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Histochemical staining and estimation of zinc content in pancreatic β-cells by using of Dithizon and 8-para(toluenesulphonylamino)quinolin

It is known that zinc in pancreatic β-cells take part in processes of formation of deposited form of insulin. Assessment of its contents allows to estimate functional state of β-cells. It is necessary to use a precise and high specific histochemical methods for identification of zinc in β-cells. Authors presented results of experimental improving of methods for qualitative and quantitative estimation of zinc content in pancreas tissue. Authors showed that: 1) decrease of thickness of sections of pancreas tissue 1,5 times — from 6–7 mcm to 4 mcm have best results for investigate of histotopography of zinc in islets using of Dithizon method; 2) similar result is obtained by decrease of dose of Dithizon njected to animals for 2–2,5 times; 3) quantitative estimation of zinc content is possible by using of histofluorimetric complex; 4) formation of a complex of dithizon with zinc in the form of the painted granules significantly improve results of analysis of histotopography of zinc in islet; 5) regarding preparing of frozen sections of pancreas tissue: best results were obtained using of temperature of freezing of tissue from -18° to – 22 °C; 6) more precise result were obtained using of additional method staining of zinc by TSH which is more sensitive for revealing of minimal concentration of zinc as 10^{7}-10^{-8}. Thus, obtained results allow to ex- pand and significantly improve possibilities of histochemical Dithizon and TSH methods for estimate concentration and disposition of zinc in pancreatic islets.

Keywords: pancreas, pancreatic islets, β-cells, histochemical methods, dithizon, 8-para(toluenesulphonylamino) quinolin, zinc, insulin, histotopography, quantitative analysis.

Pancreatic islets of human pancreas contains, as well as islets of many mammals (rabbits, dogs, cats, pigs, mice, horses, hamsters), a large amount of ions of zinc [1–3] participating in formation of zinc-insulin complex, a deposited form of hormone in β-cells. Histological methods are unsuitable for estimate functional state as for to estimate content of deposited insulin in β-cells. More preferable is using of specific and sensitive histochemical methods of staining ions of zinc in B-cells.

Diphenylthiocarbazon (Dithizon) and 8-para(toluenesulfonilamino)quinoline (8TSH) possess two important properties for this purpose: 1) to form with zinc in β-cells chelat complexes highly specific for zinc; 2) complexes with 8TSH have bright green fluorescence that allows to observe visually of zinc in β-cells and estimate content by measuring of intensity of fluorescence by using of fluorescent microscopy; 3) complexes of zinc with Dithizon revealed in cells as bright red granules using of dark microscopy. As these complexes at the same time are toxic both substances possess diabetogenic properties and are capable within 15–30 min. after intravenous injection selectively result destruction and death of the majority of the β-cells that accompanied by development of a diabetes mellitus 1 type within the first 2–3 days [4–7].

Previously it was possible to estimate zinc content in β-cells by visual analysis only using of fluorescent or dark microscopy. Quality of obtained results of histochemical identification of zinc by means of both

methods was remained insufficient for investigate histotopography of zinc in islets as well as for the quantitative assessment of its contents that was caused both by not high quality of staining technologies and of quality of preparing sections of frozen tissue of pancreas most suitable for such researches. The research aim of work consists for improving of staining procedures, preparing of frozen sections of pancreas tissue and development of method of assessment of measuring result of staining of zinc in β-cells.

Materials and methods

12 rabbits weighing 2250–2700 g and 14 white mice weighing 30–35 g were divided for 2 groups: 1) rabbits and mice were killed 6–8 min after intravenous injection of solutions of dithizon and 8TSH (46–49 mg/kg and 38-42 mg/kg); 2) rabbits with diabetes caused by Dithizon (48–50 mg/kg). Control: intact animals.

Preparing of Dithizon solution: 30 ml of distilled water added 0,6 ml of 25 % of solution of ammonia, 400 mg of Dithizon. Mixing on a water bath (+70 °C) for 10 min., filration. The received filtrate represented 1% water ammonia liquor of Dithizon solution was injected intravenously to rabbits into an ear vein and to mice in a tail vein. Preparation of solution 8TSH for injections: 25 mg. powder 8TSH (Institute of high pure reagents, Moscow, Russia) dissolved in 70% ethanol at a temperature + 70 ^{o}C within 6–7 min. on a water bath then injected intravenously of 38–42 mg/kg.

