ORAL LONG-ACTING PHARMACEUTICAL FORM OF INSULIN ON THE BASIS OF SELF-ORGANIZING KVASI-LIVING SYSTEM OF COMBINATORIAL PEPTIDES

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Diabetes mellitus is one of the most common serious diseases that is based on absolute or relative lack of hormone of the pancreas – insulin[1].

Therapy by insulin (insulin administration from the outside) is a traditional and single method of treating the disease allowing to compensate for the lack of insulin in the body [2,3].

The most common method of insulin administration is by a subcutaneous injection. This method is inconvenient, traumatizing for patients (especially children), causing physical and emotional suffering, but most importantly, it may itself exacerbate the pathology of the disease. The latter is due to the fact that with subcutaneous injection of insulin normal blood glucose levels are achieved through systematic hyperinsulinemia in peripheral tissues, whereas the liver (the main place of activity of the endogenous insulin produced in the body), is lacking insulin [4,5].

The only way to prevent the complications inevitably associated with insulin injection, is by achieving whenever possible a complete simulation of the natural pathways of hormone supply in a living organism – i.e., to simulate the physiological difference in the insulin levels in the portal and peripheral circulatory systems [6].

From this point of view, the oral (by mouth) way of insulin delivery is the most favorable [7].

The main obstacles hindering the creation of the oral forms of insulin are the hormone low resistance to the action of proteolytic enzymes in the gastrointestinal tract and low permeability of insulin through the epithelial tissue of the intestinal wall into the bloodstream that is due to low lipophilicity, and large size of hormone macromolecules [8].

Over the past decades there have been numerous attempts to create oral forms of insulin, but no one have succeeded in developing an effective drug that could compete with intravenously injected insulin on the therapeutic action [9].

Among the pharmaceutical forms of oral medications the most attractive and promising is the solid form [10], since it is the most comfortable and convenient in application, as well as in storage. In addition, the production technologies of these forms are relatively inexpensive and sufficiently developed [11,12].

We have proposed the new kvasi-living self-organizing system for the purpose of creating pharmaceutical oral forms of insulin. The system is a mixture of insulin oligopeptides with artificially increased negative charge of the molecules [13]
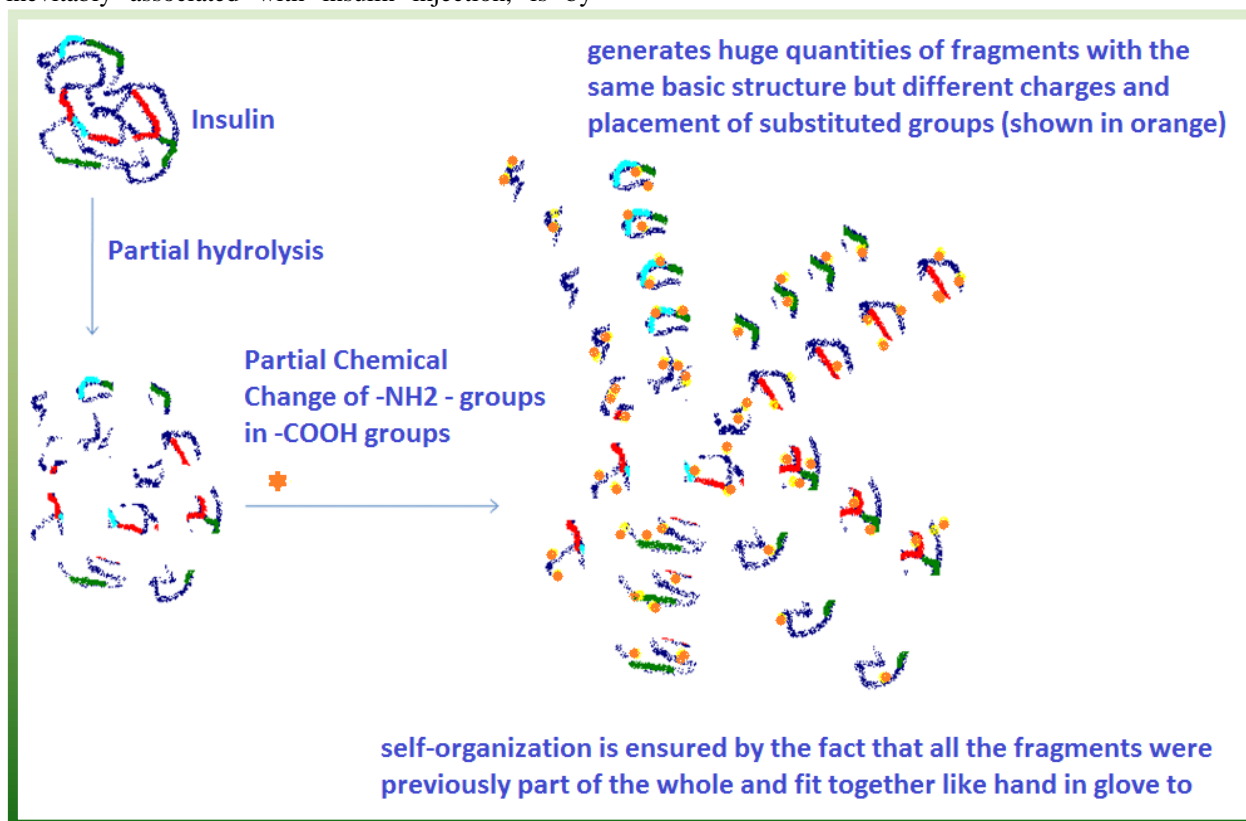
(Fig. 1)

