only insulin content.

Key words: insulin contents, immunohistochemical, immunofluorescent, pseudoisocyanine and aldehyde fuchsine methods, B-cells.

The are a few histochemical and immunohistochemical methods staining of insulin in pancreastic B-cells: immunofluorescent (IF), immunohistochemical (IG), aldehydefucshine (AF) [1], Victoria-4 methods [2, 5–8] and diethylpseudoisocyanine (PS) [7, 9].

Advantage of fluorescent IF and PS methods determined by more high sensivity of fluorescent stainings: minimal concentration of substances as 10^{-7}–10^{-8} maybe revealed using these methods. Meanwhile histological sections past staining maybe used for microscopy within short time as 0,5–1 h. Using fluorescent methods not possible to estimate state of histostructure of pancreatic islets. Immunohisto-chemical method as IF is more high specific for staining of insulin comparatively with all other methuds and now widely are used in the world.

Aim of work: to compare results of insulin staining in sections of rat's and rabit's pancreas tissue using staining by Victoria-4 method [V4] comparatively with immunohistochemical [IG], aldehydefucshine [AF] and pseudoisocyanine [PS] technologies.

Materials and methods

Pancreas tissue of 12 rats Vistar and 2 rabbits were used. Fixation in Bouin 24 h. Paraffin sections 4 mcm were used. Staining methods for insulin revealing in B-cells: Victoria-4 [V4], immunohistochemical [IG], aldehydefucshine [AF], pseudoisocyanine [PS] and staining by Dithizon [DZ].

The reagent Victoria blue 4R, diphenylnaphtylmethane derivative, mol. wt. 520 ($C_{34}H_{34}N_3Cl$), color Index 42563, «MERCK» (Germany) was used for staining of insulin in B-cells [3]. We have used fixation of pancreas tissue in Bouin, permanganate oxidation of sections prior staining using V4 as a 0.05 % acid alcoholic solution [4, 5].

Mixture for oxidation: 0,3 % aqueous potassium permanganate — 50 ml, 0,3 % Sulpuhric acid — 50 ml. Victoria Blue 4R main solution: 96º alcohol — 100 ml, Victoria Blue 4R — 1 g. Victoria Blue 4R staining solution: Victoria Blue 4R main solution — 25 ml, 96º alcohol — 100 ml, Glycerin — 300 ml, 1 % acetic acid glacial — 25 ml [2].

Maximum light absorbtion of V4 solutions depended on the concentration of the reagent. It is suggested that V4 forms molecular aggregates with maximum of absorbtion at 593 nm which could represent monometric dye particles and minimum absorbtion at 540 nm dimeric dye molecules [6]. The nature of the fixation in Bouin on staining of B-cells is not clear. Perhaps this is dependent on the formation of waterinsoluble insulin picrate [4]. Without Bouin fixation crystalline bovine insulin stains by V4 only after oxidation [4, 5].

Insulin content in B-cells was estimated by measuring of absorbance by photometry of B-cells located in central part of islets. Parameter K was calculated. For Pseudoisocyanine and Immunofluorescent methods: K=IN1/IN2; IN1-intensity of fluorescence of B-cells, IN2-intensity of fluorescence of exocrine tissue. For calculation direct dependence was used: increasing of amount of insulin in cells accompanied by higher intensity of fluorescence. For Immunohistochemical, Aldehydefucshine and Victoria-4 methods: K2=AB1/AB2; AB1-absorbance of light by exocrine tissue, AB2-absorbance of light by B-cells. For calculation inverse relationship is used: more intensive staining of cells for insulin result reduction of light amount accepted by photometer. By each method 20–25 pairs measurements endocrine/exocrine tissue was used. The average values of K1 and K2 parameters for exocrine tissue was accepted for 1.00.

Results

Insulin in pancreatic B-cells was identified using of all histochemical methods (Table 1, Fig. 1).