8TSH formed fluorescent complexes with zinc and cadmium. But cadmium is absent in pancreatic β-cells. The complex Zn^{+2}-8TSH in ultraviolet light at of 360–370 nanometers fluoresces bright green light (Fig. 1B), and the Cd-8TSH complex – bright yellow. That is why 8TSH for β-cells is high specific for staining of zinc ions. Method is sensitive for revealing of zinc concentration as 10^{-7}–10^{-8}. The reagent was offered by Institute of High Pure Reagents (Moscow) as high specific for revealing of zinc in liquids and tissues. Y.A. Lazaris and coll. was used it for identification of zinc in tissues of animals, including pancreas tissue [5, 7–9].

Aldehyde-fuchsine method was used for staining paraffin sections of pancreas tissue [10]. Blood glucose concentration in animals of group 2 was carried out daily within first 5 days in animals of, and then twice per week. In 10 days animals were destroyed by an air embolism. Frozen sections 4–5 mcm of pancreas of animal from group 1 were investigated using dark-field microscopy after intravenous administration of Dithizon and of luminescent microscopy for histochemical luminescent identification of zinc in β-cells after staining by 8TSH. 0,4 % aceton solution 0,4 % of 8TSH was used: several drops of which applied on sections for 10-12 sec.; washing of sections later by bidistilled water.

Zinc content n B-cells was estimated by a histofluorimetric method in the relative units (r. e.) by measuring intensity of fluorescence of complex Zn^{+2}-8TSH in β-cells and of density of concentration of granules of Zn^{+2}-Dithizon [11,12] by calculation of parameter «K» based on direct dependence between intensity of a fluorescence (8TSH) and of density of staining (Dithizon) of β-cells and content of zinc. Calculation of parameter K for a 8TSH-luminescent method of identification of Zn^{+2}-ions in β-cells: IF1/IF2, where: IF1-luminescent emission of B-cells, and IF2-intensity of luminescence of exocrine tissue (absence of color, as 1.00). Calculation of parameter K for Dithizon method of identification of Zn^{+2}-ions in β-cells: IF1/IF2, where: IF1- density of staining of β-cells and IF2- density of staining of exocrine tissue (absence of color, as 1.00).

Results and discussion

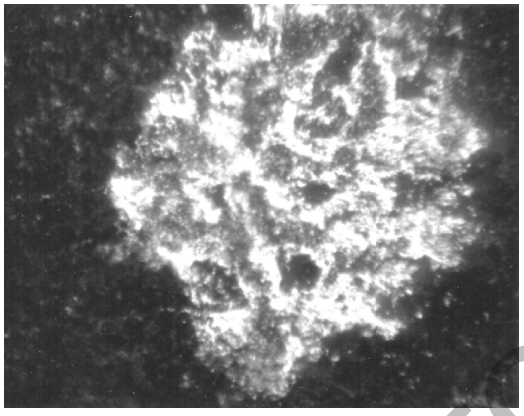
Obtained results demonstrate that the highest content of ions of Zn+2-was observed in pancreatic islets of intact rabbits (table 1). In sections of pancreas of the animals of group 1 showed positive Dithizon reaction for zinc in the form of red granules of Zn+2-Dithizon complex (Fig. 1A) filling cytoplasm of β-cells. In the sections of animals group 2 with diabetes reaction for zinc is negative (Table 1, Fig. 1B) that is caused by almost complete selective destruction of β-cells. The similar results obtained using of 8TSH reaction: a large amount of zinc in β-cells of intact animals — the intensive bright green luminescence of a complex Zn+2-8TSH (Fig. 1B) in compared with expressed negative reaction in β-cells of animals with diabetes (Fig. 1G, Table 1) was observed. Diabetes in animals of group 2 was confirmed by using of aldehyde-fucshin method of staining: destruction and death of majority β-cells and marked decreasing of insulin content in β-cells.

Table 1

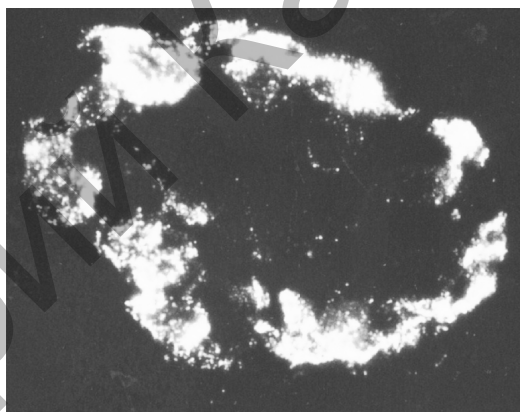
Insulin content in pancreatic β-cells in animals with experimental diabetes in relative units (r.e.)