Figure 1. - Synthesis of the kvasi-living self-organizing system based on insulin peptides for the purpose of creating an oral pharmaceutical form

As seen in Figure 1, the first stage of modification is the enzymatic hydrolysis of insulin molecule (in this case, pepsin). Next, the structure of the synthesized oligopeptides is partially modified in order to replace a part of positive charges in amino groups of lysine and histidine for the carboxyl residues of dicarboxylic acids. Partial modification is actually a combinatorial synthesis that leads to the formation of thousands of different peptides with different structure and specificity. Such a system is protected from the action of intestinal proteolytic enzymes, as it has been already hydrolized, and consists of small oligopeptides. It is freely absorbed from intestine due to small size of its molecules and like a complement can be collected on the insulin receptors into insulin-protein assembly [14].

The mechanism of such an assembly is well described in [15].Self-assembly of supramolecular peptide systems is also well studied in bacteriophages [16]. Initially, the number of modified peptides is redundant to ensure the process of self-organization in the insulin receptor. If the body of a diabetic has insulin antibodies, or receptors do not match the insulin structure (tolerance to insulin in diabetic type 2); also, if the number of insulin receptors is insufficient the kvasi-living system is capable of self-organization and self-assembly. It automatically picks up from excessive peptides only those components of the "mosaic" [17] that lead to the establishment of a truly effective kvazi-insulin on the receptor [18]. Antibodies do not effect these peptides, since the structure of peptides differs from that of insulin. Small size of the composite oligopeptides and excess negative charge of molecules block generation of antibodies and contribute to the long-term effect of the drug and provides the opportunity to apply such systems orally. Previously, α- and γ-interferons have been also modified by us with the application of kvasi-living systems' technology and they have shown completely new properties [19,13] ,.

The purpose of the research was to obtain an oral form of insulin on the basis of kvasi-living self-assembled and self-organized system of acylated peptides derived from enzymatic hydrolyzate of insulin (MI), and to study the effectiveness of the resulting system on the model of alloxan diabetes in rats.

