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G.G. Meyramov[1*], K.-D. Kohnert[2], A.Zh. Shaybek[1], F.S. Abikenova[3], K.S. Temireeva[4], Zh.Zh. Blyalova[1], K. Elshina[1], A.G. Meyramova[4]

[1]*Karagandy University of the name of academician E.A. Buketov, Karaganda, Kazakhstan;*
[2]*Institute of Diabetes "Gerhardt Katsch", Karlsburg, Germany;*
[3]*Medical University Karaganda;*
[4] *Academy Bolashaq, Karaganda, Kazakhstan*
**Corresponding author: meyramow@mail.ru*

# Indirect method of histochemical assessment of insulin content in pancreatic β-cells

Methods for analysis the state of histostructure and insulin and zinc content in pancreatic tissue allow to estimate either a state of histostructure alone or visual insulin content in ß-cells. Zinc content indicating the content of deposited insulin and the ability of ß-cells to form this form of hormone is possible using an additional method that increases the volume of work and complicates investigation. Authors have proposed a method that allows to simultaneously evaluate both the state of the histostructure and the quantitative content of deposited insulin and zinc in ß-cells of the pancreas. It has been experimentally confirmed that both the aldehyde-fucshsin method of insulin staining and Dithizone method for staining of zinc-ions in ß-cells can be separately used simultaneously for quantitative analysis of the content of deposited insulin and zinc in ß-cells. It has been experimentally confirmed that both the aldehyde-fucshsin method of insulin staining and the Dithizone method of detecting zinc in ß-cells can be separately used simultaneously for quantitative analysis of the content of deposited insulin and zinc in ß-cells. The use of both methods allows not only to assess the state of the histostructure, but to assess the content and functional ability of ß-cells to synthesize insulin, as well as to form its deposited form using zinc.

*Keywords*: pancreas, ß-cells, insulin, zinc, Dithizon, histostructure, insulin synthesis.

## *Introduction*

Pancreatic islets of a number of animals (rabbits, dogs, cats, hamsters, mice) contain significant amounts of zinc ions [1]. There are indications that zinc has an important role in the formation of a deposited form of insulin in β-cells [2, 3], so that the synthesized hormone is stored in cells and excreted into the blood depending on the level of glucose in the blood. Meanwhile, today there are no direct experimental data confirming the formation of the Zn-insulin complex in β-cells. There are several methods for selectively assessing the histostructure of pancreatic islets, the content of deposited insulin and zinc in β-cells, the use of which in combination greatly complicates the conduct of research.

*The purpose of the study*: to study the relationship between the content of zinc and insulin in β-cells of the pancreas, followed by the justification of the choice of one of the histochemical methods, which makes it possible to assess as a condition.

*Aim of work*: to investigate the relationship between the content of zinc and insulin in β-cells of the pancreas.

## *Experimental*

Animals: 12 rabbits, 2150–2400 g were used. Aim of work:

i) to investigate histotopography of the localization of zinc and insulin in β-cells of pancreatic islets, comparing the results of visual research;

ii) assess the content of zinc and insulin in cytoplasm of β-cells using quantitative histophotometric analysis by comparing the obtained data;

iii) in experiments using the complete elimination of insulin from the cytoplasm of β-cells to estimate the content of zinc and insulin visually and quantitatively comparing the results.

