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THE MULTIDIMENSIONAL EVOLUTION OF INSULIN FROM THE MOLECULAR TO THE THERAPEUTIC LEVEL

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Accepted

100 years since the discovery of insulin represents a very special event, which marks a crucial moment in the evolution of medicine worldwide, to which a major contribution was made by the famous Romanian Professor Nicolae Constantin Paulescu.

Related to this moment, this paper will point out some of the most important steps that have taken place over time, until now, without being able to accurately include all the information regarding the contribution that all those concerned have had on this theme.

The period preceding the discovery of insulin consists of a succession of events that took place over time, marked by contrasts and disputes between researchers, failures, disappointments and hopes, but also by discoveries and rediscoveries of certain phenomena in the context of that time.

Keywords: insulin, pancreas, β-cell, diabetes.

INTRODUCTION. DISCOVERY OF INSULIN

The discovery of insulin has truly revolutionized both the therapy and the prognosis of diabetes. This is one of the most studied diseases in the history of medicine, whose mentions are found in a collection of Egyptian medical texts written in the Ebers papyrus discovered in Thebes (1550 I.H.), as well as in ancient Indian and Chinese textbooks. [1,2] The term "diabetes" was introduced by Appollonius of Memphis and comes from the Greek word "diabainein" meaning "to pass through" which refers to the polyuria of patients. Indian doctor Sushruta, surgeon Charaka used the term "madhumeha" which referred to sweet taste of the urine. Aretaeus of Cappadocia was the first who made a complete, documented presentation of this disease. Claudius Galenus used the terms "diarrhea urinosa" and "dipsakon" and Avicenna in the Canon of Medicine described the abnormal appetite and gangrene in diabetic patients. Paracelsius in the 16th century established that the urine of patients contained a substance that after evaporation appeared as a white powder. The attribute mellitus derived from the Latin word "mel" and was added to the term diabetes by John Rollo. Since ancient times, it has been considered that the site of the disease is inside the kidneys a concept that will dominate medical thinking for the next 1500 years, given the polyuria and the sweet taste of diabetic urine. [3–6] Although diabetes was well known to the Egyptians, Hindus, Chinese and Greeks, a link between the disease and the pancreas was not made until the 19th century. The pancreatic islands were first identified in 1869 by Paul Langerhans. A few years later, Gustav Laguesse suspected the autocrine function of the island he called Langerhans[7,1,5] In 1907 Lane using histological staining techniques identified the β-cell in the structure of the pancreas. In 1938 – it was experimentally proven that the β-cell is a definite source of insulin.[8]

Before the 1920s there were no effective pharmacological agents for the management of diabetes and from this reason type I diabetes was a fatal disease. Things changed radically with the discovery of insulin. A major contribution to the

discovery of insulin was made by the Professor Nicolae Paulescu, who named the pancreatic extract "pancrein" and published the results of his studies in 1921 in the journal Archives International des Physiologie. In January 1922, the first human insulin injection was given. [9]

Biochemist James Bertam Collip developed a new protocol for the process of extracting and concentrating pancreatic extract. In 1923 Banting and MacLeod were awarded the Nobel Prize in Physiology or Medicine for their discovery of insulin. [10,11]

The award raised a lot of controversy and Best, Collip and Paulescu were excluded, and to compensate for this Banting and MacLeod decided to share the prize with Collip, while Noble and Paulescu were officially excluded from the discovery of insulin. [10]

The scientific community highlighted the efforts and merits of Professor Nicolae Paulescu, who was not sufficiently recognized for his contribution to the discovery of insulin. He showed that the pancreatic extract contained certain substances capable of producing an antidiabetic effect. [9,12]

The term insulin was mentioned in 1909 by Jean de Meyer, and it was also used by Edward Sharpey-Schafer in 1913 and MacLeod in 1923.[7,13]