Table 1

Intensity staining of B-cells and of exocrine tissue using various methods and insulin content (parameter K) in pancreatic B-cells

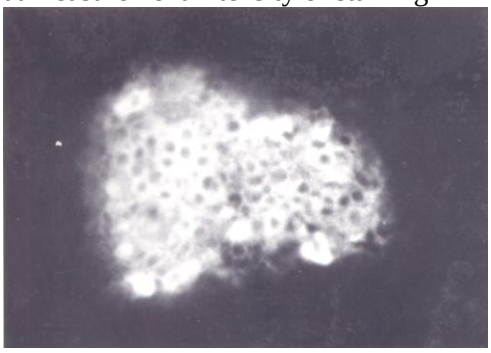
№	Pancreas tissue	Staining technologies				
		AF	IG	PS	V4	IF
1	Rats: B-cells; exocrine tissue; results staining of histostructure of islets	1.94±0,05 1.00±0,02 high	1.79±0,04 1.00±0,03 insufficient	2.02±0,11 1.00±0,08 insufficient	1.83±0,08 1.00±0,04 high	2.08±0,12 1.00±0,02 insufficient
2	Rabbits: B-cells; exocrine tissue	1.98±0,04 1.00±0,04	– –	2.07±0,12 1.00±0,07	1.96±0,05 1.00±0,03	– –
3	Rats; diabetes: B-cells; exocrine tissue	 1.07±0,07 1.00±0,06	 1.04±0,04 1.00±0,03	 1.02±0,03 1.00±0,07	 1.16±0,09 1.00±0,05	 1.09±0,03 1.00±0,04
4	Density staining of exocr. tissue V4/AF, V4/IG	1.14±0,04	1.07±0,03	–	–	–

Table 2

Practical characteristics methods of insulin staining in B-cells

№	Characteristics of methods	Staining technologies				
		AF	IG	PS	V4	IF
1	Specifity for insulin staining	Specifity for staining a few hormones; for B-cells specific for insulin	Absolute specifity for insulin staining in any tissues	High specifity for staining of A-chain of insulin	High specifity for staining of A-chain of insulin	Absolute specifity for insulin staining
2	Staining of histostructure of is lets and of exocrine tissue	Staining in detail; suitable for detail analysis of state of histostructure	Staining not in detail; not suitable for detail analysis of state of histostructure	Not staining of histostrusture; not suitable for analysis of state of histostructure	Staining in de tail; suit able for detail anaysis of state of by histostruccture	Not staining of histostrusture; not suitable for analysis of state of histost ructure
3	Reagents	Reagents for staining are produced by many firms	Kits for insulin staining are produced by many firms; method is widely used as uni versal for staining many hormones	Diethylpseudoisocyanine for staining is produced only by SERVA	V4 is produced by a few firms	Anticorps are produced by a few firms as by research laboratories
4	Practical using	Long time stored histological paraffin sections of tissue	Long time stored histological slides	Slides stored a short time (0,5–1 h)	Long time stored histologycal slides	Slides stored a short time (0,5–1 h)

Fluorescent methods showed more high deviations of value of parameter K that can be explained by more wide fluctuation intensity of fluorescence in various islets. The highest value of parameter K was obtained at measurement intensity of staining in B-cells of complex zinc-insulin by Dithizon slides (Fig. 1.7).



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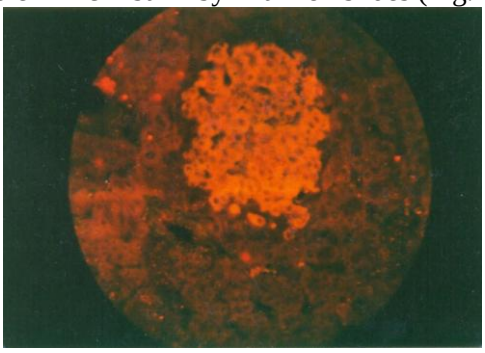

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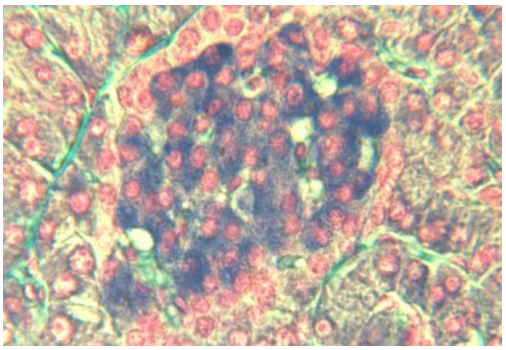

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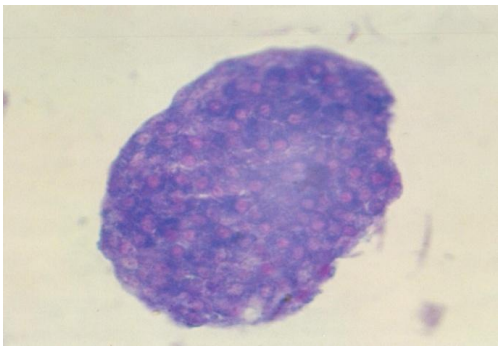

