ACTIVATION OF KNOCKDOWN INSULIN GENES REGULATING THE GENERAL CARBOHYDRATE METABOLISM IN ADIPOSE TISSUE

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In work [1] it was shown that the sugar reducing effect of the new non-insulin oral preparation RL-175 on animals with experimental diabetes is reached by restoration of succagogue insulin activity of Langerhans islets destroyed by alloxan and activation of the insulin gene in β-cells.

The experimental preparation RL-175 apparently must be referred to the most promising ones in the treatment of patients with type II diabetes mellitus. Thus, the succagogue effect of many oral drugs [2] is achieved by increasing the level of *in vitro* ATP/ADP through the β-cells membrane depolarization, whereas under the influence of RL-175 preparation the number of insulin receptor points on the surface of target cells in various organs and tissues increases. As is known, [3] insulin resistance is developed precisely due to the reduction in the number of these receptors.

Another close link – the link between reduction in the organism sensitivity to the action of the endogenous insulin and such forms of pathology as obesity, metabolic syndrome, atherosclerosis, reproductive function disorder and others – was also revealed [3]. Obesity is one of vivid manifestations of metabolic syndrome, and therefore, of the development of non-insulin dependent diabetes mellitus (IND, T_2O).

This work represents new evidence of high regulation of carbohydrate metabolism and other physiological and endocrine functions in the adipose tissue under the influence of RL-175 preparation.

Research Design and Methods

In this work we used indirect insulin assay method *in vivo*, because this method is more informative than the well-known method based on the insulin ability to stimulate glucose oxidation in vitro [4, 5].

First, the observed increase of insulin level during hunger in the presence of normal or increased glucose concentrations, as well as simultaneous increase of insulin and glucose in response to glucose administration, is methodologically unacceptable for non-pedigree rats. These animals differ from one another by different insulin-resistant forms of glucose intolerance. Secondly, animals' organisms can maintain normal sensitivity to leptyne or adipocytes (adipose tissue hormones) despite significant development of obesity in some forms of genetic disorders [3].

In the experiment 160 white non-pedigree male and 30 female rats of 30 to 35 days of age and of the same weight were used. The animals were divided into 16 groups of 10 animals in each, which were kept in similar zoo-veterinary and feeding conditions in accordance with the requirements of the National Research Council.

Four hours before the experiment the animals were starving. Water intake was unrestricted. Determination of ketone bodies (acetone and β-hydroxybutyrate, in terms of acetone) was carried out in the morning hours when non-pedigree rats were starving for 4 hours under Peden method [6]. Development of knockdown diabetes in animals was characterized by a marked urination, thirst, weight loss and atony.

The first experimental group was orally administered with RL-175 preparation at a dose of 100mg/100g/ml dissolved in 50ml of 30% alcohol. The second group served as the physiological control. The first experimental and the second control groups

of rats were daily administered with the preparation and alcohol for 14 days. The third group served as the intact control.

The blood for testosterone and corticosterone content analysis was taken from the cervical vessels under ether narcosis before the beginning of the experiment and then every seven days for three weeks after the end of animals feeding with RL-175 and the solvent. Plasma was obtained by 15 minutes blood centrifugation in heparin at 3000 rpm. and it was stored at - 20^0C.

Testosterone was determined by direct radioimmunoassay, and corticosterone – by competitive protein-binding assay [4, 5]. Calculation of steroid hormones content in plasma and statistical results processing with the use of Student's method was performed on D-3-28 computer with the use of specifically designed program. The results of hormones content change in the animals' blood is represented in Fig. 1.

The rats from experimental IV-VIII and control IX-XIII groups were used as a test for studying RL-175 effect on the reproductive function and the body weight growth intensity.

Experimental and control groups of animals after the 4-hour starving were on daily basis weighed in the morning at one and the same time on the electronic analytical balance. The effect of RL-175 on the weight of seminal glands and appendages (Table 1) and the kinetics of spermatogenesis in the seminal canals of rats (Table 2) was measured with the use of standard method [7].

RL-175 effect of on the animals' weight gain was determined in doses: IV - experimental - 100mg/100g/ml, V - 50mg/100g/ml, VI - 20mg/100g/ml, VII - 10mg/100g/ml, and VIII - 5mg/100g/ml. Accordingly, IX-XIII groups of animals served as physiological control.