№	Experimental conditions	Insulin content in pancreatic β-cells (r.e.)		Initial blood glucose level (мМ)	Blood glucose level 8 days after inject-tion of Dithizoни (мМ)
		8-TSH reaction (zinc)	Dithizon reaction (zinc)		
1	Intact rabbits	2,02±0,05● (n=26)	1,94±0,08▫ (n=23)	4,9±0,4	-
2	Diabetes caused by Dithizon (rabbits)	1,08±0,03● (n=18)	1,04±0,02▫ (n=19)	5,2±0,5	16,3±3,5
3	Intact mice	1,95±0,03* (n=24)	1,82±0,04+ (n=24)	4,1±0,3	-
4	Diabetes caused by Dithizon (mice)	1,03±0,03* (n=16)	1,05±0,02+ (n=18)	3,8±0,4	9,7±0,8

● * ▫ + p≤0,001; n — number of measurements.



1А



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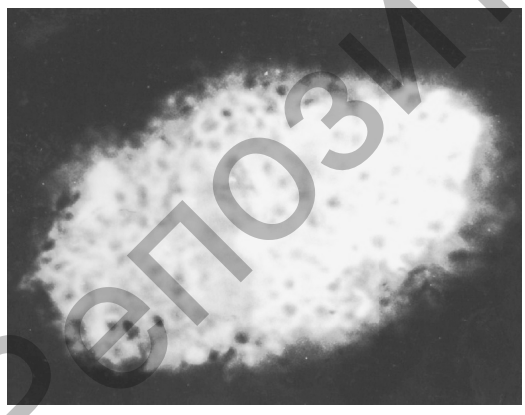

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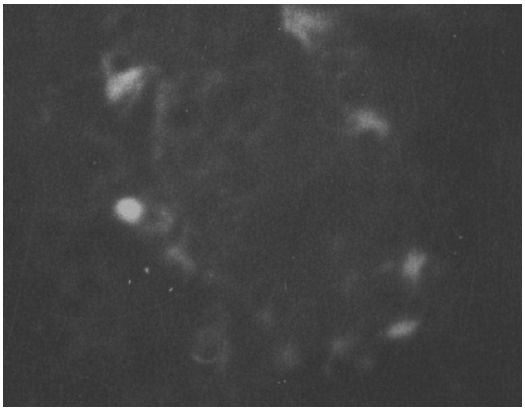
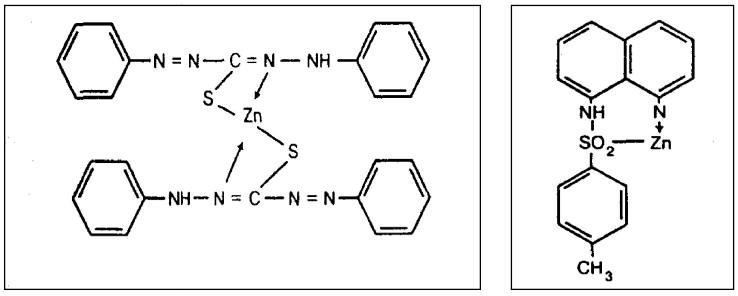


1Г

1А- Intact rabbit. Injection of Dithizon, 49,8 mg/kg. Red granules of complex Zn+2-Dithizon in cytoplasm of β-cells. Dark microscopy; x280; 1Б – Rabbit. Diabetes caused by Dithizon 7 days after injection. Absence of granules of complex Zn+2-Dithizon in destroyed β-cells; on periphery A-cells. Dark microscopy; x280;1В- Intact rabbit. Injection of 8TSH, 39,4 mg/kg. Bright green fluorescence of complex Zn+2-8TSH in β-cells. Fluorescent microscopy; x280; 1Г- Rabbit. Diabetes caused by Dithizon 7 days after injection. Negative reaction for zinc in β-cells. Fluorescent microscopy; x280

Figure 1. Histochemical reaction for zinc in β-cells in experimental diabetes

It is known that in process of formation of the Zn^{+2}-complex with diabetogenic derivatives of 8-oxyquinolin atom of zinc is fixed between S or O atoms in position 8, and N or O atoms — in positions 1 or 2 (Fig. 2).



Dithizon 8-TSH

Figure 2. Chelat complexes Zn^{+2}-Dithizon and Zn^{+2}-8TSH

Diabetogenic derivatives of 8-oxyquinolin have in the 8 position of quinolin ring active OH^- radical or other radicals contains atoms of S, N or O. Six isomers of 8-oxyqunolines not contains in this position of such radicals or atoms or if these radicals were extracted from a molecule — were not capable to form complex salts with zinc and not possess completely diabetogenic properties [13, 14]. It was necessary to return the active radicals in position 8 for to restore diabetogenic activity of substance [13, 14]. Formation of the chelat-complex by atoms of O and N result formation of pentagon or hexagon rings [13].

