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EDITORIAL

Role of vitamin C in diabetic ketoacidosis: Is it ready for prime time?

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Abstract

Diabetic ketoacidosis (DKA) is life-threatening acute metabolic complication of diabetes mellitus (DM) that is characterized by acidosis, ketosis, and hyperglycemia, currently affecting mostly patients under 30 years of age with diabetes mellitus type 1. In both, DM and DKA, a pro-inflammatory state exists. This clinical entity occurs as a result of hyperglycemia-induced disturbances, resulting in an increased oxidative metabolism. For the latter reason, the use of vitamin C seems promising in DKA due to its antioxidant role in reducing the superoxide radicals that are consequence of the oxidative stress. This can decrease the pro-inflammatory state and avoids complications. Vitamin C, or also known as ascorbic acid, has been widely used in several illnesses, such as common cold, tissue healing, fertility, atherosclerosis, cancer prevention, immunity restoration, neuro-degenerative disease and also has been suggested to decrease the risk of DM, and this reason is giving place to believe that vitamin C can have an important role in treating diabetic complications such as DKA. In order to counteract these oxidative disturbances in DKA patients, we analyzed the current data regarding vitamin C and evaluate its role in any type treatment of this complication in the near future.

Key words: Vitamin C; Diabetes complications; Ascorbic acid; Diabetic ketoacidosis; Diabetes mellitus

Core tip: The use of vitamin C in diabetic ketoacidosis (DKA) has remained controversial due to insufficient clinical data. The lack of concrete evidence, and no randomized controlled trials available on the use of vitamin C for DKA has caused significant controversies and debate. Some preliminary data, however, has shown a decrease in lipid peroxidation and limitation of endothelial damage. There is a significant need for a large randomized clinical trial to evaluate the role of vitamin C in patients with diabetes mellitus and specifically in those with DKA.

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INTRODUCTION

Diabetes mellitus (DM) continues to present a global challenge, with a large number of individuals being diagnosed daily around the world. It is estimated that the number of patients with DM in the world will be 366 million, or approximately 4.4% of the population by the year 2030[1]. A life-threatening complication of DM is diabetic ketoacidosis (DKA), which is an acute metabolic complication marked by acidosis, ketosis, and hyperglycemia. It results from lack of insulin, or insulin resistance along with increased levels of cortisol, glucagon, catecholamine and growth hormone. In addition, this clinical entity may be precipitated by an inadequate insulin administration, infection or other comorbidities (such as acute myocardial infarction, hyperthyroidism, stress)[2].

In the United States, most patients with DKA (54%-76%) are less than 30 years of age and have type 1 DM, with a mortality rate of less than 1% in hospitalized patients[3]. In these critically ill patients, an increase in the oxidative metabolism is commonly seen[4].

Ascorbic acid, most commonly known as vitamin C, is a water-soluble antioxidant, which has a role in scavenging superoxide radicals, and has been reported to inhibit low-density lipoprotein oxidation and stabilize the endothelium[4,5]. Vitamin C is essential for the normal physiological function of the body by playing a role in the synthesis and metabolism of tyrosine, tryptophan and folic acid, in addition to hydroxylation of proline, glycine and catecholamine. This vitamin also helps in lowering the cholesterol level by conversion of cholesterol into bile acid[6,7]. Vitamin C has also been widely used in the treatment of common cold, tissue healing, fertility, atherosclerosis, cancer prevention, immunity restoration, and neurodegenerative disease and has been suggested to decrease the risk of developing DM[7]. Furthermore, vitamin C is known to participate in the regeneration of antioxidants molecules such as tocopherol, glutathione, carotenes and urate[8].

DISCUSSION

Diabetes is characterized by a pro-inflammatory state, which leads to oxidative stress that results in the production of free radicals[9]. This has been studied in the context of DKA. For example, Lee *et al*[4] studied the degree of oxidative stress by determining the levels of fatty acids in six patients before, during and after DKA, as well as, the levels of vitamin A, C and E during these periods. In this study, lipid peroxidation was noted 24 to 72 h after correction of DKA; In addition, the levels of vitamin C and E were also decreased 24 to 72 h post correction of DKA. These authors suggested that vitamin C and E may play and important role in the presence of oxidative stress in DKA[4].

Recently, vitamin C has been shown to be beneficial in-patient with septic shock, opening a new era of interest in the role of vitamin C on many other diseases. There are several studies that have clearly documented vitamin C deficiency among patients who are critically ill with sepsis and septic shock[10-12]. To our knowledge, no randomized clinical trial analyzing the role of vitamin C in DM complications, such as DKA, is being done. Prior studies have shown that vitamin C ingestion interferes with testing devices that monitor glucose and ketones, giving false-positive results[13].

Ceriotti *et al*[14] showed that vitamin C exhibited falsely elevated readings for glucose and beta-hydroxybutyrate in hospitalized patients. Moreover, the use of vitamin C in diabetic patients has remained questionable due to a prior study performed by Beckman *et al*[15] showing that oral intake of vitamin C achieved a low concentration of plasma level, being unlikely to scavenge extracellular superoxide anion.

CONCLUSION

The use of vitamin C in DKA has remained controversial due to insufficient data collected in recent years. For the latter reason, it has not been applied in the clinical field. We believe that based on the data mentioned above vitamin C supplementation may have a role in patients with DKA. A large randomized controlled clinical trial aimed to identify if vitamin C supplementation in patients with DKA modifies their outcome is needed.

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REVIEW

Treatment approach to type 2 diabetes: Past, present and future

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Abstract

Type 2 diabetes mellitus (DM) is a lifelong metabolic disease, characterized by hyperglycaemia which gradually leads to the development and progression of vascular complications. It is recognized as a global burden disease, with substantial consequences on human health (fatality) as well as on health-care system costs. This review focuses on the topic of historical discovery and understanding the complexity of the disease in the field of pathophysiology, as well as development of the pharmacotherapy beyond insulin. The complex interplay of insulin secretion and insulin resistance developed from previously known "ominous triumvirate" to "ominous octet" indicate the implication of multiple organs in glucose metabolism. The pharmacological approach has progressed from biguanides to a wide spectrum of medications that seem to provide a beneficial effect on the cardiovascular system. Despite this, we are still not achieving the target treatment goals. Thus, the future should bring novel antidiabetic drug classes capable of acting on several levels simultaneously. In conclusion, given the raising burden of type 2 DM, the best present strategy that could contribute the most to the reduction of morbidity and mortality should be focused on primary prevention.