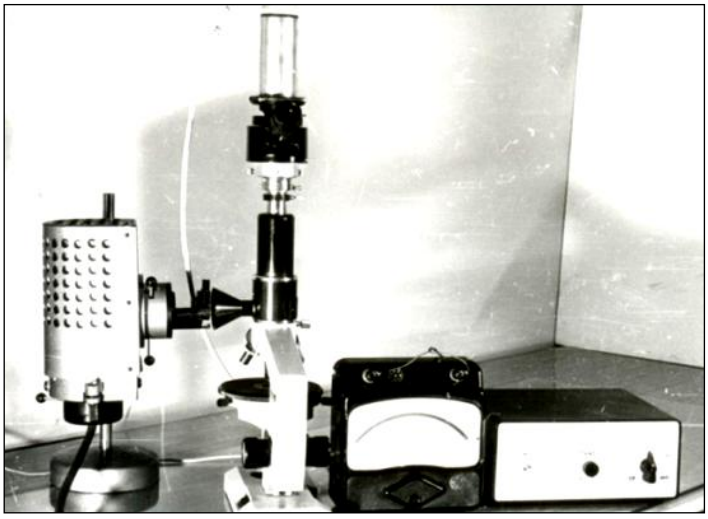
2 groups of animals: Group 1 — injection of 2 % water-ammonium solution of Diphenylthiocarbazone (Dithizon, DZ), which forms the red granules of Zn-DZ chelat complex in β-cells observed in the sections of the frozen gland of pancreas using of technique of dark field microscopy, which was previously confirmed by the method of spectral analysis of the absorption spectra of extracted from β-cells of the complex in com-

parison with the artificially obtained in vitro similar complex [4]; Group 2 — rabbits; elimination of deposited insulin from β-cells by repeated oral administration of Glibenclamide, 25 mg/kg within 3 days. 2 parts of pancreas tissue were used: the first was frozen in cryostat with the subsequent microscopy of frozen sections in a dark field. The second part was fixed for 24 hours in the Bouin liquid.

To evaluate the content of insulin in paraffin sections of the pancreas, an aldehydfuschine color method is used, which allows to evaluate both the content of insulin in β-cells and the state of the islands of the islands. The most important advantage of this method in comparison with others is the possibility of studying the histotopography of insulin in β-cells. Slices with a thickness of 4 μm were painted on insulin with aldehydfoxin [5], zinc content in β-cells was evaluated by a quantity assessment in the relative units of the granite of the ZN-DZ complex.

For a quantitative assessment of the content of deposited insulin in β-cells of light absorption, for which the microfluorimetric complex [6, 7] was used based on FEU-31, combined through a micrograph with a microscope (Fig. 1).

The obtained digital data were processed statistically using Student's t-test.



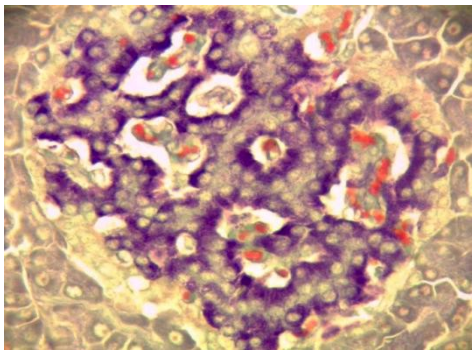
Composition of the complex: ultraviolet lamp; microscope; micro-meter; power supply;
FEU-31 photo-electronic multiplier; microphotonage with the adjustable diaphragm built into the field,
which locks the central part of the islets containing β-cells

Figure 1. Hystophluorimetric complex for the quantitative assessment of the content of insulin and zinc in V-cells

Photometry was subjected to β-cells localized in places of hormone exocitosis around the islet capillaries, which contain the largest hormone. The assessment of insulin coatings in the pancreatic islands was carried out in relative units (O.E.) in terms of indicator of light absorption K, determined by the formula: K = A2/A1, where A1 is the size of the photo flow that arose when measuring the coloring density of insulin aldehydfuxine in β-cells (in MKA) and A2 — the size of the photograph that arose when measuring the glow of the cells of exocrine tissue. When using an aldehydfoxy — a new method, it was evaluated by the formula with the reverse dependence (K1 = A2/A1, where A1 is the intensity of the light absorption with β-cells containing insulin and A2 — weakly absorption of exocrine tissue cells that do not contain insulin). That is, the denser the coloring — the more light it is absorbed by a photometer, respectively, the lower the size of the phototock and the higher the insulin content. When using a dytison method of zinc coloring, a direct proportional dependence was used (K2 = A1/A2, where A1 — gluttonization of painted β-cells and A2 — Iltoy glow of the surrounding fabric). That is, the more intense the color of the β-cells-the more light goes on a photometer and vice versa [7, 8]. For each option, 30 measurements of zinc and insulin accumulations were performed in β-cells located around the intra-regional capillaries.