Pentagonic rings are evidently more stable. In case if atoms of sulfur participate in formation of chelates and then most stable are quadrangular rings (Fig. 2). Electrons of the lone pair of electrons are displaced from nitrogen donor-atom located in the first position to zinc atom. In experiences with various isomers of 8-oxyquinolin dependence according to which the maximal toxicity possess isomers which are forming chelates of structure 1:1 with metal and have a stability constant logarithm equal 7,6 and above to 9,4 [13]. The complexes of derivatives of 8-oxyquinolin possess high toxicity for B-cells formed with zinc have a high rate of logarithm of a constant of stability, equal 8,5. G. Weitzel and coll. [15] confirmed that the complex of structure 1:1 contains 1 molecule of 8-oxyquinolin and 1 atom of zinc is most toxic for cells.

High durability of the Zn^{+2}-Dithizon complex 2:1(Fig. 2) determined by space elongation of molecule of Dithizon and disposition of two phenolic rings on the ends of a molecule that does not prevent the atoms of sulfur and nitrogen located in the center of a molecule to approach zinc atom. Besides, atom of zinc is located between two atoms of nitrogen and sulfur, regarding to which affinity of zinc is very high and exceeds affinity to oxygen. At last, two molecules of Dithizon having totally larger number double connections increases toxicity of the Zn^{+2}-Dithizon complex.

Isoniazid, a anti-tuberculosis drug, is capable to form strong five-membered chelates with zinc. Perhaps, it is exist some relation between this property and facts of marked xanturenuria and more high frequency of diabetes in patients treated by Isoniazid taking into consideration that Isoniazid is antagonist of pyridoxal-5-phosphate [16].

High specificity of Dithizon for identification of zinc confirmed by results of comparative spectral analysis of spectrum of absorbance of complex Zn^{+2}-Dithizon extracted from B-cells with the similar artificial complex formed in vitro. The maximum of absorption of both ranges was identical and made 530 nanometers [7].

Thus, both methods of histochemical identification of zinc in cells of tissues of bodies allow to reveal zinc-chelator complexes in cells using of dark microscopy and luminescent microscopy with quantitative measuring. Advantage of 8TSH method is more high sensitivity. The most important advantage of Dithizon method is ability to form the Zn^{+2}-Dithizon complex in β-cells not in the form of homogeneous color but in the form of the bright red granules which are settling down in the form of various density of scatterings in cells accordingidirect dependence of concentration of zinc in various parts of islets and β-cells.

It, in turn, give the exclusive chance for precise studying of character of a histotopography of zinc in β-cells which are settling down in various parts of pancreatic islets that is essential for investigation in detail of mechanisms of diabetogenic action.

Regarding preparing of frozen sections of pancreas tissue. We obtained better results keeping the following conditions: 1) temperature in frozen camera of cryostate to keep between -18 and – 20 ^{o}C. At temperature more than -23…-24^{0}C pancreas tissue over is frozen; 2) metal holder of tissue must be cooled in cryostate camera before fixation on it of pancreas tissue; not using water on a holder for fixing tissue as ice will complicate preparing of sections; 3) for removal of section it is enough to straighten of it and to touch carefully it with a warm microscope glass, having waited so far it well will finish then glass with sections to remain in the same place, in a freezing camera; 4) for investigation of histotopography of zinc in pancreatic islets the more suitable sections of pancreas gland is thickness equal of 4 mcm, and for the quantitative assessment 6–7 mcm.

Some restriction for both methods is need of use of frozen sections. This restriction this same time have advantage as freshly frozen sections is not exposed to any types of processing procedures (fixing, dehydration and others) which could to influence on revealing of zinc. Both methods are single highly specific concerning identification of zinc not only in pancreas tissue, but also in prostate and in the salivary contains a large amounts of zinc.

Meanwhile 8TSH-method can be used also in work with the fixed samples of tissues, however it is possible only using as fixing liquid of 70^{0} ethanol enriched with the H_2S allowing to besiege zinc ions in β-cells.