Key words: Type 2 diabetes mellitus; Physical activity; Hyperglycaemia; Insulin resistance; Hypoglycaemic agents

Core tip: Type 2 diabetes mellitus (DM) is a global burden disease and one of the leading all-cause mortality causes due to cardiovascular (CV) complications. The rapid raise in the understanding of its pathogenesis resulted in treatment approach options beyond insulin that also provide beneficial CV effect. We discuss this scientific pathological and pharmacological development through a comprehensive historical approach. The wide spectrum of therapeutic agents currently used in type 2 DM treatment result in a CV mortality reduction which is not exclusively in correlation with glucose-lowering potency but is linked to its mechanism of action.

Blaslov K, Naranđa FS, Kruljac I, Pavlić Renar I. Treatment approach to type 2 diabetes: Past, present and future. *World J Diabetes* 2018; 9(12): 209-219
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INTRODUCTION

Diabetes mellitus (DM) is chronic, lifelong progressive metabolic disease characterized by hyperglycaemia due to absolute or relative insulinopaenia. There are several different types of DM and each are caused by a complex interplay between genetic predisposition and environmental factors. The metabolic dysregulation that contributes to hyperglycaemia includes diminished insulin secretion, impaired glucose utilization or increased glucose production, and eventually causes pathophysiological changes in multiple organs and organ systems[1]. Despite all the scientific advances in the field of pathophysiology, diagnosis and treatment, the prevalence of DM has shown a dramatic rise over the past 200 years. Nowadays, DM represents a global burden disease with fatal consequences on human health, and significant impact on health-care system costs. It is estimated that in 2017, there were 451 million people (ages 18-99 years) with diabetes worldwide[2], and this number is expected to rise, mostly due to type 2 DM. Thus, we review herein the longitudinal history and therapeutic approaches beyond insulin for type 2 DM.

HISTORY AND CLASSIFICATION OF DM

The first documented symptoms of DM were recorded by ancient physicians in 1552 B.C. in a 3rd Dynasty Egyptian papyrus, being described as a rare mysterious disease characterized by excessive urination which leads to emaciation and death[3,4]. Around year 150 of the new era, the term "diabetes mellitus" meaning "honey" and "siphon" was introduced by an ancient Greek physician Aretaeus, reflecting the sweet urine taste in affected individuals[5]. However, its recognition as what is called a "clinical entity" - a condition that has separate and distinct existence from any known underlying cause or specific treatment option - occurred in an 1822 publication in the *New England Journal of Medicine and Surgery*[6].

The idea beyond this disease was not clarified until 1889, when Josph von Mering and Oskar Minkowski found that pancreatomy performed on a dog resulted in fatal diabetes[7,8]. In 1910, Edward Albert Sharpey-Schafer hypothesized that this might be due to the lack of a single pancreatic chemical, which he called "insulin"[6]. His hypothesis was confirmed by the discovery of insulin in 1921 by Frederick Banting and Charles Best[9,10]. After initially reversing diabetes in a dog using an extract from pancreatic islets of a healthy dog, together with James Collip and John Macleod they purified the hormone from bovine pancreas and used it to treat diabetes in humans[11].

From that point of time, DM has represented a fertile ground for scientific research[12]. Since 1923, 10 scientists have received a Nobel prize for diabetes-related investigation[6]. Over the past two centuries it became clear that DM does not represent a unique clinical condition with a common pathophysiological background. Namely, insulin transformed the lives of children and young adults with diabetes but had limited impact upon the survival of those diagnosed at the age of 50[6].

The classification of DM is primarily based on the pathogenic process that results in hyperglycaemia. In brief, it is now well known that severe insulin deficiency accounts for about 10% of all DM cases and is characterized by selective autoimmune destruction of insulin producing pancreatic β-cells, which are classified as type 1 DM, usually occurring in younger, lean individuals[1]. The majority of patients, however, belong to the group with insulin resistance as the core pathophysiological disorder rather than insulin deficiency[1], classified as type 2 DM. This type of DM is phenotypically often accompanied by central obesity, hypertension and dyslipidaemia.

The different pathophysiological background of hyperglycaemia was first proposed by Himsworth[13] in the year 1936, who tested the ability of injected insulin to clear an oral glucose load from the circulation. He concluded that there were insulin sensitive patients whose diabetes was due to insulin deficiency and insulin insensitive patients whose diabetes was due to resistance to insulin. These findings were strengthened by the research of Claude Bernard, who showed that blood glucose is also regulated by the non-glucose precursors driven by the liver[14-16], which probably represent the core of diabetes classification that was, however, not adopted until the 1970s[17] under the terms "maturity onset diabetes" and "non-insulin-dependent diabetes" (NIDDM). Those were abandoned in favour of the newer terminology between 1980 and the 1990s. The categorization of DM involving two principal groups, namely type 1 and type 2 DM, raises its own concerns,

especially in terms of type 2 DM because of diversity in clinical presentation and the natural course of the disease requiring an individual therapeutic approach[18].

PATOPHYSIOLOGY OF TYPE 2 DM: WHAT HAVE WE LEARNED IN THE LAST CENTURY?

Type 2 DM (formerly known as NIDDM) is a common metabolic disorder characterized by insulin resistance, relative impairment in insulin secretion, and certain degree of genetic predisposition, the prevalence of which markedly rises with the degree of obesity[1]. It is often accompanied by hypertension and dyslipidaemia: high serum low density lipoprotein concentrations and low serum high density lipoprotein concentrations that increase cardiovascular (CV) risk. The constellation of these clinical conditions is referred to as metabolic syndrome. Although, the risk factors associated with this type of DM were observed as early on as the 1920s and the term "metabolic syndrome" was coined in the 1950s when the French physician Jean Vague noticed that upper body obesity seemed to be associated with an increased risk for the conditions of atherosclerosis, diabetes, kidney stones and gout[19]. He also noticed that these patients show significant improvement in their diabetes, high blood cholesterol and high triglycerides following a low-calorie and low-carbohydrate diet[20]. The term became commonly used in the 1970s, and in the 1988, Gerald Reaven hypothesized that insulin resistance could be the underlying factor linking this constellation of abnormalities, which he went on to name "syndrome X"[21,22].

Indeed, it is now well known that type 2 DM usually presents with varying degrees of insulin resistance, consequent relative insulin deficiency, and hyperglycaemia which further impair pancreatic β-cell function, resulting in a vicious cycle of metabolic state worsening[23]. In addition, it is now well known that the majority of type 2 DM patients have genetic risk for its development. Its importance is supported primarily due to the observation that normoglycaemic offspring of type 2 DM parents have reduced non-oxidative glucose metabolism associated with increased muscle intracellular lipid content and reduced muscle glycogen synthesis[1]. This is due to a complex interaction among many genes and environmental factors (*i.e.*, a complex polygenic interplay which finally results in insulin resistance, namely decreased insulin sensitivity) that represents a core pathophysiological factor in type 2 DM development.