Materials and methods

Synthesis of MI on the basis of insulin's succinylated peptides. Crystalline insulin (Indar, Ukraine) in the amount of 100 mg was dissolved in 1 ml of 0.1 M hydrochloric acid and then enzymatically hydrolyzed by incubation with pepsin (Fluka, 400 ED / mg) at room temperature for 1 hour. Then, while stirring the solution the powdered succinic anhydride (7.5 mg) was added slowly and incubated with stirring for 60 minutes. The resulting peptides were purified of salts in column Sephadex G-25, with TRIS-hydrochloride as the eluent. The yield of protein was contralled by the absorption of the eluate in the UV region of the spectrum, at 280 nm. Salt-free peptides were poured into vials and lyophilized. Further the hypoglycemic effect of MI on the model of alloxan diabetes was studied in rats: at rest and during glucose load. Input control of insulin was provided using the microfluidic method at bioanalyzer Agilent-2100, chip Protein-80 [20]. The chromatogram shown in Figure 3. MI was analyzed using high pressure liquid chromatograph at Millichrom-A-02 (Novosibirsk, Russian Federation) in the Microcolumn [21, 22], Hypersil-18 at a pressure of 30 kPa 5% ACN, 50 mM ADHP to 60% ACN, 50 mM ADPH. The chromatogram of peptides shown in Figure 4. MI was dissolved in 0.9% sodium chloride solution to form the solution, equivalent to 4.3 mg protein / ml, then 0.5% polysorbate stabilizer was added. 0.04% benzalkonium chloride was used as preservative.

The study of hypoglycemic action of MI administered orally to a model of alloxan diabetes

The experiments used 80 white (albino) rats of Vistar, males weighing 180-220 g. The care of the animals was provided in standard vivarium conditions. were maintained in standard environmental conditions of temperature (22-25°C), relative humidity (60 - 70%), dark/light cycle, and fed a standard diet and water ad libitum. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as guideline of the Animal Welfare Act. Diabetes was induced by single intraperitoneal injections of alloxan monohydrate at a dose of 120 mg / kg, freshly prepared from 0.9% sodium chloride solution. The animals were deprived of food for 24 hours before the injection of alloxan [23].

Diabetes was fully developed in rats 72 hours after the injection of the toxin, as evidenced by the level of glucose in the blood serum. For the experiments the rats were selected with a fasting glucose content above 11.1 mmol / l (fasting blood glucose). Glucose content in blood , taken from the tail vein was determined using glucometer "One touch Ultra" (USA) [24].

In the first set of experiments, without glucose, the animals were distributed into 4 groups of 10 animals per each:
1 - intact control (saline administration);
2 - intact rats injected with the modified insulin (MI);
3 - diabetes control (infusion of saline solution);
4 - animals with diabetes injected with MI.

Rats were fed for 18 hours before and 3 hours after administration of insulin and placebo. Modified insulin was administered in a dose of 50 U / kg, that is 5 times higher than the doze effective in rats (10 U / kg) according to references. The drug was dissolved in saline at the rate of 25 IU / ml and was administered through an intragastric probe in a dose of 0.2 ml/100 g. The control animals were injected saline solution in similar doses. Glucose content in rats blood was assessed prior to drug administration and then in 0.5, 1, 2, 3 and 24 hours thereafter.

In the second experimental setup, with a load of glucose, animals were distributed into 2 groups of 10 animals per each:
1 - diabetes control (infusion of saline solution);
2 - diabetic animals injected with MI.

The animals were deprived of food for 18 hours before the start of the experiment. Feeding was provided after taking blood samples for the three hour experiment. MI was given per os, in a dose of 50 U / kg, animal control group received saline. After 15 minutes, rats were injected with glucose in a dose of 3 g / kg (40% solution, 0.75 mg/100 g). In the experiments, the drug Glucose was used - the 40% injection solution in vials of 20 ml, manufactured by JSC "Farmak" (Kiev, Ukraine). Immediately before MI injection and 0.5, 1, 2, 3 and 24 hours after the load the glucose content in blood serum was determined.

The research results are processed with the method of variational statistics using Student's test, with significance level $P \le 0,05$
The data are presented in Table 1 and Figure 5

Results and Discussion

Figure 2 shows the diagram of insulin enzymatic hydrolysis by pepsin, and places accessible to succinic anhydride attack. As seen in Figure 3, hydrolysis results in seven oligopeptides. Partial acylation of these peptides is calculated according to the laws of combinatorics to obtain the maximum number of peptid derivatives. The ratio of insulin moles that should be modified to moles of anhydride is calculated according to combinatorial equation [24]:

$$m=(2^n-1),\qquad(1)$$

where:

m-number of molecules (and moles) of insulin, which must be modified to obtain the maximum amount of various insulin derivatives, this value for insulin is equal to 131,071

n-number of amino acid residues available for modification by anhydride in one insulin molecule (it is conditionally accepted that insulin is not hydrolyzed, and represents the whole molecule)

$$k=\frac{n(2^n-1)+n}{2}=n2^{(n-1)}\qquad(2)$$

where:

k- number of moles of succinic anhydride, which is necessary for the modification of a protein molecule containing n groups available for modification.