*Results*

In painted by aldehydefucshine pancreatic tissue drugs of intact animals, saturated purple color indicates the presence of a large amounts of deposited insulin in the cytoplasm of β-cells (Fig. 2.1), which concentrates in the places of hormone around the islet capillaries.



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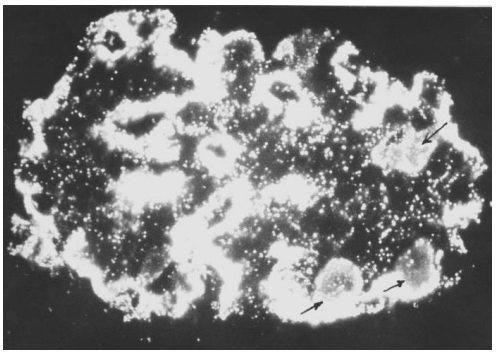

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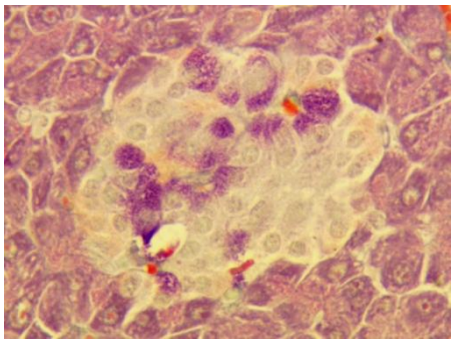

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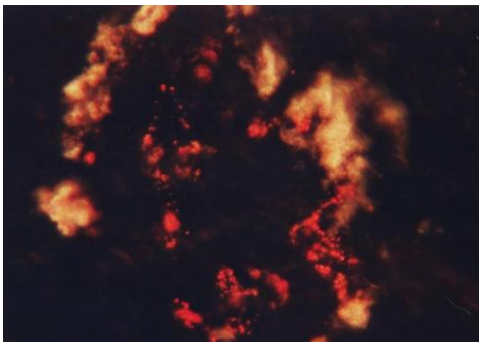

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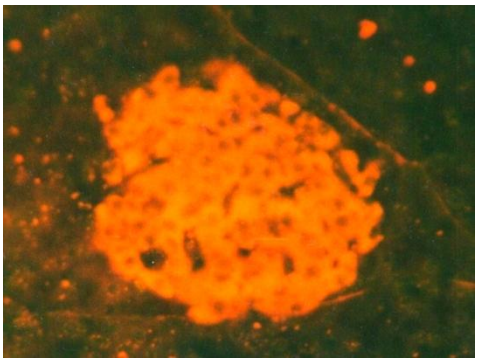

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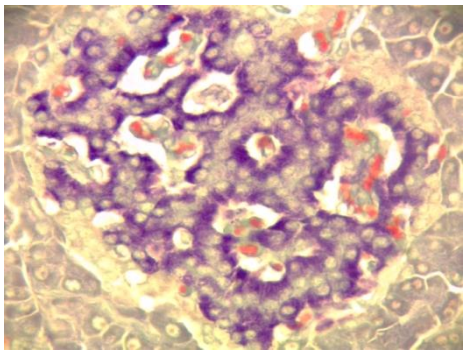

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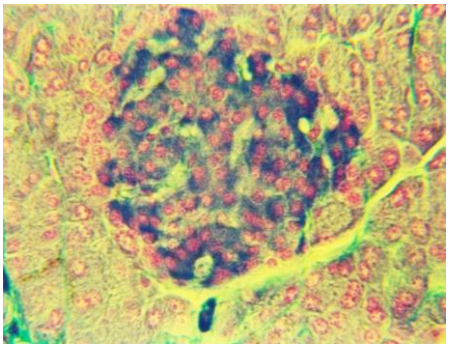