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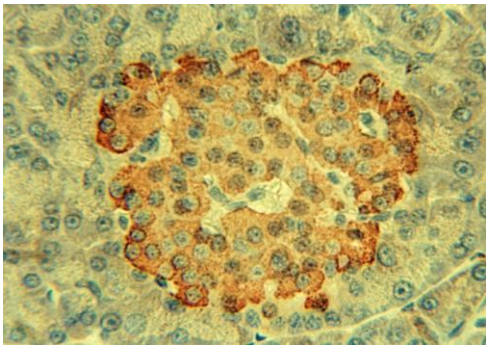

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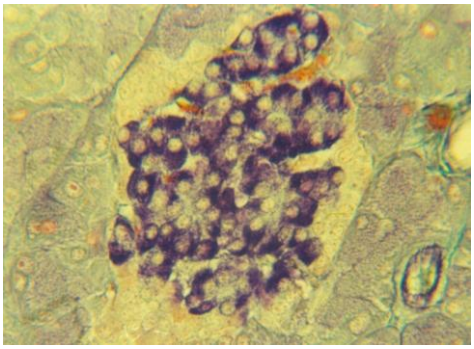

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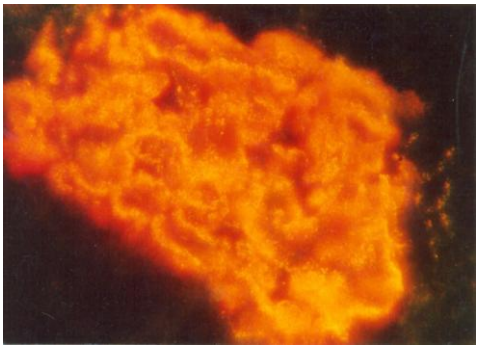

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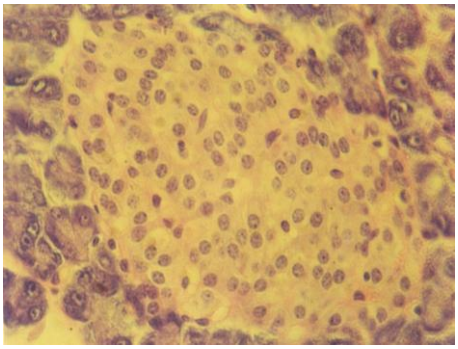

1.8

1.1 Intact pancreatic islet. Insulin. Intensive fluorescence. Immunofluorescent staining method; 5×40.
1.2 Intact pancreatic islet. Insulin. Red fluorescence of A-chair of molecule of insulin. Diethylpseudoisocyanine staining method; 3×40.
1.3 Intact pancreatic islet. Insulin. Dark blue color of insulin.Victoria-4 staining method; 7×40.
1.4 Isolated intact pancreatic islet. Dark blue color of insulin.Victoria-4 staining method; 7×40.
1.5 Intact pancreatic islet. Insulin. Brown color of insulin Immunohistochemical staining method; 7×40.
1.6 Intact pancreatic islet. Insulin. Dark violet color of hormone. Aldehyde fucshin staining method; 7×40.
1.7 Intact rabbit's pancreatic islet. Insulin-Zn^{+2} red coloring complex. Staining by Dithizon; 7×40.

1.8 Intact pancreatic islet. Insulin. Hematoxylin and eosin staining method; 7×40.

Figure 1

High fluctuations of absorption in sections painted by Victoria-4 comparatively with AF and IG methods are obviously caused besides the following reasons: 1) evidently more intensive staining of other structures of pancreatic islets as wall of blood vessels, nucleus, connecting tissue and of exocrine tissue that result more intensive absorbtion of comparatively with IG and AF methods; 2) density of staining of exocrine tissue is more high too comparatively with AF and IG methods and approximately same, as well as using staining by Hematoxyline and Eosin (Table 1; Fig. 1.3–1.6, 1.8).

It was reported that a possible explanation for the ability of B-granules to react with V4 past oxidation only determined by structure of insulin. Oxidation result dissociation of disulfide bonds between two chains of molecule of insulin. It is suggested that reactivity of B-cells with V4 is dependent on the staining of oxidised A-chain of insulin. The sulphonic acid groups in the A-chain give conditions favourrable for staining by V4 [10].

Analysis of characteristics of methods of insulin staining (Table 2) showed that V4 and AF methods are more preferable for estimate as of insulin content as state of histostrusture of pancreatic islets and exocrine tissue. Chemicals specific of V4 method is more high comparatively with AF method. Immunofluorescent staining method [IF]. We have obtained same results of staining by IF as using of sections of pancreas tissue (Fig. 1.1–1.3). IF is high specific method for revealing of Insulin in B-cells. Decreasing of Insulin content in B-cells of islets past action direct action of Streptosotozin was evidently demonstrated by this method (Fig. 1.3).