MORPHOLOGY AND ULTRASTRUCTURE OF PANCREATIC β-CELLS

The islets of Langerhans become visible by perfusion of pancreas with neutral red, method that facilitates their counting. It was reported a number up to 1,800,000 of islets of Langerhans, prevailing in the tail of pancreas. On histological sections stained with Mallory azan method, the β-cells are recognized after the brownish-orange colour of their granules, as well as after their localization towards the interior of the islet. [14] It was thus estimated that 60–70% of an islet consists of β-cells.[15] On ultrathin sections, the β-cells appear in transmission electron microscopy as polygonal cells with large, euchromatic nuclei, containing in cytoplasm important amounts of rough endoplasmic reticulum and abundant Golgi apparatus. However, the main ultrastructural feature of these cells is represented by numerous cytoplasmic granules. The secretory granules contain, in man, a rectangular electron-dense crystalline core, surrounded by an electron-transparent region. In rat, the dense core of these granules may have more or less round shapes (Figure 1).[16] The mean size of the secretory granules in rat was reported to be about 460 nm, with dense cores of about 260 nm. [17]

Insulin biosynthesis takes place in the β-cell, following the model of polypeptide hormone synthesis. The process is diagrammed in the image on the top left. The insulin molecule is synthesized by the ribosomes attached to the rough endoplasmic reticulum as preproinsulin (a chain of 110 amino acids and 12,000Da), that becomes proinsulin – a molecule of 86 amino acids (of about 9,000 Da) after the removal of an N-terminus amino acid sequence (required for synthesis initialization, and molecule internalization into the endoplasmic reticulum lumen). The proinsulin also receives a G-shape configuration based on two disulfide bonds. Once transported to Golgi apparatus, the molecule is further processed by proteolysis, resulting three peptide chains: two forming the insulin and a third one known as peptide C. The chemical structure of the two-chains of the mature human insulin molecule was determined by Frederick Sanger in the early 1950s. The pioneering study showed that human insulin B-chain consisted of 30 amino acids and the A-chain of 21 amino acids, with the B- and A-chains being held together by the two disulfide bonds CysB7 to CysA7 and CysB19 to CysA20, with a third intra-chain disulfide bond linking CysA6 to CysA11. [18] Steiner identified proinsulin as the larger single-chain precursor of insulin, determined that proinsulin is the origin of C-peptide, and showed that the insulin and C-peptide were secreted from the β-cell in equimolar proportions. In 1968, Ronald Chance at Lilly Research Laboratories published the porcine sequence of the proinsulin molecule. [3]

After synthesis, the insulin is packed into vesicles known as secretory granules. Mature secretory granules contain biologically active insulin. Each β cell contains approximately 13,000 secretory granules, being in their way to the plasma membrane. The secretory granules have a higher density at the vascular pole of the β-cells. Through the phenomenon of Ca^{2+} – dependent exocytosis, the release of insulin from β cells in the interstitial space takes place, and from here, the hormone moves to the fenestrated capillaries of the islet. Once exocytosis occurs, the membranes of the secretory granules are recovered by clathrine-mediated endocytosis. Insulin exerts its main effect on blood sugar by facilitating the transport of glucose across the cell membrane. [19–22]

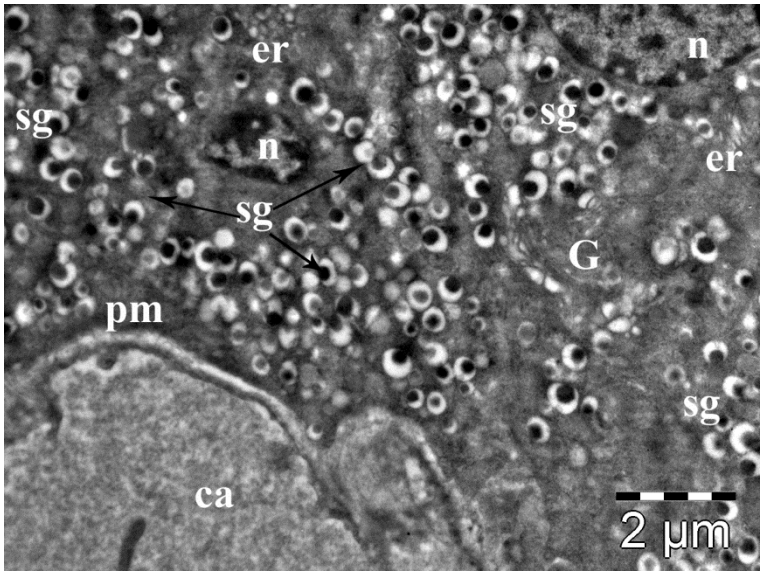

Figure 1. Transmission electron microscopy image of a normal rat pancreatic β-cell. ca=blood capillary; er=endoplasmic reticulum; G=Golgi apparatus; n=nucleus; pm=plasma membrane; sg=secretory granules. (personal archive Adrian Florea).