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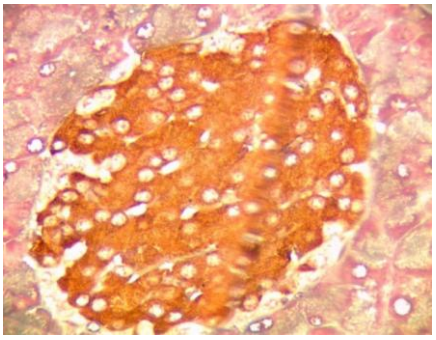

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*1* — Pancreatic islet of intact rabbit. Aldehyde fucshine staining. Violet color of insulin; maximum concentration of hormone in β-cells at the contact with the endothelium of blood capillaries; ×280. *2* — Pancreatic rabbit islet after administration of 47.2 mg/kg Dithizone; histotopography: Zinc-DZ granules are maximally concentrated around the capillaries, adjacent to the endothelium; ×280; *3* — Pancreatic rabbit islet after insulin elimination from β-cells by Glibenclamide, 15 mg/kg repeatedly within 3 days. Aldehyde fucshine staining; the absence of violet color indicates almost complete absence of insulin in β-cells; ×280; *4* — Pancreatic rabbit islet after elimination of insulin by Glibenclamide and followed administration of 48.6 mg/kg of Dithizone; almost complete absence of Zn-DZcomplex in β-cells; there are only single granules of the complex; ×280; *5* — Pancreatic island intact rat. Painting with Victoria 4R reagent. Insulin is colored dark blue; in pink around the periphery — A-cells; ×280; *6* — isolated intact pancreatic islet. Painting with Victoria 4R reagent. Dark blue colored insulin; ×280; *7* — Pancreatic island intact rat. Coloration by immunohistochemical method. Insulin is colored brown. Histostructure unchanged; ×280; *8* — isolated intact pancreatic islet. Coloration by immunohistochemical method. Insulin is colored brown. Histostructure unchanged, ×280

Figure 2. Accumulation of insulin in β-cells

In frozen sections of pancreas of intact animals after administration of 47.6 mg/kg of Diphenyltiocarbazone (DZ) 5 min after injection, a large amounts of chelat complex of Zn-DZ visible as bright red granules that filled the cytoplasm of β-cells (Fig. 2) were formed in β-cells.

A visual study of the histotopography of zinc and insulin in the pancreatic islets showed: the maximum amount of zinc and insulin were concentrated at the poles of β-cells that directly contact with the endothelium of capillaries (Fig. 2.1 and 2.2). Visually the localization of zinc and insulin did not differ. To obtain more convincing objective data, a quantitative assessment of the results was carried out

The results of the quantitative investigation of the content of insulin in β-cells did not reveal any reliable differences between the content of zinc and insulin in β-cells (Table), which is like confirmation of existing assumptions regarding the fact that insulin forms in β-cells a deposited form in the form of a complex with zinc.

To obtain additional direct evidence, experiments were carried out with selective elimination of insulin from β-cells by Glibenclamide. The results convincingly indicate that the excretion of insulin, accompanied by a sharp lowering of its content, up to the complete absence in β-cells, was also accompanied by the removal of zinc ions from β-cells in the same quantities (Table 1, Fig. 2), as evidenced by a negative reaction to zinc in β-cells. There were no accurate differences at the same time.

T a b l e

**Quantitative assessment of the content of deposited insulin and zinc in β-cells (in relative units, O.E.)**

| № | Groups of animals | Concentration of granules of complex $Zn^{+2}$-DZ in cytoplasm of β-cells ($A^1/A^2$ index) (o.e.) | Concentration of granules of deposited insulin in the cytoplasm β-cells ($A^2/A^1$ index) (o.e.) |
|---|---|---|---|
| 1 | Group 1 | 6.84±0.42<br>*n* = 28 | 7.08±0.29<br>*n* = 30 |
| 2 | Group 2 (mobilized by insulin from β-cells by Glibenclamide | 1.31±0.09<br>*n* = 26 | 1.28±0.09<br>*n* = 32 |