Diethylpseudoisocyanine chloride fluorescent method [PS], a high specific for revealing A-chair of molecule of Insulin, showed same result comparatively using of sections of Pancreas tissue (Fig. 1.4–1.6). Time for staining of sections in 0,4 % solution of Diethylpseudoisocyanine was reduced from 20 min till 15 min as was reduced time for washing of sections past staining procedures. This method showed marked decreasing of Insulin content in damaged B-cells (Fig. 1.5, 1.6) in com pared with intacts.

Aldehydefucshine method showed analogical results. A significant differences are revealed of state of histostructure as of Insulin content in damaged isolated islets comparatively with intacts (Fig. 1.8). Aldehydefucshine method [AF] contrary to IF and PS is not belong to high specific because colours other hormones too. But for pancreatic B-cells not contained other hormones AF is specific for Insulin.

Discussion

Analysis of results showed that using of histological and histochemical methods for staining of sections of isolated pancreatic islets have similar or equal to similar results obtained in pancreas tissue past staining by same methods. Fluorescent histochemical methods as Immunofluorescent reaction for Insulin as method using of Diethylpseudoisocyanine are more sensitive and identify the very low concentrations of investigated substances as 10^{-7}–10^{-8}, that has been confirmed by our results. Meanwhile both these methods have a common fault: histological sections past completing of staining procedures are not permanent and must be investigated within short time. Both methods are belong to high specific for staining of Insulin or of A-chair of molecule of Insulin. These methods are more precise for measuring intensity of insulin staining in B-cells because no other structure of islets are stained.

More suitable for practical using is Aldehydefucshine technic. Histological sections of pancreas tissue as of isolated islets stained by this method are permanent and can be stored for a long time. Aldehydfucshine method is not belong to high specific for Insulin staining. It is known that some pituitary hormones can also be stained by Aldehydefucshine method. Meanwhile for pancreatic islet's B-cells this method you can be measured as specific for insulin because the other hormones in B-cells are not synthesized. Method Victoria 4R is high specific for Insulin and as Aldehydefucshine technic gives an opportunity to obtain permanent histological sections. Quantitative estimation of insulin content in stained sections is based on measuring of absorbed by B-cells of light. However, both of these methods are belong to histological methods too and result staining not only of Insulin, but also other structures of B-cells which absorbed light as Insulin. Therefore, results of estimation of Insulin content in the B-cells by measuring of absorbance is not so precise as using fluorescent histochemical methods for Insulin staining.

We used significantly reduced time for fixation of Islets in Bouin from 24 h for pieces of pancreas tissue up to 15–30 min for isolated Islets. Time for staining of sections of isolated islets by Diethylpseudoisocyanine was reduced to 15 min comparatively with 20 min for sections of pancreas tissue.

Conclusions

1. V4 and AF methods evidently are more suitable for to estimate state of histostructure of pancreatic islets and of exocrine tissue, not only the content of insulin in B-cells.

2. V4 method is more specific for staining of insulin comparatively with AF method.

3. V4 method is more precise for quantitative estimate of the insulin content in B-cells comparatively with Pseudoisocyanine and Immunofluorescent methods and less precise in compared with Immunohistochemical and Aldehyde-fucshine methods.

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А.П.Андреева, Б.Такк, А.М.Айткулов, А.А.Кикимбаева, Г.Т.Тусупбекова

«Виктория-4» әдісінің көмегімен В-жасушаларда инсулин құрамын бақылаудың гистохимиялық әдісі

Бұл жұмыста Victoria-4 гистохимиялық әдіс (мәліметтер) деректері иммуннофлюоросцентті, иммунногистохимиялық, жаңа псевдоизоцианил және альдегидфуксинды басқа гистохимиялық әдістер қолдану нәтижелерімен салыстырмалы тұрғыда берілген. Авторлардың көрсетуімен бұл әдіс жоғарыдағы әдістердің болу тығыздығының сандық бағасының дәлдәгін беруінде жеткіліксіз болғанымен, инсулинге қатысында спецификалық артықшылығымен ерекшеленеді. Бұл әдістің құндылығы панкреатит аралшарының әртүрлі құрылымдарын сапалы бояу негізінде В-жасушалардағы резервты инсулиндік анықтаумен қатар аралшалардың гистоқұрылымдық күйін сапалы бағалау мүмкіндігін береді.