INSULIN PHARMACOLOGICAL FORMS DEVELOPMENT

Insulin was discovered by Sir Frederick G Banting and Charles H Best at the University of Toronto in 1921 and together with James Collip and John Macleod purified the hormone insulin from bovine pancreases. Hans Christian Hagedorn founded the Nordisk Insulin Laboratorium. In 1922 the Nordisk laboratory successfully extracted a small quantity of insulin from a bovine pancreas and the first patients were treated in 1923. [18,23]

In 1935 Dorothy Crowfoot Hodgkins took the first diffraction images of insulin crystals. These studies were concretized in 1969 at the University of Oxford with the first crystal structure of a peptide hormone and showing that insulin was a hexamer composed of three heterodimers. [3,24] In 1936 slowly absorbed insulin was produced by combining the hormone with protamine. In 1939 David Aylmen Scott created the insulin – protamine-zinc complex, whose glucose lowering effect lasted up to 48 h. In 1951–1952 were developed the amorphous "lente" insulins (IZS), semilente, lente, and ultralente. [10]

The first description of radio-labeled insulin binding to liver cell membranes was by the Australians House and Weidemann in 1970, followed by more detailed reports from two USA laboratories in 1971. [25,26]

In 1975, fully synthetic insulin (CGP 12 831) was produced. Genentech rDNA human insulin obtained in 1978 from combination of A and B chains individually expressed in *E. coli*. [10,27]

Crystal structures of zinc-free insulin dimers described by Caspar and colleagues demonstrated that the T2 substructure in the 2-Zn insulin hexamer does not require hexamer assembly or zinc-ion coordination. [28]

The first commercial insulin formulations were made with animal insulins, primarily beef and pork insulins, which had pharmacokinetics and pharmacodynamics properties very similar to those of human insulin in spite of differences in their amino acid sequences. [29,30]

By the 1990s, the first form of rapid-acting insulin analogue was approved (insulin lispro), followed by the first truly long acting insulin analogue (insulin glargine) in 2000.[7]

It is now recognized that human insulin is part of a larger family of sequence-linked hormones that includes insulin, the insulin-like growth factors IGF-I and IGF-II, the relaxin peptides relaxin-1, -2, and -3 and the insulin-like peptides INSL3, INSL4, INSL5, and INSL6. [31]

Table 1 describes the various forms of Insulin analogs – "analogs" of human insulin – "redesigned" insulin molecules ("designer insulins") by changing their primary structure or by attaching other molecules to obtain the "prandial" and "basal" effect of endogenous insulin.

INSULIN ADMINISTRATION

The main pathway of insulin administration is subcutaneous injection around the abdomen with the formation of a subcutaneous depot. For human

insulin the selection of the injection site is very important, the fastest absorption is in the abdomen as opposed to the thigh area. For an action similar as possible to the physiological state, it is preferable to administer prandial insulin in the abdomen and basal insulin in the thigh. Due to technological advancement, the absorption of second-generation insulin is not influenced by the injection site. In case of emergencies, intravenous administration by multiple injections or continuous infusion is preferred. Back-up administration in this cases may be by the intramuscular way.[32] The inhaled route is mentioned in studies with lower efficacy compared to insulin aspart and lower risk of hypoglycemia, its use being limited due to adverse effects and is contraindicated in patients with chronic lung disease or asthma.[33] Insulin patches may be another route of insulin administration. Insulin-laden microemulsions are currently the most effective, although they are still in the preclinical phase. Transdermal application is an interesting alternative to insulin injection.