In our case, n = 17, k = 1114112. Thus, for the modification of 131 071 mol of insulin, 1,114,112 mol of succinic anhydride are required. This results in 131 071 different molecules of succinylated insulin. The molar ratio of anhydride to insulin is 8.5: 1. In this case, the synthesis will be observed of the maximum number of different insulin derivatives capable of interaction and self-organization into the supramolecular structure of kvazi-insulin on the insulin receptor.

Figure 2 shows the chromatogram of the industrial insulin Indar separation in bioanalyzer Agilent-2100 that operates on the microfluidic principle. As can be seen in the figure, insulin is presented in the form of two isomers with similar molecular weights that is characteristic of microbial proteins with different folding pathways. This chromatogram confirms presence of insulin in the initial preparation in case of its relatively high purity, and allows to yield MI.

Figure 4 shows the HPLC chromatogram of the final MI product, - namely, the mixture of acylated peptides after proteolysis of insulin by pepsin. As can be seen in the chromatogram, instead of the original seven peptides the significantly greater number of succinylated peptides is synthesized that confirms completion of the combinatorial synthesis reaction. This chromatogram can be further used as the primary method of quality control for the medicines based on kvasi-living systems.

Table 1. Dynamics of glucose content in blood of rats with alloxan diabetes after single oral administration of insulin peptide supramolecular assembly

Groups of animals	Glucose content in blood serum, mmol / l					
	Initial level	0,5 h	1 h	2 h	3 h	24 h
Control	4,68±0,20	4,76±0,23	4,61±0,19	4,62±0,15	4,51±0,20	4,71±0,18
Control+MI	4,57±0,18	4,58±0,19	4,67±0,18	4,71±0,13	4,67±0,13	4,56±0,15
Diabetes	19,48±1,77	19,83±1,53	18,24±1,34	17,58±1,36	16,23±1,43	19,49±1,29
Diabeted + MI	18,82±1,00	15,50±1,2[2]	11,99±1,22[1,2]	9,24±1,34[1,2]	7,74±1,56[1,2]	1,28±1,39[1,2]

Notes: [1] - Statistically significant differences relative to baseline values;
[2] - Statistically significant differences between groups of Diabetes and Diabetes + MI (p <0.05).

The modified insulin hypoglycemic action was studied in experiments conducted on rats with alloxan - induced type I diabetes. The results were compared with action of placebo and with intact control. As follows from the data in Table 1, the introduc-

tion of the modified insulin into intact animals (without diabetes) did not result in statistically significant changes in blood glucose levels. At the same time the introduction of the modified insulin to diabetic animals caused a significant change in this indicator. In the diabetes control group a gradual slight decrease in

blood glucose was observed associated with the lack of food in animals. It is known that in diabetes blood glucose is not a stable and tightly controlled parameter, as it is the case in healthy animals. Within 30 minutes after MI introduction a decrease of glycemia was detected. In all subsequent periods the glucose level decreased, and the differences were significant, both in relation to the original data, and to diabetes control. By 3 o'clock the figures reached almost normal levels and were more than 2 times lower than in the diabetes control group. The rates were significantly lower and 24 hours after MI administration.