The total of 30 male and 30 female rats were also under experiment related to the study of RL-175 influence on the animals' reproductive function. Out of non-pedigree rats of both sexes three groups of 10 families couples in each were formed with the account of their age and body weight on the basis of analogues couples. All 30 family couples (in three XIV-XVI groups) were put in separate cages of 10 family couples in each, where they were kept during the entire experiment (92 days) in similar conditions of normal feeding, water and vivarium keeping regimes. The only difference was that the male and female rats in experimental group XIV were orally administered with RL-175 preparation at a daily dose of 10 mg/100g/ml once a day for 14 days. Groups XV and XVI served as physiological and intact controls. The results of the experimental preparation effect on the animals' reproductive functions are presented in Table 3.

In the experiment 52 4-months old pigs of the large white pure breed of one of the farms of North Ossetia-Alania were also used. The animals were selected on the basis of analogous couples with the account of their age and body weight. Four groups of 13 animals in each were kept in similar feeding and breeding conditions.

All animals received the same basic ration for fattening. The animals were fed twice a day with porridge-like feed. The first (1) control group of piglets received feeding diet without RL-175 preparation. The second (2) experimental group was given RL-175 preparation at a dose of 1.4 grams per head for the dry weight of the ration together with the feed. The third and the fourth groups of piglets were administered 1.7 g/head and 2.1 g/head, respectively, of the experimental preparation together with the basic diet. Administration of RL-175 was carried out on daily basis for 25 days.

The pigs were kept in groups in a pig-house on wooden floors with sawdust bedding in the microclimate conditions required by veterinary standards.

When the animals reached their 8-months age, the monitoring slaughter was carried out. Its results are presented in Table 4. The data on the body weight change of the experimental and monitor pigs for 120 days of feeding (without the preparation) are presented on Fig.3.

Results

In this work we used the method based on insulin ability to stimulate glucose oxidation in vivo. As is well known [4, 5], the cells of adipose tissue of the rats' appendage testes demonstrate particularly high sensitivity to this hormone. The biological activity of adiponektin and leptin hormones, which are secreted into the blood by the cells of adipose tissue of the rats' appendage testes, in stimulating the normal regulation of carbohydrate metabolism and other physiological and endocrine functions (burning fat in the body, secretion of pituitary growth hormone and some gonadotrophic hormone) is known [3].

This high activity of the living tissue hormones of the animals with knockdown diabetes was identified by the increase in the number of receptors of target cells to insulin and the conductivity of this hormonal signal to the transcriptional genes.

In particular, the content of ketone bodies in the blood of experimental animals served as the indicator of the knockdown level of the insulin-receptor gene in adipose tissue of appendage testes. After 4-hour starving of non-pedigree rats followed by oral administration of RL-175 preparation to the Ist experimental group at a dose of 100mg/100g/ml, the acetone content reached 0.89 ± 0.05 mg/% and β-oxybutirate – 2.5 ± 0.19 mg/% vs. 0.3 ± 0.03 mg/% and 1.8 ± 0.8 mg/%, respectively, in II and III control groups of rats (n = 160).

Under the influence of RL-175 preparation there was observed significant activation of testes in ontogenesis with the non-pedigree male rats, which resulted in more rapid and intensive growth of their weight exceeding the weight of testes of control rats of the same age and weight: with the rats beyond 6 months of age – 1.8-2.0 times, with adult rats (over 12 months of age) – 300-500 mg (Table 1).

Table 1. RL-175 preparation effect on the weight of seminal glands and appendages of the rats of different age composition and correlation of the organs weight and body weight (10 rats in each group, $p<0.05$)

Group composition	The weight of seminal glands and appendages of the rats of different age, mg					
	47-51 days			120-140 days		
	Seminal glands	Appendages	Correlation of the gland mass to the body weight	Seminal glands	Appendages	Correlation of the gland mass to the body weight
1. Experimental	700.0 ± 18.3	121 ± 6.2	7.8 ± 0.8	2077 ± 64.7	183.5 ± 6.3	8.3 ± 0.4
2. Physiological control	555 ± 38.5	48,0 ± 2.5	5.0 ± 0.5	1200 ± 44.5	92.0 ± 9.6	6.6 ± 0.4
3. Intact control	560 ± 63.2	67.0 ± 4.4	4.8 ± 0.4	1200 ± 93.1	110 ± 10.5	6.5 ± 0.5

The number of emerging spermatids in the body of one Sertoli cell of the experimental animals reached 40-60 pcs. and in the control ones – from 15 to 20 emerging spermatids. (Table 2).