The exact molecular mechanism leading to insulin resistance has not been elucidated so far. Although the amount of insulin receptor expression on target tissues is diminished due to insulin's cellular internalization and reduced tyrosine kinase activity, the different expression probably represents the secondary and not the primary defect. It is considered that the post-receptor alterations in insulin receptor substrate-1 (IRS-1), regulating phosphorylation and dephosphorylation, might play a predominant role in this condition. Precisely, there is an imbalance between IRS-1 tyrosine and serine phosphorylation (Figure 1)[24]. Diminished IRS-1 tyrosine phosphorylation results in reduced translocation of glucose transporter type 4 (GLUT-4) to the plasma membrane, which enables glucose influx into the cells. Simultaneously, enhanced IRS-1 serine phosphorylation activates mitogen-activated proteins, whose action is not involved in metabolic but in mitotic insulin activity and proinflammatory pathways activation which result in intramitochondrial stress and further enhance insulin resistance. It is also implicated in the diabetes-related micro- and macrovascular complications' development. In brief, insulin resistance consists of two tightly coupled mechanisms: lack of suppression of glucose production and lack of glucose uptake by peripheral tissues, primarily muscles. Skeletal muscles usually utilize more than 80% of the circulating glucose in the presence of circulating insulin, while in the condition of insulin resistance this effect is diminished[19-22,24,25].

Furthermore, during the overnight fast, there is a substantial (1.8-2.0 mg/kg per min) glucose production, essential to meet the needs of the brain and other neural tissues whose uptake accounts for 50%-60% of total glucose disposal and is insulin independent. In euglycaemic individuals, the hepatic glucose production is suppressed following the glucose influx into the portal vein due to rise in insulin and inhibition in glucagon release[1]. In type 2 DM, this mechanism is diminished, which then results in both fasting as well as postprandial hyperglycaemia. The mechanisms involved in hepatic glucose production include hyperglucagonaemia, increased levels of circulating glucose precursors, free fatty acid oxidation, enhanced sensitivity to glucagon and decreased sensitivity to insulin[1,24].

A gradual increase in insulin resistance requires a notable higher amount of insulin in order to overcome hyperglycaemia. Consequently, as pancreatic β-cells start to release insulin from its secretory granules, a higher amount of amylin appears in higher concentration in circulation but also in pancreas itself[1,23]. Circulating amylin decreases glucose uptake in peripheral tissues, *i.e.*, enhances insulin resistance, while the pancreatic amylin further decreases pancreatic insulin secretion contributing to the hyperglycaemic state in both cases[1]. Thus, this complex interplay of insulin secretion and insulin resistance in the liver and the skeletal muscle, also known as "ominous triumvirate"[1], represented the first proposed fundamental mechanism of type 2 DM development and progression over the last two decades, *i.e.*, ever since it was established by a prospective study carried out by Jallut *et al*[26] in 1990 and later supported by many prospective studies carried out in diverse ethnic populations[27,28].

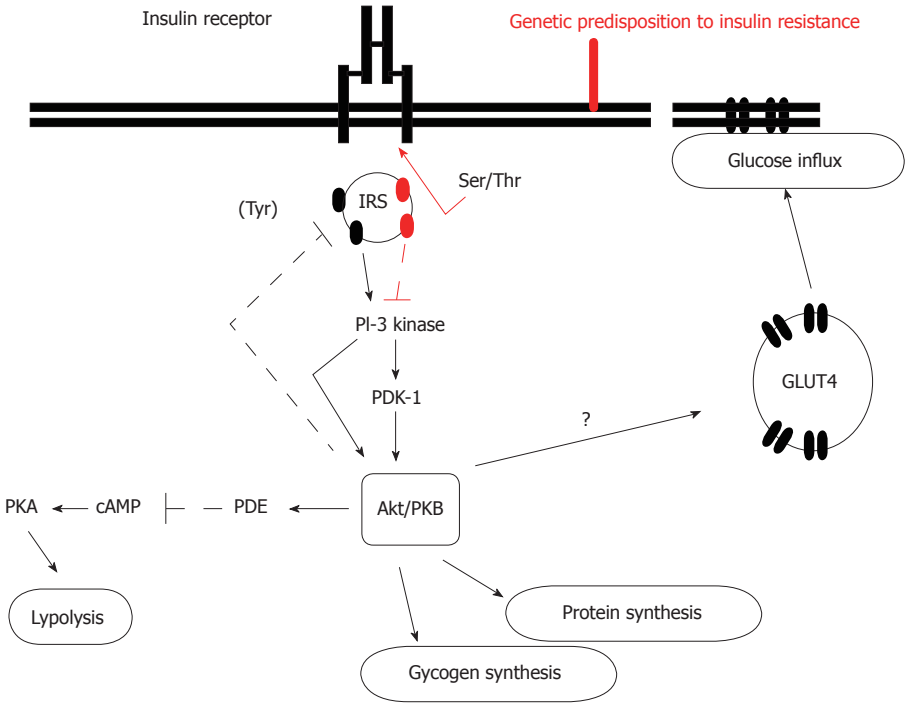

Figure 1 The molecular mechanism of insulin resistance. In insulin resistance, the binding of insulin to its receptor does not result in serine phosphorylation of insulin receptor substrate-1 and activation of the cascade of intracellular substrates' activation which result in glucose influx, glucagon and protein synthesis, and lipolysis inhibition. IRS: Insulin receptor substrate; Ser/Thr: Serine/threonine protein kinase; Tyr: Tyrosine kinase; PI-3: Phosphatidylinositol 3; PDK-1: Phosphoinositide-dependent protein kinase-1; Akt/PBK: AKT serine/threonine kinase 1 (protein kinase B family); PDE: Phosphodiesterase; cAMP: Cyclic adenosine monophosphate; PKA: Protein kinase A; GLUT4: Glucose transporter type 4.