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«Виктория-4» гистохимический метод окраски инсулина

В работе приведены данные использования гистохимического метода Victoria-4 в сравнении с результатами применения других гистохимических методов: иммунофлюоресцентного, иммуногистохимического, псевдоизоциани нового и альдегидфуксинового. Авторами показано, что данный метод, являясь специфичным в отношении инсулина, уступает по точности при количественной оценке плотности окраски вышеперечисленным методам, но отличается преимуществом в стоимостном отношении. Его преимуществом также является достаточно качественная окраска различных структур панкреатических островков, что дает возможность достаточно качественно оценивать состояние гистоструктуры островков помимо определения содержания в В-клетках депонированного инсулина.

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Long time prolonged elimination of zinc-insulin complex from B-cells not result dysfunction of cells

Authors showed that almost complete elimination of zinc-insulin complex from cytoplasm of B-cells caused by 3 days prolonged administration of Glibenclamide to animals accompanied by complete disappearing of insulin and zinc-ions from B-cells. Next 6–7 days free of using of Glibenclamide result parallel complete recovery of amount of insulin and zinc in B-cells without any changes of histostructure of islets and function of B-cells.

Key words: rats Vistar, histology, insulin, B-cells, dissociation of complex, secretion, pancreatic islet, histostructure.

Pancreatic B-cells of many sorts of animals and of human contained a large amount of zinc-ions which take part in process of forming deposited form of insulin in cells which concentrated in B-granules of cells. In pancreas tissue B-cells contain large amounts of zinc. The major role of zinc is the binding of insulin in hexamers [1]. Zinc ions and insulin create a hexameric, crystalline structure, comprising 2 zinc ions and 6 insulin molecules, which is stored in the secretory granules until secreted in response to metabolic demands [2]. Zinc in B-cell secretory granules is involved in the storage and stabilization of the insulin hexamere in B-cells [2, 3]. Zinc ions appear to play important significance in process of microcrystallization of the precipitated insulin granules. May be it is advantage in condensing the stored hormone [2].

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It is known that prescription of Sulphorea result elimination of complex insulin-zinc from B-cells [4]. It was showed that 1 week past of partial or almost complete elimination of zinc-insulin complex from B-cells, function of cells are restored. It is not investigated question: are eliminated from B-cells zinc and insulin, insulin only or insulin and part of zinc-ions. Previously it was reported that binding of zinc-ions in B-cells by diabetogenic or not diabetogenic chelat active chemicals result a complete binding of zinc-ions and dissociation of complex 1,5–2 h later: chemicals are removed from cells and zinc remains in the cytoplasm of B-cells [5].

Meanwhile now is not cleared what is insulin and zinc content in cytoplasm of B-cells more long time after elimination of insulin from B-cells.

Aim of work: to investigate insulin and zinc ions content as state of histostructure of pancreatic islets more long period later, as 30 days, after almost complete elimination of zinc-insulin complex from B-cells.

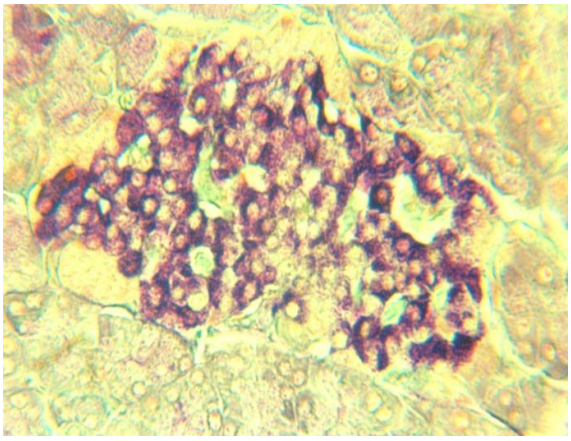
Materials and methods

26 rats Vistar 170–185 g were used. 2 % starch suspension of Glibenclamide (GB) used for peroral administration to animals in doses 10 and 25 mg/kg for 6 days 1 time daily. Blood Glucose control: 3 h, 24 h, 3 days 15 days and 30 days after past administration of GB). Histostructure of pancreas tissue and insulin content in B-cells were studied 30 days after administration of GB.