Interstitial cell (Leidig cells) producing male sex hormones (androgenic hormones) and supporting spermatogenesis, became functionally active, which resulted in the increase of nuclei volume, increase of their general size as compared to the control. Nuclear-cytoplasmic ratio was within the normal limits.

Table 2. Indicators of adipose tissue cell function of the rat's testes appendages

Indicators	Groups	
	Experimental	Control
Number seminiferous epithelium layers:	1	1
1. Spermatoblast		
2. Primary and secondary spermatocyte	4-5	2-3
3. Spermatid	6-7	3-4
Number of forming spermatids on the body of one Sertoli cell	40-60	15-20
In % value to control	267-300.0	100.0
Sperm motility, in %:		
After 4 hours	60.0	45.5
After 12 hours	33.3	28.0
Number of immotile forms of sperm, in %	21.0	25.0
Number of degenerative changed sperm, in %	7.2	8.0

Examination of spermatozoa functional state in a drop of physiological solution showed higher sperm motility of the experimental rats as compared with control ones: after 4 hours the number of active mobile sperm was 60% (in controls - 45.5%), after 12h – 33,3% (in controls - 29%). The percentage of degeneratively changed spermatozoa did not exceed control values and was 8%.

The results of testosterone and corticosterone content change in blood plasma of non-pedigree white male rats, fed orally are represented on Fig. 1. From this graphic material we can note certain decrease of testosterone in the control groups of animals by the end of the second week, and its restoration to the initial physiological level after three weeks.

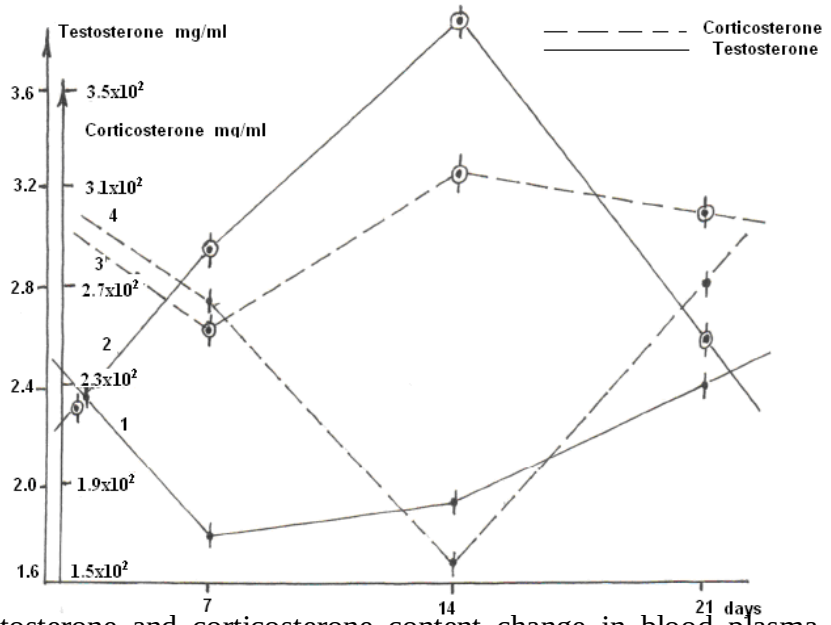

Figure 1. Testosterone and corticosterone content change in blood plasma of white non-pedigree male rats under the influence of RL-175 preparation.

Legend: 1, 4 – hormone distribution in the blood of control animals;
2, 3 – hormones distribution in the blood of experimental animals.

At the same time, with the experimental groups of animals, testosterone concentration after two weeks of RL-175 administration grew 1.5 times as compared to the benchmarks. After three weeks the hormone content decreases to the initial level.

On Fig.1 the change of corticosterone content allows making conclusions that the content of this hormone in two weeks after the experimental preparation administration to the animals twice exceeded the same control indicators. Moreover, there is a tendency for long-term conservation of high levels of corticosterone, which obviously indicates a high tension on adrenal function due to the involvement of a larger proportion of carbohydrates in general bioenergetic homeostasis of organism than in the control groups [8].

In the experiment the results of live weight and reproductive function growth of non-pedigree knockdown rats served as the criteria for measuring the effect of RL-175 on the secretion of leptyne. It was shown that in the IV experimental group of animals with the dose of 100mg/100g/ml the weight loss within 48 hours was from 20 to 36 g, after which the animals' weight restored to the norm as compared to the IX control group. On the fifth day one rat died.

In the V experimental group of animals with the preparation dose of 50mg/100g/ml there was observed weight gain in rats without preliminary weight loss. Weight gain 40% exceeded benchmark indicators of X group.