However, since 1990 and until 2009, due to technological and pharmacological advances, there was a growing body of literature suggesting that the "ominous triumvirate" was not the sole pathophysiological disturbance in type 2 DM. An exponential growth of experimental and clinical studies suggested the possible implications of other organs: primarily pancreatic glucagon producing cells (α-cells), visceral adipose tissue, gut, kidneys and central nervous system. This was finally defined by DeFronzo[29] in 2009 under the term "ominous octet". As it gradually became evident that persons affected by type 2 DM have mostly a specific adipose tissue topography, *i.e.*, that visceral obesity is often accompanying type 2 DM, the two might be considered a part of this pathogenic process. It has been shown that they are also insulin resistant, resulting in the antilipolytic effect and thus leading to daylong elevation in the plasma free-fatty acid concentration[30-36], which not only further disrupts pancreatic β-cell function[37,38] but also promotes hepatic and muscle insulin resistance[39-41] and stimulates gluconeogenesis[42,43]. Moreover, the visceral adipocytes have a secretory capacity for a number of biological active products, including the adipokines, namely adiponectin, leptin, resistin, tumour necrosis factor-α, interleukine-1β, plasminogen activator inhibitor-1, retinol binding protein 4, *etc*., which are all associated with the function of intermediary metabolism[30,44].

After the basic elucidation of these so-called "dysharmonius quartet", at the beginning of the 2000s, the concept of type 2 DM pathophysiology was further expanded to include the gastrointestinal tissues as the fifth member of the "quintessential quintet". Even back in 1932, it was observed that the administration of an extract from the upper intestine could produce a fall in blood glucose, and the presumed hormone was named "incretin"[45]. Moreover, it was noticed that oral glucose administration resulted in a much greater insulin secretion, as compared to intravenous glucose infusion in a concentration that mimicked orally-absorbed plasma glucose concentration[46-48]; the phenomena was called "incretin effect". The discovery of two gastrointestinal peptides - glucose-dependent insulinotrophic peptide secreted by the K-cells of the more proximal small intestine, and glucagon like peptide-1 (GLP-1) secreted by the L-cells of the distal small intestine - probably mediate > 99% of this "incretin effect". They have been shown to delay gastric emptying, stimulate insulin and suppress glucagon secretion in a glucose-dependent manner. In fact, the diminished "incretin effect" has been repeatedly shown in type 2 DM patients[49-52].

The sixth member of the "octet" is the pancreatic α-cell. We have already mentioned glucagon, *i.e.*, the lack of glucagon suppression in the context of hepatic insulin resistance as well as in the incretion part, so it does not come as surprise that the higher fasting plasma glucose has been documented in several clinical studies in type 2 DM individuals back from the 1970s[53-58]. It has been demonstrated that higher concentrations of fasting glucagon closely correlate with

the increase in fasting hepatic glucose production as described in detail earlier in the paper. Furthermore, the authors showed a simultaneous decrease following somatostatin infusion. Thus, it is clear that hyperglucagonemia merits being considered as one of the key features in the pathogenesis type 2 DM.

In addition to muscle, liver, pancreatic α-cell and β-cells, adipocytes and the gut, the kidney is one of the two most recent members implicated in the pathophysiology of type 2 DM. Although excessive urination was one of the first described characteristics of DM[3-5] and the effort of its reduction was one of the first therapeutic approaches to DM while the sweet urine taste represented one of the first diagnostic approaches for DM, its pivotal role in type 2 DM pathogenesis was only recently explained. Physiologically, a total of approximately 162 g of glucose is filtered by kidneys on daily basis, and 90% of that amount is reabsorbed by the high capacity of sodium-glucose-transporter-2 (SGLT2) in the renal proximal tubule, while the remaining 10% of the filtered glucose is reabsorbed by the SGLT1 transporter in the descending part of the proximal tubule[59]. The result is that no glucose appears in the urine in healthy individuals. During the last two decades it became evident that in both type 1 and type 2 DM the maximal renal tubular reabsorptive capacity is increased[60,61]. This mechanism is due to markedly increased levels of SGLT2 mRNA and protein itself in the proximal renal tubular cells in type 2 DM[62]. In conclusion, the overactivation of an adaptive response by the kidney to conserve glucose seems to play an important role in hyperglycaemia development in type 2 DM.

Finally, the last component of the "ominous octet" is the central nervous system. Following functional magnetic resonance imaging investigations, it became evident that certain hypothalamic areas, precisely those which are involved in appetite regulation, show diminished inhibitory response in insulin-resistant type 2 DM individuals compared to normal glucose tolerant subjects, even though the plasma insulin response was noted as markedly increased in the obese group. This indicates that besides peripheral tissues, the insulin resistance, a common feature of all type 2 DM subjects, exists is the central nervous system.

Therefore, it is evident that the last few decades provided better and comprehensive knowledge on the pathophysiology of type 2 DM, which certainly had repercussion on the pharmacological treatment approach.

LONGITUDINAL HISTORY OF TREATMENT OPTIONS IN TYPE 2 DM

From the very beginning, even back in the times when frequent and excessive urination was considered a hallmark of DM, physicians tried to understand how it could be managed. They initially used the methods that could decrease this process, and there are documents reporting that horseback riding often was one of the first proposed methods[5]. Centuries later, Rollo[63] established the link between consumed food and the amount of glucose in the urine. He observed that carbohydrates increased glucose levels, while animal products' consumption resulted in less glucose[3,4,64]. Thus, he proposed that DM treatment should be based on a high fat and protein rich diet with low carbohydrates. This modification of diet became the recommended treatment for diabetes until the discovery of insulin[65].

Since the first proposal in 1877, a series of modifications and even individualized approaches, such as "oat-cure", "potato therapy" and the "starvation diet", persisted until 1916. This was the year when a Boston scientist, Elliot Joslin, was established as one of the leading diabetes experts upon creating *The Treatment of Diabetes Mellitus* textbook, wherein he reported that fasting diet combined with regular but moderate physical activity could significantly reduce the risk of death in diabetes[66]. Although the diet and physical activity concept underwent numerous changes, especially over the last two decades, they still represent two official fundamental treatment approaches for type 2 DM, beyond pharmacological treatment[67], and probably will remain so in the future.

The pharmacological treatment options in type 2 DM, however, did not occur until after the 1900s, and in the century of their development resulted in a wide spectrum of insulin and non-insulin hypoglycaemic agents experiencing an exponential growth. Although the discovery of insulin is considered the beginning of the pharmacological "DM-treatment" era, it is less known that the discovery and the use of oral hypoglycaemic agents (OHL) started 60 years ago, with an OHL class of biguanides that now represent the basic, first-line pleiotropic agent known as metformin[67]. Namely, during the medieval times, the French lilac plant (*Galega officinalis*) was used to relieve DM symptoms in Southern and Eastern Europe[68]. At the beginning of the 20th century, the anti-hyperglycaemic compound of the plant, guanidine, was isolated, synthesized and named Synthalin[69]. The further development of Synthalin and other guanidine homologs was stopped since they were hepatotoxic, and completely ended with the discovery and global use of insulin[70]. However, a resurgence of interest in the biguanides occurred several decades later, as the pathophysiology of DM as well as the difference in its clinical presentation (*i.e.,* beforementioned classification) became clearer. Although metformin was introduced in 1959, its wide clinical use started two decades after and it was not approved in the United States until 1990[68-71]; meanwhile, other agents from this class - phenformin and buformin - resulted in a significant number of lactic acidosis, which led to their withdrawal[70]. Nowadays, metformin represents the only clinically significant biguanide whose

primary mechanism of action is the ability to reduce hepatic gluconeogenesis and glycogenolysis due to enhancement of insulin resistance[72].