The important aspects of MI action are:
1) Gradual pattern of changes, which exclude formation of diabetic hypoglycemia observed with the introduction of injectable forms of insulin. If our hypothesis is correct, this can be explained by the length of the process of insulin molecule self-assembly. This may also explain the lack of glucose reduction in intact animals treated by MI . The duration of the process allows activation of compensatory mechanisms that support stable glucose level in healthy organism (glucagon production, etc.).
2) Exceptional duration of the effect – is up to 24 hours. The phenomenon can be explanated by the following considerations. First, the structure of modified peptides of insulin may differ from the structure of native insulin. This makes them inaccessible to the action of the first enzyme that metabolizes insulin - hepatic glutathione- insulintranshydrogenase that is characterized by a high substrate specificity [25]. Secondly, the modified peptides of insulin, as noted above, may be unresponsive to insulin antibodies - their production does not occur, leaving MI active for a long time.
The action of MI is most clearly manifested under a standard glucose load in the background of fasting for 18 hours. The data in Figure 4 show that introduction of MI drastically alters the glycemic curves characteristic of diabetes. There is no distinct increase in glucose level within the first 1-2 hours after the load. The curve in this period is smoothed, and within 3 hours glucose level is reduced to almost normal values. As in the previous experimental setup, 24 hours after administration of the MI blood glucose was also significantly lower than in the diabetes control. Consequently, MI not only reduces glycemia smoothly within 24 hours, but also "takes care" of its postprandial increase.

Thus, MI has the following advantages:
1. mild action, absence of evident hypoglycemia;
2. prolonged effect;
3. smoothing of postprandial hyperglycemia.

Of course, the present paper provides only limited explanation of the obtained phenomena, and further research and testing is required. It is also necessary to evaluate the possibility of long-term (in a course) introduction of MI, and to identify the effective dose for this mode of introduction (it can be possibly reduced).