Table 1

Year	Major event
1982-1983	Commercialization of the first insulins utilizing rDNA technology, Humulin® R (rapid) and N (NPH, intermediate-acting)
1996	The first insulin analogue (Humalog-Eli Lilly)
2000	Novo-Nodisk registered insulin Aspart (NovoRapid) The first long-acting analogue-insulin Glargine (Lantus, Sanofi-Aventis)
2004	Sanofi-Aventis registers insulin glulisine (Apidra)
2005	insulin detemir (Levemir, Novo-Nordisk)
2006	Approval of Exubera, the first inhaled insulin (Sanofi-Aventis and Pfizer)
2015	Approval of Degludec (Tresiba, Novo-Nordisk)
2019	Approval of Fiasp by FDA
2020	Lyumjev (from Elu Lilly)

Different insulin regimens have been developed that require adaptation to the patient's glycemic profile. The basal regimen consists of a basal insulin that ensures plasma insulin concentration both during the day and at night. The basal plus regimen requires the addition of a fast or ultra-fast prandial insulin, which controls the postprandial glycemic spike with less risk of late hypoglycemia. Prandial insulin is given before main meals with the addition of one prandial insulin in the basal plus 1 regimen and two in the basal plus 2 regimen. The basal bolus is the closest administration to physiological insulin secretion and is the insulin of choice in type 1 diabetes. Premixed insulin is fixed mixture of prandial and basal insulin, but it is not recommended because of difficult titration with consequent hindrance in reaching glycemic targets.[34,35]

Continuous subcutaneous insulin infusion is a substitute for basal and prandial insulin – only one type of short-acting or fast-acting insulin is used – low-dose subcutaneous (basal) and bolus (prandial) administration and association with continuous self-monitoring is absolutely necessary. It is recommended in children and adults with T1DM who have difficulty managing blood glucose levels by blood glucose monitoring and multiple insulin injections, those who cannot easily recognize hypoglycemic episodes, have a history of severe hypoglycemia, are susceptible to nocturnal hypoglycemia or are afraid of hypoglycemia episodes. [36] The first hybrid closed loop system approved by the FDA in 2016, is the Medtronic 670 G pump in patients over 14 years of age; with basal rate limitation (not below 8 IU/day basal rate). Advantages are suspended before low mode (stops 30 min before low limit, then restarts by itself) and auto mode (adjusts basal rates every 5 minutes. [37] Continuous self-monitoring (CGM) is a non-invasive, painless method that shows real-time blood glucose values and trends in blood glucose variability throughout the day and prevents hypo- and hyperglycemia. Compared to self-monitoring, CGM significantly reduces glycosylated hemoglobin values, glycemic variability and improves patients' quality of life.[38,39]

Management devices are disposable syringes, pen – advantage in use by patients with manual problems or visual disturbances (noise per unit insulin), insulin pumps – regulation by the patient and transdermal insulin injection devices.

"Smart insulin" – is a concept in the research stage, according to which an insulin particle could be modified so that it can be activated by a sufficiently high concentration of glucose in the blood.[40] The focus is now on the latest advances in the development of oral insulin formulations. Oral administration is much more appropriate and attractive compared to the subcutaneous route, but unfortunately can not be used due to poor epithelial permeability and enzymatic degradation in the gastrointestinal tract. Extensive research has been conducted to explore possible ways of administering insulin based on new methods, such as liposome, microsphere, nanoparticles, dissolving

bands in the oral cavity, sprays that allow oral and pulmonary absorption. These next-generation therapies could help improve the quality of life of diabetic patients on insulin therapy.