The sulfonylureas (SUs) represent the second and latest class of OHL, the discovery of which was triggered by the observation of an accidental hypoglycaemic effect. All the other OHL classes developed rapidly during the last two to three decades, accompanied by a deeper understanding of the complex pathophysiology of type 2 DM at the molecular level. The history of SUs starts in 1937, with the observation of hypoglycaemic activity of the synthetic sulphur compounds[73] and which was further confirmed by hypoglycaemia occurrence in typhoid patients treated with antibiotic para-amino-sulfonamide-isopropyl-thiodiazole[74]. In 1946, Auguste Loubatieres confirmed that aryl SU compounds stimulated release of insulin and, therefore, they require the remaining pancreatic β-cell function to achieve the effect[73,74]. Soon after, in the 1950s, the first SU - tolbutamide - was marketed in Germany[74], followed by the introduction of the other first-generation SU agents-chlorpropamide, acetohexamide, and tolazamide. The next advancement in SU therapy was the release of the more potent second-generation agents, back in 1984 - glipizide, glyburide, and gliklazide - and the third-generation agent glimepiride which was released in 1995[73-75]. Nowadays, the SUs are widely used, since they are generally safe, inexpensive, and relatively predictable, with hypoglycaemia as the primarily use-limiting side effect. In addition, due to research advances in seeking an agent that could be used with less fear of hypoglycaemia, the class of meglitinides was released on the market in 2000[76,77]. The meglitinides act similar to SUs, *i.e*., they enhance insulin secretion but their effect is diminished at low glucose concentrations. Thus, they might cause hypoglycaemia but less frequently and less severe than the "conventional" SUs[74].

The class of thiazolidinediones (TZDs) was initially introduced to the market in the middle of 1990. As a peroxisome proliferator–activated receptor-γ drug class activator, they were recognized soon after the discovery that the activation of this precise cell surface receptor enhances skeletal muscle insulin sensitivity and reduces hepatic glucose production[31,78]. This class of agents was thought to possess a similar but more durable effect than metformin. Troglitazone was the first TZD approved by the United States' Federal Drug Administration (FDA), and was soon accompanied by pioglitazone and rosiglitazone[79]. Trosiglitazone was removed from the market 4 years after its release, in 2000. This was due to the FDA having received reports of 63 hepatic failure cases with lethal outcome in patients treated with troglitazone[80]. Two other TZDs, rosiglitazone and pioglitazone, have been linked to fluid retention, which limited their use in patients with congestive heart failure. Pioglitazone has been shown to have a potentially modest beneficial impact on CV disease but has also been associated with a possible increase in the incidence of bladder cancer[79]. However, the wide use of rosiglitazone was soon associated with an increased risk of myocardial infarction, which led to its temporary market restriction, *i.e*., it remained available only in the United States. However, in November 2013, there was a change in position based on the findings of the large "Rosiglitazone Evaluated for Cardiovascular Outcomes and Regulation of Glycemia in Diabetes" (RECORD) study, demonstrating that individuals treated with rosiglitazone did not have an elevated risk of myocardial infarction compared to those taking other antihyperglycemic agents[81,82]. Despite this, pioglitazone remains the most used TZD at the present time, taking its place in all the available diabetes management guidelines as the first to second option, but one cannot speculate whether rosiglitazone will dominate the market in the near future.

Before the endocrine role of gut itself in the "incretin effect" became a clear and important pathophysiological player in type 2 DM development (which then led to introduction of so called "incretin" group of antihyperglycaemic agents), a class of drugs targeting gut, precisely small intestine α-glucosidase and thereby decreasing glucose absorption, was introduced into clinical practice[74-78]. The first drug in this category of α-glucosidase inhibitors that reached the market was acarbose, back in 1995, followed a year later by miglitol. Despite the logical background of drug development, neither drug has ever been used widely, probably because of the modest impact on A1C and their gastrointestinal side effects. However, following the discovery and elucidation of the incretin-insulin pathway, the "incretin based" class of agents became an intriguing and rapid growing area of research and development in the pharmaceutical industry.

At first, researchers became interested in the development of dipeptidyl-peptidase inhibitors - agents that could be taken orally and would prolong the circulating half-life of endogenous incretins[45-47,74]. First among these was sitagliptin, in 2006[83]; this was soon followed by saxagliptin, linagliptin, vildagliptin and alogliptin, comprising a separate OHL class, the "gliptines". Obviously, these drugs showed good results in the post-market clinical trials, as nowadays they are recommended even as second-line diabetes therapy[67]. Parallel to those, GLP-1 analogues were developed. The first analogue, exenatide was produced from exendin-4, which was isolated from the salivary gland venom of the Gila monster (*Heloderma suspectum*)[45-47,74]. This drug become available for clinical use in 2005[74]. A second GLP-1 receptor agonist, liraglutide, was approved in 2010. Soon after, *i.e*., over the last 8 years, the market of GLP-1 analogues has grown exponentially, starting with the once-weekly form of exenatide, dulaglutide, lixisenatide and albiglutide, to lately with semaglutide[74]. Although these drugs major advantage is weight loss, they also show cardio- and neuro-protective effects[67,84,85]. However, the exact mechanism of these post-market results remains to be elucidated.

And finally, after the "ominous octet" was completed with the elucidation of overactivation or renal tubular glucose reabsorptive capacity[59-62] due to a markedly increased level of SGLT2 in the proximal renal tubular cells[86], the selective SGLT-2 inhibitors were developed. More precisely, when SGLT-2 is antagonized, the excess of glucose in the renal tubules is not reabsorbed but instead secreted in the urine. Canagliflozin was the first SGLT-2 inhibitor to be approved by the FDA, in March 2013[87], which was followed by dapagliflozin in early 2014 and finally by empagliflozin.