In the VI experimental group with the preparation dose of 20mg/100g/ml the weight gain overran the benchmarks of XI group by more than 50%. In VIII experimental group of rats with the preparation dose of 10mg/100g/ml the gains overran the indicators of XII control group by more than 50%. In IX experimental group with the preparation dose of 5mg/100g/ml the gains overran benchmarks of XIII control group by 45-50%.

The results presented in Table 3 allow concluding that in rats that were born from parents fed by the experimental preparation (XIV group), the true accelerated puberty was revealed. The terms of the first litter appearance in the experimental group decreased from 116-118 days in control groups XV and XVI to 89-80 days.

Table 3. Characteristics of the reproductive function of the control and experimental non-pedigree rats (Registration of litter in the "families" was carried out for 92 days from the time of the first litter, $p<0.01$)

Group composition	Number of families in a group	The age of rat families with the first litter, days	Number of born infant rats within 92 days			The interval between two litters, days
			In the first litter	For 92 days	Average per family	
XIV. The mal and female were orally administered RL-175 (in spore solution)	10	89 ± 3.0	98	239	2.4	38.3 ± 1.2
XV. Control female and male (physiological control)	10	118 ± 1.0	81	88	0.9	93.0 ± 0.8
XVI. Control female and male (intact control)	10	116 ± 1.7	86	119	1.2	92.0 ± 0.5

Counting of litter in «families» was carried out during 92 days from the date of the first animal yield. During the stated period the litter in control families of group XVI amounted to 119 infant rats, in XV control group - 81 animals. In XIV experimental group the litter in the «families» amounted to 239 infant rats.

Differentiation of seminiferous epithelium cells in the seminal canals of control and experimental groups of non-pedigree rats is presented on Fig. 2.

A B

Figure 2. Differentiation of seminiferous epithelium cells in seminiferous tubules with white non-pedigree rats. On the cross section of seminiferous tubules of XVI control group animals (A) there are five generations of gametal cells. While in XIV experimental group (B) on the cross section of seminiferous tubules there are at least nine layers of gametal cells generations.

Increased of fertility of non-pedigree white rats due to reduction of the interval between litters, as well as the emergence of early puberty under the influence of RL-175 preparation was observed with 5 generations (the observation period).

During the observation period we have not noted any marked aging of the rats' reproductive function. Physical development of the infant rats born in experimental «families» within the first month of postnatal life did not differ from that in control groups. The weight gain, eyes opening, acquisition of hair-coat covering with the rats in experimental families happened in accordance with physiological standard.

The analysis of metaphase plates of the rats' bone marrow showed the absence of chromosomal aberrations. All chromosomes were acrocentric, normal, helimerization was not disrupted.

As has been shown above in the Tables 1 and 2 under the influence of RL-175 preparation there occurs significant activation of testes and their appendages, resulting in rapid and intense growth of their mass, which exceeds the weight of testes and appendages of control animals of the same age and weight.

Felgen and Brachet histological study of the rats' testes showed significant increase in thickness of the convoluted seminiferous tubule walls amounting to 250-320 microns (in the control group 150-200 mc), increase in all sectors of seminiferous epithelium.

Activation of knockdown insulin receptor genes in the adipose tissue by RL-175 preparation is of extended nature, because the animals' generative function even at the age of 2.4 years is active [9, 10]. This is despite

the fact that the maximum life age of normal mice is 33-36 months, and the duration of reproductive function in males is 1-1.5 years, and in females is 1.5-2 years.

It was shown in special experiments on animals intensive fattening that in the experimental groups administered with different doses of RL-175 preparation, the increase of live weight of piglets in relation to benchmark indicators is as follows: by 5 months of age up to 18%, by 7 months of age up to 25%, by 8 months of age up to 22% (Fig. 3).