THE ARTIFICIAL PANCREAS

The first insulin pumps and devices for continuous glycemic monitoring appeared in the 1960s. Since then there have been attempts to create a system in which glycemic information could automatically influence insulin administration.[41] The artificial pancreas also known as advanced hybrid closed loop is used in management of T1DM. It has shown an effective time in range glucose values without increasing the risk of hypoglycemia, especially during night.[42] Their effectiveness may also reflect in good glycemic control in perioperative states associated with lower numbers of hypoglycemia and decreased variability of glucose levels.[43]

Bihormonal pump (insulin + glucagon) is a research topic performed in patients with T1DM. Currently, studies are being performed to optimize the action of insulin and the progressive automation of insulin pump therapy. The bihormonal pump can reproduce the physiological endocrine pancreas secretion. Compared with open loop control, in closed loop control system, time in range was achieved in 86.6% *vs.* 53.9% of time.[44,45]

The discovery of β-pancreatic cells and their decisive role in carbohydrate metabolism was an important milestone in the pathology of diabetes. They have a remodeling ability with an essential role in maintaining glycemic homeostasis. Normally there is a balance between the process of forming new β-cells and their apoptosis. [46,47] A reduced number of β-cells was reported in patients with diabetes. The cells die by apoptosis or necrosis. They are usually replaced by collagen fibers which lead to fibrosis of the pancreas. [48] In the experimental streptozotocin-induced diabetes in rats, the apoptosis of β-cells was also reported as highly increased, the remaining intact cells displaying important ultrastructural alterations, including smaller secretory granules (of about 275 nm) without the dense core, or with smaller cores (about 195 nm). [17] The origin of a new adult β-cell is a crucial question that scientists must answer in order to identify treatments that restore the mass of β-island cells in diabetic patients. Finally, future regenerative therapies should aim to stimulate the replicative capacity of β-cell-remaining cells in diabetic patients and / or induce neogenesis, stem cell therapy can be a promising treatment for diabetic patients.[49,50]

NON-INSULIN PHARMACOLOGICAL AGENTS

Synthesis of pharmacological agents, in which insulin therapy is a basic pillar in the treatment of this disease. Regarding other pharmacological agents used in the therapy of T2DM we can mention biguainides (metformin) and thiazolidinediones (pioglitazone, rosiglitazone) that have the effect of decreasing insulin resistance, also with encouraging effects on obesity and cancer.[51] Sulphonylureas (glimepiride, glibenclamide, glyburide) and glinides (repaglinide, nateglinide) increase insulin secretion from pancreatic cells, reduce glucagon production and improve insulin sensitivity of peripheral tissues. [52,53] Alfa-glucosidase inhibitors (acarbose, miglitol) have their action on elevated postprandial glycemic values and dipeptidyl peptidase-4 inhibitors (sitagliptine, saxagliptine) works on incretine hormones with increasing insulin and decreasing glucagon. The new pharmacological classes that have received the most attention in recent studies are sodium-glucose cotransporter protein-2 (SGLT-2) inhibitors (dapafliflozin, empagliflozin) and glucagon-like peptide-1 (GLP-1) receptor agonists (dulaglutide, semaglutide, liraglutide) with major benefits on cardiovascular and renal risk associated in patients with T2DM. [54,55]

Key messages. The discovery of insulin has really revolutionized both the therapy and the prognosis of diabetes. Insulin remains one of the most effective means of balancing disrupted metabolism in diabetes.

Insulin therapy aims to remove the symptoms of endogenous insulin deficiency by obtaining a glycemic control, as operative and sustainable as possible. Optimal metabolic control is reflected in the absence of clinical signs and severe hypoglycemia, as well as the absence of symptomatic hyperglycemia and ketoacidosis.

Therapeutic targets must be permissive, established in agreement with the doctor and the patient, with the gradual increase of their requirements. Insulin use requires motor and cognitive skills, therapeutic education. Achieving the set goals leads to avoiding the occurrence of

chronic micro and macrovascular complications and improving the patient's quality of life.

CONCLUSIONS

Insulin treatment is considered one of the most important advances in medicine and is currently one of the challenges of the daily practice of the diabetologist.

We are currently witnessing a large-scale concerted technological revolution in insulin injections and how they are being administered using highly ingenious and efficient devices.

β-cell cell regeneration is a major goal of antidiabetic therapy, with researchers pedaling in this direction.

Understanding the mechanisms that govern the growth and maintenance of β-cell mass represents an important issue that has persisted for a long time in the attention of the scientific community.

The performances reached in antidiabetic therapy would not have been possible without the perseverance of scientists, some more or less known, others more or less recognized, including Nicolae Paulescu, to whom we pay tribute today for his exceptional scientific merits.

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