Thus, here we described a wide spectrum of non-insulin therapeutic agents currently used in type 2 DM treatment. The official recommendations on their efficacy and safety, indications and contraindications, and effective combinations change almost on a yearly basis; yet, simultaneously we are experiencing an increase in diabetes-related complications, especially those leading to CV death[2,67]. This would, however, come as surprise if we kept in mind the results from the "Action to Control Cardiovascular Risk in Diabetes" trial[81] which clearly demonstrated that aggressive glycaemic control does not reduce the risk of CV death, despite the reduction in myocardial infarction.

NON-INSULIN THERAPEUTIC APPROACH AND CV RISK

As aforementioned in detail, due to the complex pathophysiological background of type 2 DM with the wide spectrum of CV risk factors that coexist in addition to the hyperglycaemia itself. It is important to emphasize here that the promising effects in terms of CV risk reduction were first published back in the late 90's, as an observation in the "STOP-NIDDM Trial" (an international study on the efficacy of an alpha-glucosidase inhibitor to prevent type 2 DM in a population with impaired glucose tolerance)[88]. The primary outcome of the study that clearly demonstrated that acarbose can prevent or delay the progression of impaired glucose tolerance to type 2 DM was recently confirmed in the "Acarbose Cardiovascular Evaluation" (ACE) trial[89], but it showed no effect in CV risk reduction. Thus, the "Empagliflozin Cardiovascular Outcome Event Trial in type 2 DM Patients–Removing Excess Glucose" (EMPA-REG OUTCOME) was the first clinical study that demonstrated superiority of a glucose lowering agent, in CV disease, heart failure, and renal and mortality endpoints compared to placebo[90]. Given the glycated haemoglobin (HbA1c) reduction of 0.45%, blood pressure in approximately 5/2 mmHg, and reduction in body weight by approximately 2%, the question arose: Could these results have been predicted based on what we knew about the mode of action?

Thus, shortly after the EMPA-REG OUTCOME trial was published, Ferrannini *et al*[90] developed a so-called "thrifty substrate" hypothesis which posits that in conditions of mild but persistent hyperketonaemia, β-hydroxybutyrate is taken up by the heart and oxidized into fatty acids. This selection of substrates improves the transduction process of oxygen consumption into work efficiency in the myocardiocytes. In addition, the enhanced oxygen release to the myocardium through haemoconcentration-driven by the diuresis[91,92] might affect a powerful synergy with the substrate shift. The rationale for this hypothesis came from experimental studies in diet-induced obese rats treated with dapagliflozin[93], ipragliflozin[94], or tofogliflozin[95] that demonstrated accelerated lipolysis and increased circulating ketone body levels, especially in the fasting state or when animals were fed in pairs. In addition, this was also confirmed in patients with type 2 DM following a 4-wk course of treatment with 25 mg of empagliflozin[96]. Concomitantly, both fasting and post-meal plasma β-hydroxybutyrate concentrations were increased 2-fold to 3-fold and these changes were similar in time course, though attenuated in extent, in a group of non-diabetic volunteers receiving the drug. This hypothesis of the thrifty substrate might also explain the similar outcome obtained by the "Combined results from the Canagliflozin Cardiovascular Assessment Study" (CANVAS)[97]. Nevertheless, the post-market period has been too short to support any general conclusion at this time.

And finally, some of the GLP-1 agonists, *i.e*., liraglutde[98] and semaglutide[99] have shown a significant relative risk reduction compared with placebo for the 3-point major adverse CV event primary outcome and relative risk in CV as well as in all-cause mortality, having low-to-moderate between-trial statistical heterogeneity. However, the concerns about their potential in pancreatic cell proliferation observed in experimental studies has not been elucidated so far, due to the relatively short post-market period[100].

CONCLUSION

In this narrative review, we described the rapid development of the pathophysiology type 2 DM concept accompanied by lifesaving treatment options beyond insulin that have dramatically enhanced the quality of life and life expectancy of affected individuals. The nephroprotective effects of angiotensin-receptor blockade, angiotensin-converting enzyme inhibition and protein restriction have been shown[99-104], while laser photocoagulation has preserved the vision of millions of patients with diabetic retinopathy[105]. The target hyperglycaemic agents' development has resulted in better glycaemic control, which has increased the focus of their potential in the context of development of diabetic complication preventive strategies.

It is now known that better gluco-regulation results in development and progression of microvascular complications, according to the large, population-based studies of Diabetes Control and Complications Trial[106] as well as United Kingdom Prospective Diabetes

Study[107]. Additionally, the follow-up ACCORD study has showed reduced myocardial infarction with improved glycaemic control, but it didn't provide an all-cause CV mortality rate. This finding raised awareness that an exclusively glucose-centric approach to diabetes will most likely not lead to reduction in all-cause CV disease mortality[98]. This finding was further strengthened by the Steno-2 trial[108,109], which demonstrated up to 50% CVD mortality with a multifactorial, instead of gluco-centric, control.

Thus, the present position statement for type 2 DM treatment comprises the simultaneous approach to control of glucose along with lipids, blood pressure and obesity[109,110]. Since obesity accompanies more than 80% of the type 2 DM population and contributes to other targeted factors' improvement, its treatment strategy should be a priority in the comprehensive assessment of diabetes care. According to the American Diabetes Association Standards of Care from 2016, bariatric surgery should be considered in obesity management, in addition to behaviour modification and pharmacotherapy[111].

However, despite all the knowledge and all the pharmaceutic agents that are available, there will always be a need for more effective treatment options in order to affect the disease in an even more precise pathophysiological pathway, in the near future. For instance, we can expect a completely novel antidiabetic drug class-oxidative phosphorylation blockers, currently represented by imeglimin[112]. The underlying mechanism of action of this drug class consists of balancing bioenergetics in mitochondria and consequent insulin resistance to result in balance of insulin secretion and utilization as well as hepatic gluconeogenesis suppression. Thus, it is important to emphasize that this is not the sole promising novel drug class. This is indicated by findings from the research on the first-in-class drug, an adenosine monophosphate (AMP)-activated protein kinase activator, targeting one of the key players in the process of energy balance preservation, especially during caloric disturbances[113], and, finally, findings on the first monoclonal antibody, bimagrumab, that blocks the myostatin type Ⅱ receptor, which results in fat reduction[114].

In conclusion, given the raising burden of type 2 DM and CV mortality due to diabetes, despite all of the therapeutic options that are available or will become available in due time, we should be focused on primary prevention, *i.e.*, targeting preventive public health policies and in the rigorous evidence-based initiatives to introduce dietary products that will address metabolic disturbances.