Conclusions: The kvasi-living system based on combinatorial acylated derivatives of hydrolyzed insulin has shown high biological activity when administered orally in rats with alloxan diabetes. The system promoted reduction in glucose level to 10 mmol/L on average, and maintained this level within 24 hours after a single application. It can be considered a candidate for development and implementation in the capacity of oral insulin. Efficiency of the preparation was confirmed in animals by using both fasting and glucose load.
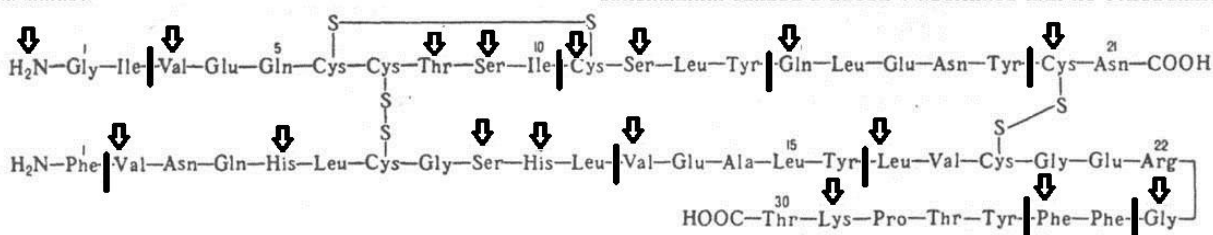
Figure 2. Black bars show places of insulin hydrolysis, when it is treated with pepsin: only seven peptides are produced, the amino group that should be attacked by anhydride are shown by black arrows (the number of groups available for acylation-n = 17).
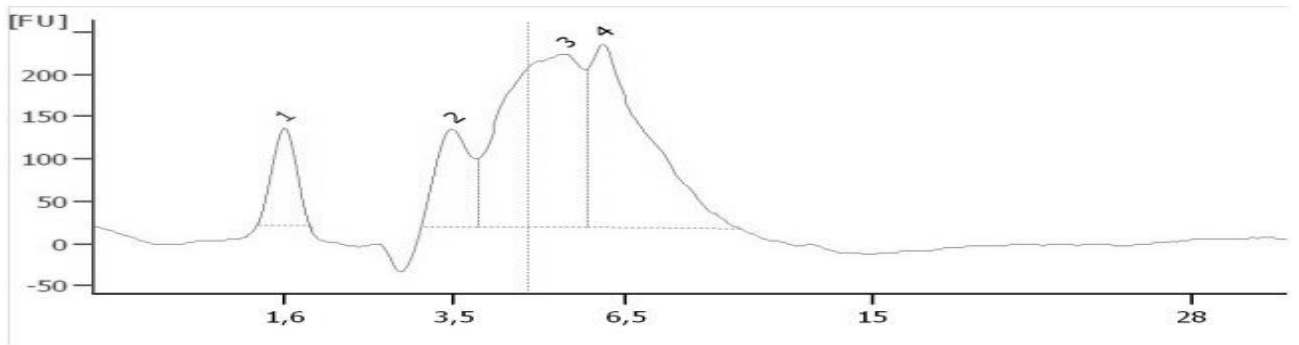
Figure 3. - Chromatogram of the input control of insulin. Separation in bioanalyzer Agilent -2100 Lab-on-chip Protein-80. Insulin peaks are represented by 3 and 4, with molecular masses of 5,5 ± 0,2 kDa and 6,1 ± 0,2 kDa
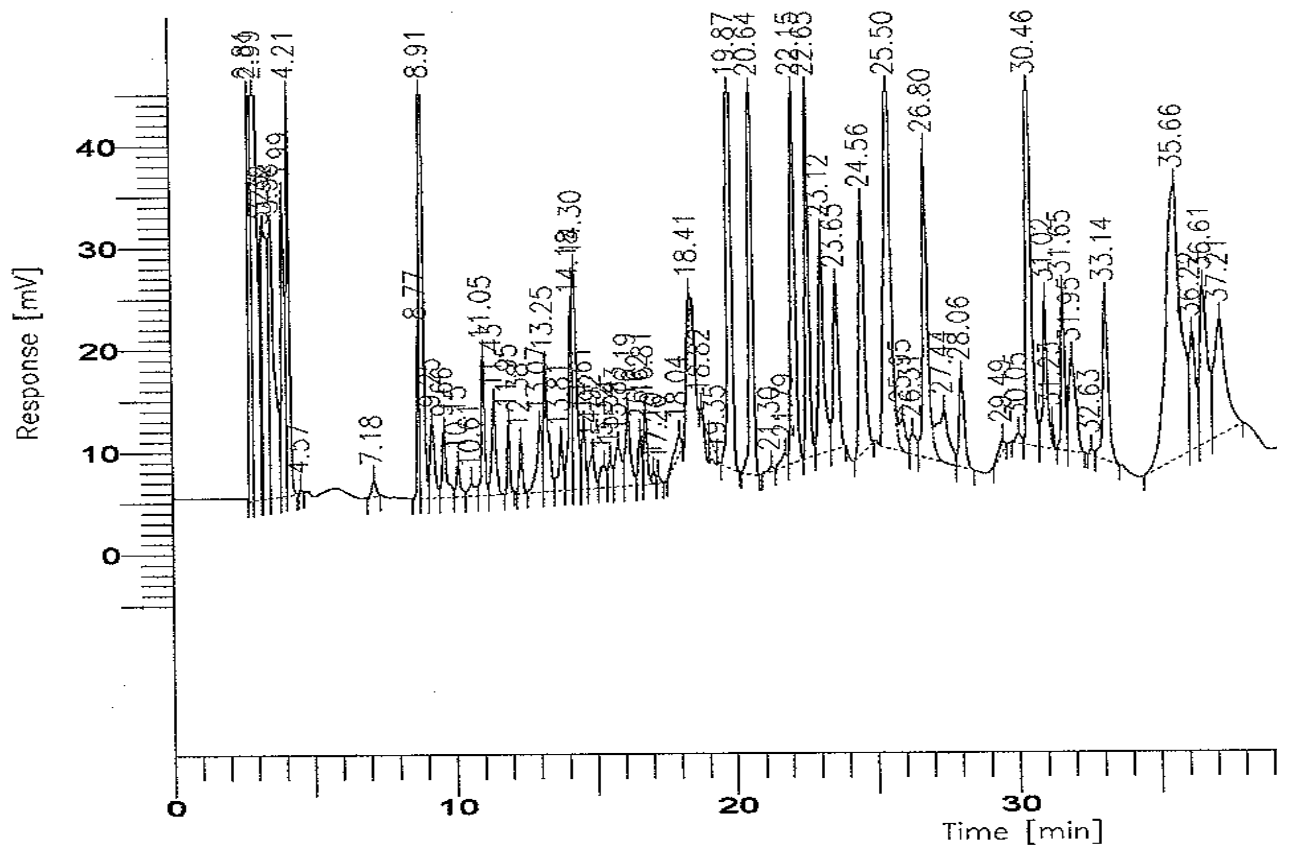