Histology. Pancreas tissue were fixed in Bouin. Sections 4–5 mcm were stained by hematoxylin and eosine; insulin staining by aldehyde fucshine [6], pseudoisocyanine [7–9] and zinc-ions — by 8-para(toluenesulphonylamino)quinolin (TSQ) [9]. Intensity of fluorescence of insulin and of zinc-ions was measured by fluorescent histofluorimetric complex constructed by G.G.Meyramov [10]. For transmission electron microscopy samples of pancreas tissue were fixed in 2,5 % Gluthar-aldehyde. Ultrafine sections of tissue contrasted by Reynolds [11] and were investigated on electron microscope JEM-7A.

Results

Blood Glucose concentration past administration of GB. Results showed that maximal decreasing of BG concentration was observed 3 h past first administration of 10 mg/kg (-approx. 20 %) and of 25 mg/kg (-28 %). 24 h past administration there are some not authentically prevalence of BG level in comparison before administration of GB. Next period from 3rd days until 30th days BG concentrations was not changed.



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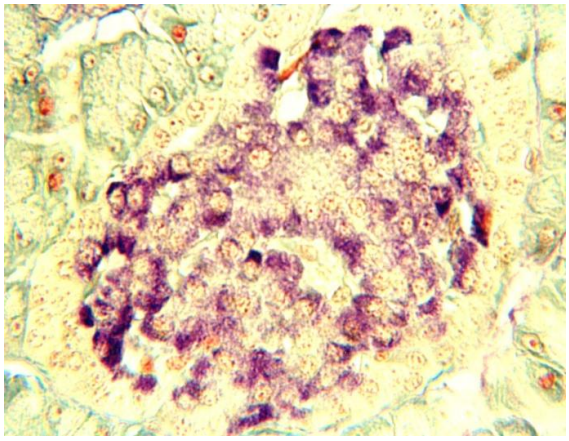

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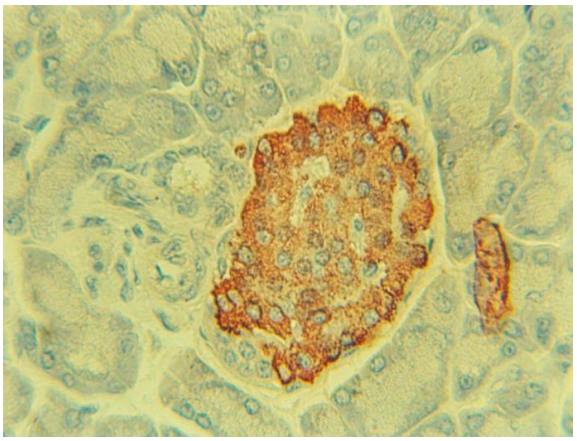

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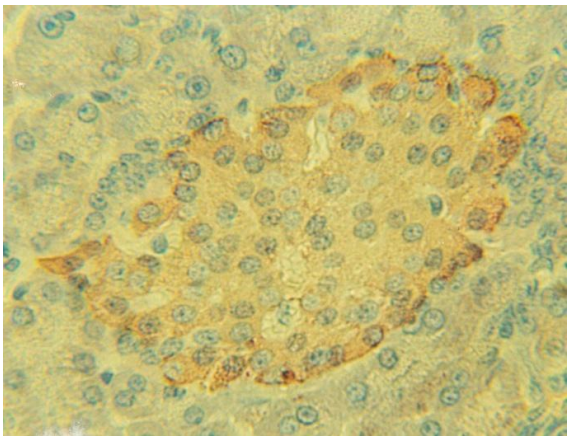

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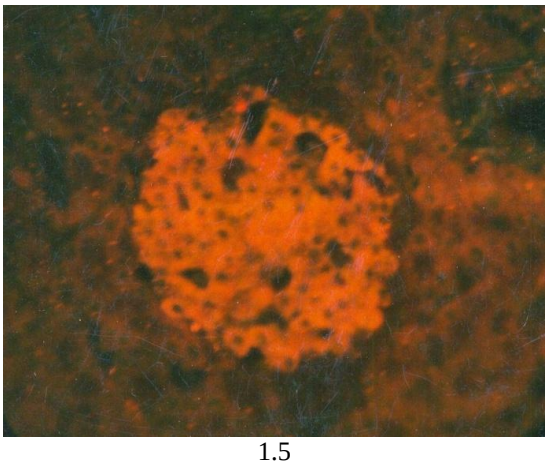