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MINIREVIEWS

Unexpected alliance between syndecan-1 and innate-like T cells to protect host from autoimmune effects of interleukin-17

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Abstract

Innate-like T cells, namely natural killer T (NKT) and γδ T cells, play critical roles in linking innate and adaptive immune responses through rapid production of cytokines. Prominent among these cytokines is interleukin-17 (IL-17), which is a potent proinflammatory cytokine that plays a critical role in host defense against fungi and extracellular bacteria. However, excessive IL-17-production promotes autoimmune diseases, including psoriasis, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, and systemic lupus erythematosus. IL-17 has also been implicated in regulating body fat, which is highly relevant given rises in obesity and type 2 diabetes. NKT cells, γδ T cells and mucosal-associated invariant T cells (MAIT) are the major sources of IL-17 involved in protection of mucosal surfaces from opportunistic infections and causing autoimmunity when become dysregulated. Given the pathogenic effects of IL-17, efforts have been directed towards understanding mechanisms that guard against IL-17 overproduction. One novel potent mechanism is mediated by the heparan sulfate proteoglycan, syndecan-1 (sdc1), which is selectively expressed by IL-17-producing subsets of NKT and γδ T cells. This unexpected role for sdc1 is uncovered by analysis of NKT and γδ T cells in sdc1-deficient mice. In this mini-review, we discuss selective expression of sdc1 by these innate T cells and consequences of its absence on IL-17 homeostasis and pathological implications.

Key words: Natural killer T cell; Natural killer T 17 cells; Tγδ17 cells; Syndecan-1; Interleukin-17

Core tip: Interleukin-17 (IL-17) is a potent proinflammatory cytokine that plays a critical role in host defense against fungi and extracellular bacteria. Excessive production of IL-17, however, has been implicated in pathogenesis of many autoimmune diseases. Our recent findings show that natural killer T (NKT) cells and γδ T cells employ syndecan-1 (sdc1), a heparan sulfate proteoglycan that is predominantly expressed by epithelia, to prevent out of control expansion of IL-17-producing subsets of NKT (NKT17) cell and γδ (Tγδ17) cells. In this mini-review, we highlight these findings and briefly discuss their significance for developing new strategies to prevent IL-17-mediated autoimmme diseases.

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INTRODUCTION

Recent data show that innate-like T cells utilize sdc1 to regulate interleukin (IL)-17 production. Significance of this alliance is uncovered by analysis of homeostasis of IL-17 production by natural killer T (NKT) and γδ T cells in sdc1-deficient mice. The results show significant increases in specific subsets of these innate-like T cells that specialized in production of IL-17 in the thymus and in peripheral organs in mice lacking sdc1 as illustrated (Figure 1). In this minireview, we briefly describe the three players forming this axis and how deficiency of sdc1 dysregulates homeostasis of IL-17 production by NKT and γδ T cells and the consequences in autoimmunity.

Syndecan family

The syndecan (sdc) family is comprised of four transmembrane heparan sulfate proteoglycans (HSPGs)[1]. These four HSPGs are sdc1, 2, 3, and 4. The structures of these sdcs are high conserved with high sequence homology in vertebrates and invertebrates[2,3]. Sdc2 is primarily expressed on cells of mesenchymal cells[4]; sdc3 is primarily expressed by neuronal tissue and cartilage[5], and sdc4 is ubiquitously expressed in most tissues[6]. On the other hand, sdc1 is a heparan sulfate that is ubiquitously expressed on epithelial cells, hepatocytes, endothelium. Sdc1 ectodomain interacts with various ligands (including growth factors, chemokines, cytokines and their receptors, and pathogens) to modulate various functions, including differentiation, migration, survival, and proliferation[7]. It is reported that sdc1 is a target of Blimp-1, the transcription factor that regulates differentiation of B cells into plasma cells. Sdc1 is also involved in the growth and metastasis of multiple myeloma *in vivo*[8]. In contrast, there is very limited information on the role of sdc1 in the adaptive immune cells except as a marker for plasma and myeloma cells and regulators of their survival[9,10]. More recently, however, we have identified sdc1 as a marker of IL-17-producing subsets of NKT cells and γδ T cells, (NKT17 and Tγδ17), respectively. The other members of sdcs family, however, in the regulation of cytokines including IL-17 are not well documented.

IL-17

IL-17 (also called as IL-17A) is a member of the IL-17 family. The family of IL-17 consists of six members: namely IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F. IL-17A is commonly known as IL-17[11], is a potent proinflammatory cytokine that has been strongly associated with pathology, especially autoimmunity. IL-17-mediated recruitment of inflammatory cells in response to bacterial or fungal infections is vital for the clearance of infections and if not discontinued it leads to the initiation of chronic inflammation and autoimmunity. Indeed, increased production of IL-17 has been associated with a wide range of inflammatory diseases, including rheumatoid arthritis[12], inflammatory bowel disease[13], diabetes[14], cancer[15], and allergic asthma[16]. Although the Th17 subset of conventional T cells was the first to be identified[17], subsequent studies identified several types of innate immune cells that are important sources of IL-17. Prominent among them are specialized subsets (NKT17 cell and Tγδ17 cell) of NKT and γδ T cells. Mucosal associated invariant T cells (MAIT) cells is another innate like T cell that is a significant producer of IL-17. They comprise up to 5% of human peripheral T cells and they express a semi-invariant TCR alpha chain (Vα7.2) which is recognize antigens in the context of the nonpolymorphic major histocompatibility complex (MHC)-related protein 1 (MR1)[18]. Production of IL-17 by MAIT cells has been implicated in the pathogenesis of various diseases like multiple sclerosis[18,19].

Here we will discuss the selective expression of sdc1 on innate-like T cells and its potential implications.