*Discussion*

The histological and specific histochemical methods of insulin staining in β-cells allow estimating its content mainly on the basis of visual analysis using light-optical or luminescent microscopy. Equally important is the parallel analysis of the zinc content in β-cells, given the existing ideas that zinc is involved in the formation of a deposited form of hormone in β-cells. In zinc-deficient conditions, or after zinc elimination together with insulin from β-cells by Glibenclamide mobilizing from β-cells together with insulin and zinc, may be created to disrupt the formation of a deposited form of insulin.

Meanwhile, two main methods are used for histochemical detection of zinc ions: Dithizone staining with the formation of a chelat complex Zn-DZ (4) in β cells in the form of bright red granules, or by using of fluorochrome toluene (sulfonylamino) quinoline (TSC), which forms a high specific complex Zn-TSC luminescent with bright green fluorescence [8]. Both methods are high specific to the recovery of zinc ions. The question arose: taking into account the experience we have accumulated earlier, which suggests that there is a marked parallelism between the content of insulin and zinc in β-cells, to detect histochemically simultaneously insulin and zinc using the same method? Is it also possible to argue that zinc and insulin ions are not a hormone and metal ions localized in the cytoplasm of β-cells separately, but a single complex that is a deposited form of insulin? Now, there are positive opinions on this matter, but no specific experimental confirmations of this have been given in the literature.

The results of the histopholuorimetric investigation by us are quite convincing that the insulin content in β-cells of the pancreas not only visually, but also quantitatively coincides with the zinc content. In experiments with elimination of insulin from β-cells showed that excretion of insulin is accompanied by simultaneous excretion of zinc. If zinc and insulin in β-cells were positioned as separately localized components, the excretion of insulin should not be reflected on the zinc content. The obtained results indicate that zinc is involved in the formation of the deposited form of insulin not just as a catalyst of the process, but as a component that binds to insulin and forms its deposited form as a Zn-insulin complex. Thus, by the content of insulin in preparations stained for insulin, the content of zinc can be estimated and vice versa: by staining of zinc using a reaction with Dithizone, the content of insulin in β- cells can be estimated in parallel. With regard to

the question of whether it is possible to limit the use of only one of the methods in order to evaluate the content of the other component in β-cells, it is advisable to use both methods, taking into account the presence of certain advantages for each of them. For example, the aldehydfucshine method allows staining not only insulin, examining the histotopography of the hormone in β-cells, but also estimate of state of other cell structures of pancreatic islets and cells of the exocrine tissue, so that the value of information in describing the state of the histostructure of the tissue increases significantly. A relative disadvantage of the Dithizone method of zinc staining is the short-term existence of sections of frozen sections of pancreas tissue. Its advantage is the absence of the necessity of using a number of procedures in the process of preparation and staining of sections of pancreas tissue.

*The procedure for coloring pancreatic tissue with Gomori aldehydfuxin in our modification (time item 11.14 was specified, item 17 was added*: 1) xylene — 5 min; 2) xylene No. 2 — 5 min; 3) xylene 3–5 min; 4) abs. alcohol 100º No. 1–5 min; 5) abs. alcohol No. 2–5 min; 6) alcohol 80º — 5 min; 7) distill. water — 5 min; 8) oxidizing agent — 2 min; 9) 2 % oxalic acid solution before discoloration; 10) distill. water — 5 min; 11) aldehydfuxin ("MERCK," Germany; "SERVA," Germany) — 5–7 min; 12) 70º acidified alcohol 1-differentiate; 13) 70-acidic alcohol No. 2-differentiate; 14) Halmi mixture — 1 min; 15) distill. water — 5 min; 16) distill. water 2–5 min; 17) abs. alcohol No. 3–5 min; 18) abs. alcohol — No. 4–5 min; 19) xylene — 5 min; 20) xylene 2–5 min; 21) imprisonment in a balm. Result: insulin in β cells stains violet A-cell yellow.