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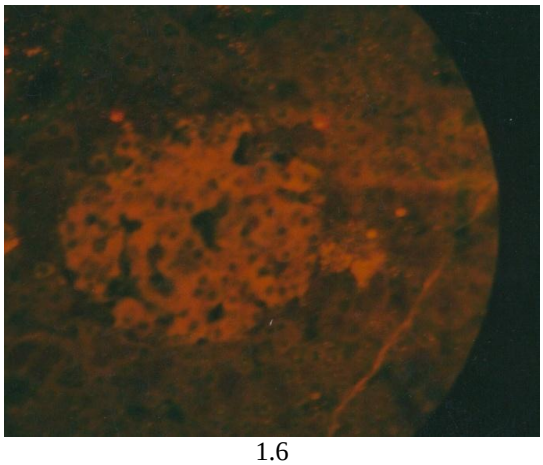


1.6

1.1 — Pancreatic islet of intact rat. Aldehydefucshine; ×280.
1.2 — Pancreatic islet 3h past action of GB 10 mg/ kg. Decreasing of insulin content in B-cells. Aldehydefucshine; ×280;
1.3 — Pancreatic islet of intact rat. Immunohistochemistry; ×280;
1.4 — Pancreatic islet 3h past action of GB 25 mg/kg. Decreasing of insulin content in B-cells. Immunohistochemistry; ×280;
1.5 — Pancreatic islet of intact rat. Pseudoisocyanine; ×200;
1.6 — Pancreatic islet 3h past action of GB 10 mg/kg. Decreasing of insulin content in B-cells. Pseudoisocyanine; ×200.

Figure 1

Table 1

Blood Glucose concentration past administration of GB

	Dose	Blood Glucose concentration, mM					
		before	3 h	24 h	3 days	15 days	30 days
1	Control (intact)	5,1±0,31	5,0±0,21	4,9±0,14	5,2±0,28	5,1±0,25	5,1±0,32
2	GB, 10 mg/kg	4,7±0,22	3,8±0,16 $p < 0,05$	4,8±0,15	4,8±0,23	4,9±0,17	4,6±0,23
3	GB, 25 mg/kg	4,6±0,15	3,3±0,14 $p < 0,05$	4,9±0,12	5,1±0,29	4,8±0,15	4,5±0,13

Table 2

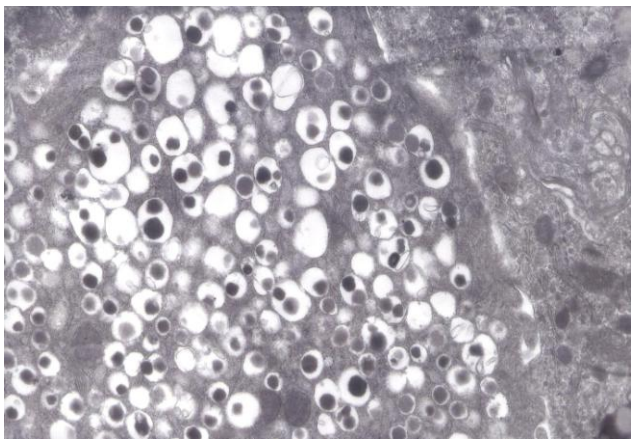
Insulin and Zinc content in pancreatic B-cells past administration of GB (parameter K)

	Condition	Insulin and Zinc content in B-cells (K)					
		before GB		3 h after GB		30 days after GB	
		insulin	zinc	insulin	zinc	insulin	zinc
1	GB, 10 mg/kg	2,04±0,05**	2,00±0,03	•1,62±0,04**	•1,71±0,03	1,94±0,06	1,87±0,05
2	GB, 25 mg/kg	1,95±0,06*	1,97±0,05	1,38±0,04*▪	1,62±0,04▪	2,00±0,07	1,96±0,06
3	Intact	2,02±0,05	1,98±0,06	1,96±0,07	1,94±0,08	1,97±0,04	2,02±0,05

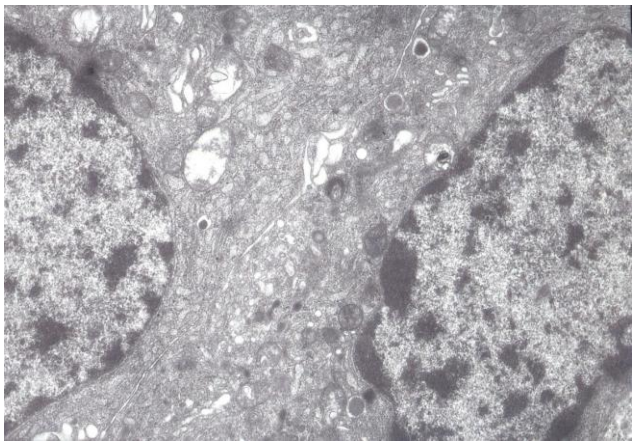
Note. * — p < 0,01; ▪ — p < 0,05; • — p < 0,05.