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Ұйқы безіндегі панкреатит аралшықтарындағы β-жасушаларындағы мырыш иондарын дифенилтиокарбазон және 8-пара(толуолсульфониламино) хинолин (ТСХ) көмегімен гистохимиялық анықтау және бағалау

Ұйқы безіндегі β-жасушаларындағы мырыш оның жасушада депо күйінде сақталуын қамтамасыз етеді. Оның құрамы β-жасушаларындағы инсулин синтездеу қабілетін көрсетеді. Панкреатиттік β-жасушаларындағы мырышты анықтау үшін ұлпадағы металдың иондар құрамын гистохимиялық әдістер талдауы нәтижесінде байқаймыз. Мақалада β-жасушаларындағы мырышты анықтауда жетілдірілген гистохимиялық әдістердің нәтижесі берілген. Зерттеу нәтижесінде көрсетілген: 1) ұйқы без ұлпасы кесіндісін қалыңдығын 1,5 есе — 6-7 мкм-ден 4 мкм дейін азайтып, дитизонды әдісті қолданғанда мырыш гистотопографиясы зерттеуде ең жоғарғы нәтиже береді; 2) жануарларға дитизон дозасын төмен енгізген жағдайда аналогты нәтиже береді; 3) авторлар бұрынырақта ұсынғандай, гистофлюориметрикалық кешенді зерттеу жасушадағы металл құрамын эмпирикалықтан сандық бағалауға көшуге мүмкіндік береді; 4) боялған түйіршік формада дитизонның мырышпен кешен құруы мырыш гистотопографиясын зерттеуде жақсы нәтижелер алуын байкатады, люминесцентті флюорохромды пайдалану барысында шашырау эффектісі байқалмайды; 5) ұйқы безін әр түрлі режимде мұздату әсері нәтижесінде кесінділерді зерттеуде ең жақсы нәтиже 18 до -22 ^{o}C температурада байқалды; 6) мырышты 10^{-7}-10^{-8} дәрежеде концентрацияда анықтауда неғұрлым сезімтал дитизонды және ТСХ әдістерін кешенді қолдану аралшықтардағы мырыш құрамының толық талдау нәтижесін береді. Осылайша, алынған нәтижелер β-жасушалары мен инсулинді синтездеу бейімділігі мен ұйқы безі ұлпасымен жұмыс барысында гистохимиялық әдістерді қолдану мүмкіндігін кеңейтеді.

Кілт сөздер: ұйқы безі, панкреатит аралшықтары, β-жасушалары, гистохимиялық әдістер, дитизон, 8-para(toluenesulphonylamino)quinolin, мырыш, инсулин, гистотопография, сандық бағалау.

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Гистохимическое выявление и оценка содержания ионов цинка в β-клетках панкреатических островков поджелудочной железы с помощью дифенилтио-карбазона и 8-пара(толуолсульфониламино)хинолина (ТСХ)

Известно, что содержащийся в β-клетках поджелудочной железы цинк принимает важное участие в образовании его депонированной формы хранения в клетке. Оценка его содержания позволяет судить о способности β-клеток синтезировать инсулин. Для выявления цинка в панкреатических β-клетках и оценки его содержания существенное значение имеет наличие совершенных методов гистохимического анализа содержания ионов этого металла в ткани. В работе представлены результаты совершенствования методик гистохимического выявления цинка β-клеток. Показано, что: 1) уменьшение толщины срезов ткани железы в 1,5 раза — с 6–7 мкм до 4 мкм — дает наилучшие результаты при исследовании гистотопографии цинка в островках при использовании дитизонового метода; 2) аналогичный результат дает снижение вводимой животным дозы дитизона; 3) использование предложенного ранее авторами гистофлюориметрического комплекса обеспечивает переход от эмпирической к количественной оценке содержания металла в клетке; 4) формирование комплекса дитизона с цинком в виде окрашенных гранул дает лучшие результаты при исследовании гистотопографии цинка, так как отсутствует эффект рассеивания, наблюдаемый при использовании люминесцентных флюорохромов; 5) исследовавание влияния различных режимов заморозки ткани железы при приготовлении срезов показало, что наилучшие результаты наблюдаются при температуре заморозки в пределах от -18 до -22 ^{o}C; 6) наиболее полные результаты анализа содержания цинка в островках дает комбинированное применение дитизонового и ТСХ-метода, являющегося более чувствительным и позволяющим выявлять цинк в концентрациях, равных 10^{-7}-10^{-8} степени. Таким образом, полученные результаты позволяют существенно расширить возможности применения гистохимических методов при работе с тканью поджелудочной железы, что позволяет получить дополнительно обширную информацию о состоянии β-клеток и их способности синтезировать инсулин.

Ключевые слова: поджелудочная железа, панкреатические островки, β-клетки, гистохимические методы, дитизон, 8-пара(толуолсульфониламино)хинолина, цинк, инсулин, гистотопография, количественная оценка.

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