Figure 4. Chromatogram of MI after insulin treatment by pepsin and further combinatorial acylation of the synthesized oligopeptides. Enormous amount of oligopeptides apear, whereas after hydrolysis there should be only seven oligopeptides. Separation conditions: Hypersil-18, 5% ACN, 50 mM ADHP to 60% ACN, 50 mM ADPH
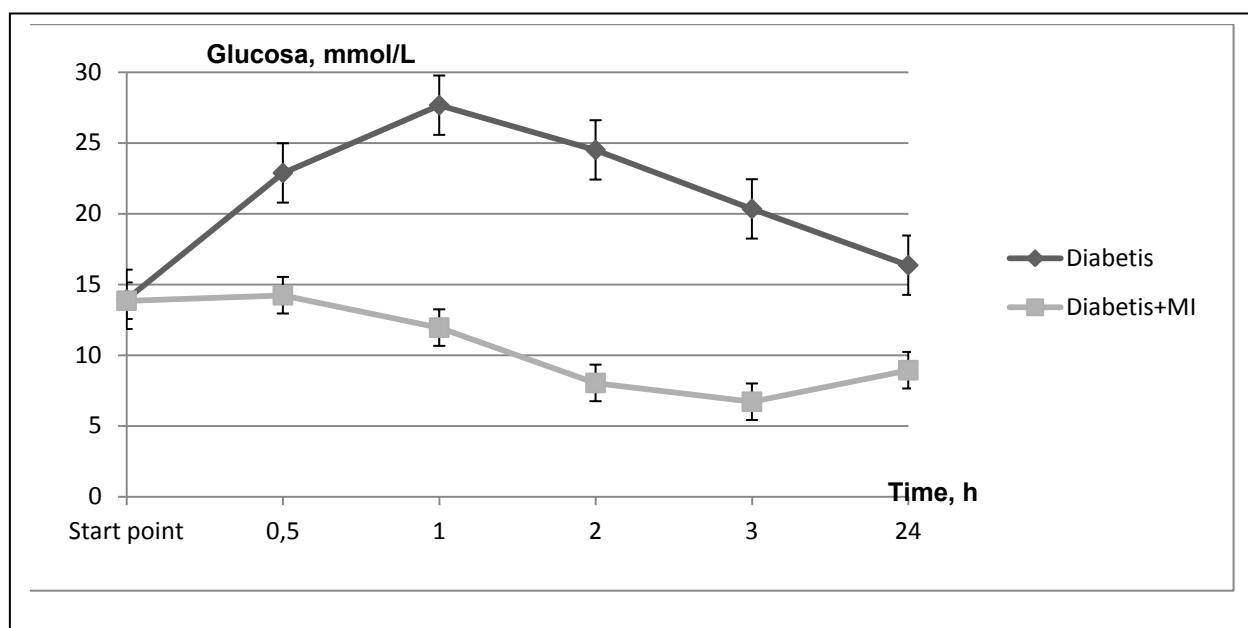
Figure 5. - The effect of MI on after-load glucose level in blood of rats with alloxan diabetes.

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The paper discusses the results of studies on physical and chemical properties of kvasi-living self-organizing insulin-based system, and on the effectiveness of its oral administration. The purpose of the studies was to modify positively charged amino acid residues into negatively charged residues of dicarboxylic acids. The process of bioorganic combinatorial synthesis produced more than 100 thousand fragments capable of self-organization in the insulin receptor. Self-organization is due to the fact that peptides were previously a part of the whole – namely, insulin molecule. These peptides had small size, and could be easily absorbed by intestines. They also had a long duration of circulation in blood and reacted with insulin receptor in a fashion similar to injected insulin. It is shown that a single oral application of such system leads to statistically significant and sustained reduction in blood glucose levels within 24 hours of application. The effect is observed in both cases: while taking the drug on an empty stomach, and with glucose and food load up to 7.11 mmol / L. A single dose of the drug led to a plateau of stable glucose levels and prevented hypoglycemia and glucose level jumps when applied to rats (control group). The kvasi-living system was obtained by partial proteolysis of recombinant insulin with pepsin, followed by partial modification of peptides with succinic anhydride.
Key words: insulin, oral form, self-assembled quasi living, succinylated

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