## *Conclusions*

1. The results of visual and quantitative assessment of insulin and zinc content in pancreatic β-cells indicate a complete correlation of results, which in turn confirms the participation of zinc as an integral part in the formation of the deposited form of insulin.

2. The most preferred method of studying the content and histotographic distribution of zinc in the cytoplasm of pancreatic β-cells is the Dithizone method of its intra-vital color in β-cells.

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Г.Г. Мейрамов, К.-Д. Конерт, А.Ж. Шайбек, Ф.С Абикенова, К.С. Темиреева, Ж.Ж. Блялова, К. Ельшина, А.Г. Мейрамова

# Панкреатикалық β-жасушалардағы инсулин құрамын гистохимиялық бағалаудың тікелейемес әдісі

Гистоқұрылымның жай-күйін және ұйқы безі ұлпасындағы инсулин мен мырыштың құрамын бағалаудың қазіргі әдістері гистоқұрылымның жай-күйін ғана бағалауға немесе ß-жасушалардағы инсулиннің құрамын көзбен көріп бағалауға мүмкіндік береді. Депонирленген инсулиннің құрамын және ß-жасушалардың гормонның осы формасын қалыптастыру қабілетін көрсететін мырыштың құрамын қосымша әдістің көмегімен бағалауға болады. Бұл жұмыс көлемін айтарлықтай арттырады және зерттеу жүргізуді қиындатады. Авторлар бір мезгілде гистоқұрылымның жай-күйін, сондай-ақ ұйқы безінің ß-жасушаларындағы депонирленген инсулин мен мырыштың мөлшерін бағалауға мүмкіндік беретін әдісті пайдалануды ұсынды. Инсулинді бояудың альдегид-фуксин әдісі, сондай-ақ ß-жасушалардағы мырышты анықтаудың дитизондық әдісі бір мезгілде депонирленген инсулиннің құрамын сандық талдау үшін пайдаланылуы мүмкін. Екі әдісті пайдалану гистоқұрылымның жай-күйіне баға беріп қана қоймай, ß-жасушалардың инсулинді синтездеу құрамы мен функционалдық қабілеттерін бағалауға, сондай-ақ мырыш көмегімен оның депонирленген формасын қалыптастыруға мүмкіндік береді.

*Кілт сөздер*: ұйқы безі, ß-жасушалар, инсулин, мырыш, дитизон, гистоқұрылым.

Г.Г. Мейрамов, К.-Д. Конерт, А.Ж. Шайбек, Ф.С. Абикенова, К.С. Темиреева, Ж.Ж. Блялова, К. Ельшина, А.Г. Мейрамова

# Непрямой метод гистохимической оценки содержания инсулина в панкреатических β-клетках

Существующие методы оценки состояния гистоструктуры и содержания инсулина и цинка в ткани поджелудочной железы позволяют оценивать либо только состояние гистоструктуры, либо, в лучшем случае, визуально содержание инсулина в β-клетках. Содержание цинка, свидетельствующее о содержании депонированного инсулина и способности β-клеток формировать данную форму гормона, возможно оценить с помощью дополнительного метода. Это значительно увеличивает объем работы и усложняет проведение исследования. Авторами предложено применение метода, позволяющего оценивать одновременно как состояние гистоструктуры, так и количественно содержание депонированного инсулина и цинка в β-клетках поджелудочной железы. Экспериментально подтверждено, что как альдегид-фуксиновый метод окраски инсулина, так и дитизоновый метод выявления цинка в β-клетках могут по отдельности использоваться одновременно для количественного анализа содержания и депонированного инсулина и цинка в β-клетках. Применение обоих методов позволяет не только дать оценку состояния гистоструктуры, но и оценить содержание и функциональные способности β-клеток синтезировать инсулин, а также с помощью цинка формировать его депонированную форму.

*Ключевые слова*: поджелудочная железа, β-клетки, инсулин, цинк, дитизон, гистоструктура, синтез инсулина.

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