Results of estimation of insulin and zinc content in B-cells showed evident decreasing amount as of insulin as of zinc-ions 3 hours after GB administration (Table 2; Fig. 1.1–1.6; Fig. 2.1, 2.2). Decreasing is more marked past administration of 25 mg/kg — for almost 30 % comparatively with approximately 20 % past ad- ministration of 10 mg/kg. Results showed that a coincidence of results between the contents in B-cells of insulin and zinc is available only for intact B-cells and after 24 h and 30 days past administration of drug. 3 hours after administration of GB results showed more intensive decreasing of insulin content comparatively with content of zinc-ions (Table 2). Results obtained before action of GB were authentically prevailed in compared with zinc ions past administration of both doses of GB. 30 days past administration of both doses of GB insulin and zinc content in B-cells were restored completely. There are not any histological changes in pancreatic islets 30 days after action of GB (Fig. 1.2, 1.4).

We found discrepancy more marked differences between results measuring of insulin and zinc-ions content 3 h past action of GB 25 mg/kg (Table 2): approximately 30 % of insulin are eliminated from B-cells and 18–20 % of zinc-ions only eliminated contrary to parallelism of results 30 days past action of GB. It is possible to suppose that part of amount of zinc-ions after dissociation of complex is eliminated from B-cells and part remain in cells (Fig. 3).



2.1



2.2

2.1 — Intact pancreatic islet. B-cells. Large number of B-granules contained zinc-insulin complex; ×3450;
2.2 — Islet 3h past administration to animal of GB 25 mg/kg. Marked decreasing of number of B-granules; ×2920

Figure 2. Transmission electron microscopy

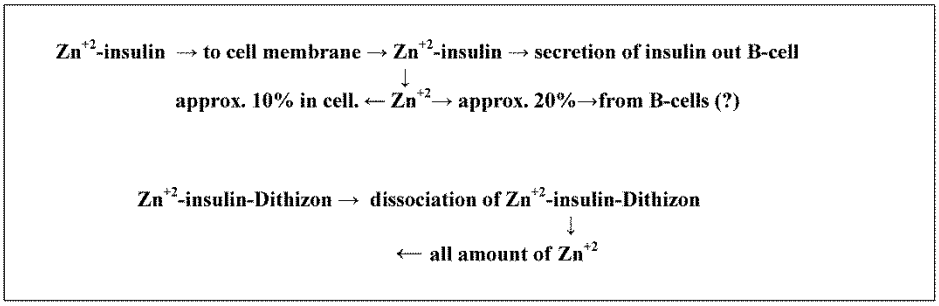


Figure 3. Dissociation of complexes Zn^{+2}-insulin and Zn^{+2}-insulin-Dithizon in B-cells

Conclusions

1. Elimination of insulin from B-cells by GB accompanied by partial decreasing of zinc-ions content 3 h after action and completely is restored 30 days later. Insulin content in B-cells reduced for approximately 30 % and of zinc-ions — for 18–20 % 3 hours after administration of 25 mg/kg of GB.

2. There are not any histological changes of histostructure of pancreatic islets 30 days past elimination of zinc-insulin complex from B-cells. Amount of insulin and zinc-ions in B-cells restored completely 30 days later.

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В-жасушалардан мырыш-инсулин кешенінің ұзақ уақыттағы элиминациясы олардың функциясының өзгерістеріне әкеп соқпайды

Авторлар Глибенкламидті үш күндік енгізуден кейін В-жасушаларынан мырыш-инсулин кешенінің элиминациясы цитоплазмадан мырыш пен инсулиннің толықтай жоғалуына әкеліп соққандығын анықтады. Препаратты енгізуді тоқтатқаннан кейін жасушалардағы мырыш пен инсулиннің мөлшері толықтай орнына келді. Панкреатиттік өсінділердің гистоқұрылысының жағдайында ешқандай өзгерістер байқалмады.

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Длительная элиминация цинк-инсулинового комплекса из В-клеток не сопровождается нарушениями их функций

Авторами установлено, что элиминация из В-клеток цинк-инсулинового комплекса, вызванная трехдневным введением Глибенкламида сопровождалась полным исчезновением из цитоплазмы инсулина и цинка. После прекращения введения препарата содержание цинка и инсулина в клетках полностью восстанавливалось. Никаких изменений состояния гистоструктуры панкреатических островков не выявлено, как и инсулинпродуцирующей функции В-клеток в последующем.