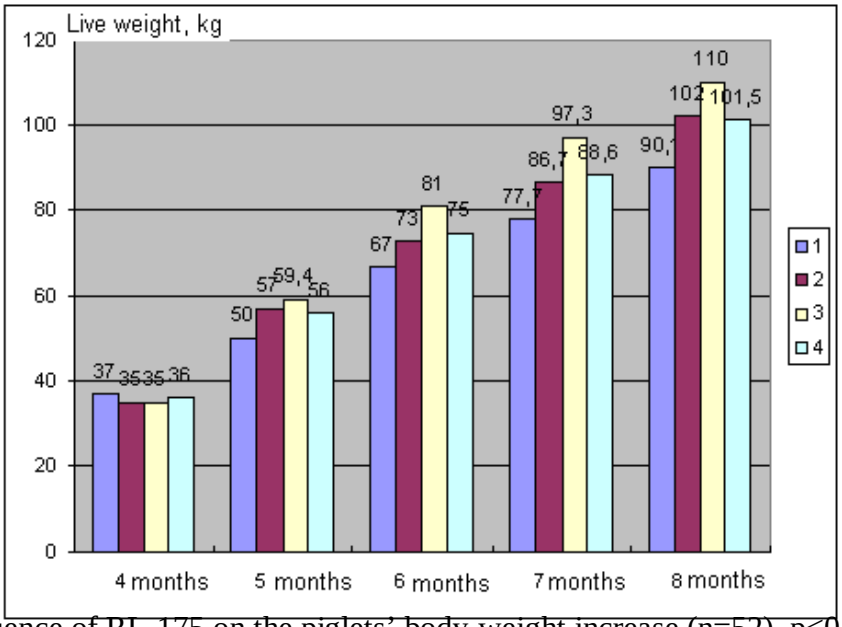

Figure 3. Influence of RL-175 on the piglets' body-weight increase (n=52), p<0,05.
Legend: 1 – Control group of animals, 2, 3, 4 – experimental groups.

The results of the monitoring slaughter of eight months old pigs (Table 4) show that the significant increase in the live weight of experimental animals takes place not only at the expense of the adipose tissue, but of the muscular tissue as well.

Table 4. Monitoring slaughter of 8 months old piglets and distribution of adipose, meat and osseous tissue in the animals deadweight (n=10 heads)

Indicators	Group	
	Control	Experimental
Average pre-slaughter live weight, kg	96.2	116.3
Average slaughter weight, kg	54.7	73.1
Slaughter output, %	56.8	62.9
% in carcass		
meat	43.5	42.1
fat	35.1	39.0
bones	21.4	18.9

Conclusion

While adipose tissue is a target of insulin action, it itself is a secreting endocrine organ and releases a number of hormones into the blood (leptyne, tumor necrosis factor α, interleukin 6, adiponektin, etc.) affecting insulin resistance. Secreting of adiponektyne by the adipose tissue is considered an impor-

tant factor in enhancing the sensitivity of tissues to the influence of endogenous insulin. Significant growth of testes and appendages, increased spermatogenesis speed and other physiological parameters (testosterone, corticosterone release) of insulin knockdown receptor cells of white non-pedigree rats as compared to the benchmarks (see Tables 1-3, Figs. 1, 2) confirm that the effect of RL -175 preparation is reflected both in genetic enhancement of insulin secreting and activation of secreting genes, adiponektyne and other hormones of adipose tissue.

Intensive growth of live weight of experimental rats and pigs as compared to the benchmarks, significant increase of rats' fertility due to reduction of the interval between litters, and distribution of fat and muscle tissue in the body weight of the specially fed pigs (Fig.3, Table 4) may also serve as a proof of high biological activity of leptyne and adiponektyne hormones under the influence of RL-175 preparation.

As is known [3], leptyne produced by adipocyte cells plays an important role in regulation of physiological and endocrine functions: it stimulates fat burn in the organism, increases the secreting of growth hormone, gonadotrophin and other hormones by pituitary.

Significant increase in sensitivity of various knockdown organs and tissues to endogenous insulin was accompanied by growth (recovery) of insulin receptors and agents of hormonal signal (Insulin Receptor Substrate - IRS 1, 2, 3, 4; Shc and GRB1; phosphatidilinositol-3-Kinase-PI3K) to transcriptional genes. It is believed that the reduction of the number of target cells receptors to insulin is the reason of epidemic spread of type II diabetes (T_2D) worldwide. For example, today there are 246 million persons suffering from T_2D in the world, and by 2030 the increase of this number to 366 million persons is anticipated [11].

Ronald P. Kahan with colleagues is the world leader in the study of molecular-genetic mechanisms of T_2D development. However, despite some progress diabetes morbidity worldwide is increasing.

In 2008 we proposed a new molecular-genetic mechanism of insulin genes activation participating in regulation of carbohydrate metabolism [1]. A new generation of highly effective non-insulin oral drugs of RL series was proposed, which leave behind the preparations known in the literature in terms of activation of insulin genes in β-cells of the pancreas as well as in other endocrine glands, protein kinase and intensification of the signal conductivity from the insulin receptor to the transcriptional genes.

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