SELECTIVE EXPRESSION OF SDC1 ON IL-17-PRODUCING NKT CELLS

NKT cells represent a distinct lineage of αβ T cells that expresses an invariant TCR and specializes in recognizing self and foreign glycolipids as antigens in the context of the CD1d MHC class1b molecule. They are experimentally stimulated using the synthetic glycolipid, αGalCer (α-Galactosylceramide)[20] and fluorochrome-conjugated αGalCer/CD1d tetramers are routinely used to stain and identify NKT cells by flow cytometry. Thus, there is fundamental differences between NKT cells

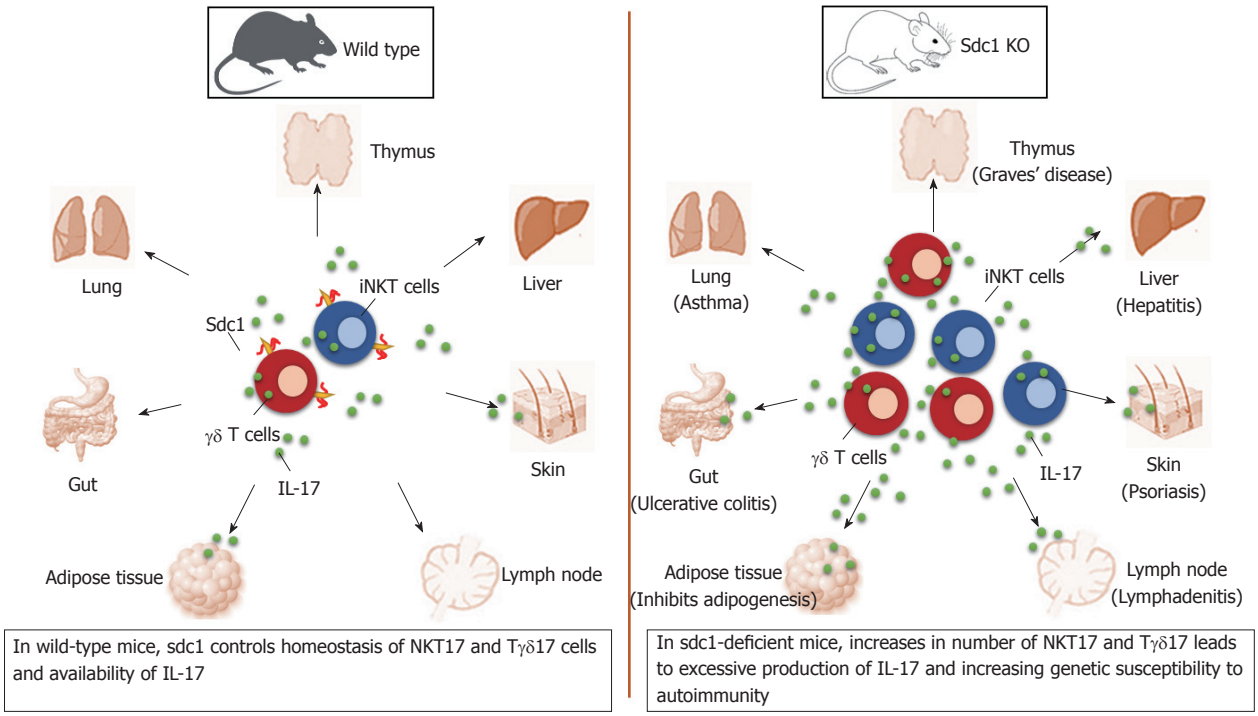

Figure 1 Innate-like T cells employ syndecan-1 to regulate interleukin-17 production. Homeostasis of interleukin (IL)-17 production by natural killer T (NKT) and γδ T cells in wild type (left) and syndecan-1-deficient (right) mice. The diagram illustrated significant increases in numbers of NKT17 and Tγδ17 subsets in the thymus and in peripheral organs thereby increasing genetic susceptibility to IL-17-driven autoimmune diseases. IL-17: Interleukin-17; NKT: Natural killer T cells; Sdc1: Syndecan-1; KO: Knockout; NKT17: Interleukin-17-producing subsets of natural killer T cells; Tγδ17: Interleukin-17-producing subsets of γδ T cells; iNKT: Invariant natural killer T cells.

and conventional T cells (which recognize peptides as antigens and express highly diverse TCR repertoire). NKT cells are considered innate-like T cells as they are selected through the agonist selection pathway that is favored by autoreactive T cell receptors (TCRs) and they acquire their effector functions while developing in the thymus by differentiating into three distinct subsets that produce interferon-γ (IFN-γ) (NKT1), IL-4 (NKT2) or IL-17 (NK17), respectively[21]. Upon stimulation, NKT cells produce massive amount of two of the most potent proinflammatory cytokines (IL-17 and IFN-γ). NKT cells are important early sources of these key cytokines that play central roles as first line of defense and in shaping adaptive immune responses, including differentiation of CD4 T cells into T helper (Th)1, Th2 and Th17 programs. These cells possess both protective and pathogenic roles in many microbial infections, autoimmune disease, allergic disease and cancer[22]. Moreover, other innate-like T cells in this regard are γδ T-cells. Both NKT cells, γδ T-cells develop in the thymus where a subpopulation specially Tγδ17 cells, acquires the effector ability to produce IL-17 rapidly[9]. Tγδ17 cells predominantly localize in peripheral lymph nodes and skin of the mice[23].

SDC1 DEFICIENCY LEADS TO EXPANSION OF NKT17 CELLS

We and others[24,25] have identified sdc1 as a phenotypic marker of NKT17 cells. Apart from being specific marker for NKT17 cell, sdc1 is a regulator of NKT17 subset. Deletion of sdc1 significantly increases the frequency of NKT17 at the expense of NKT1 cells, which was reflected in systemic increase in production of IL-17 in sdc1-knockout (KO) mice as compared to WT mice upon α-Galcer stimulation[26]. These results uncover a critical role for sdc1 expression in regulating homeostasis of NKT17 and consequently production of IL-17.

An intriguing aspect of NKT cells is their selective residence in metabolic organs with NKT1 residing mainly in liver and NKT17 cells in visceral adipose tissue[21,26]. Furthermore, whereas a great deal is known about specific roles of Th1, Th2 and Th17 subsets, the precise roles of NKT cells remain poorly undefined and the specific functions of its three distinct effector subsets and their relationships to one another remain unclear. The relationship between NKT17 cells and adipose tissue, however, has been difficult to dissect even though IL-17 inhibits adipogenesis and causes insulin resistance[27]. Moreover, attempts to understand overall metabolic role of NKT cells produced conflicting data that ranged from tolerogenic to pathogenic or no role[28]. A main likely reason, in our opinion, is the complex nature of NKT cells and studying them as one whole even though they are comprised of distinct subsets with clearly opposing functions. Therefore, our ability to sort NKT cells into viable NKT17 and NKT1 using sdc1 expression present new opportunities to study their

specific properties, how they modulate one another, and to generate adoptive hosts bearing exclusively NKT17 or NKT1 cells to examine their specific effects on VAT separately. Sdc1 deficiency is associated with reduced body fat and insulin resistance in chow-fed mice. Kasza *et al*[29] reported that sdc1KO Balb/c mice have reduced intradermal fat and that their VAT is also significantly reduced in 12-wk-old mice.

SELECTIVE EXPRESSION OF SDC1 ON Tγδ17 CELLS

γδ T cells are a population of lymphocytes expressing γ and δ TCR chains and these innate immune T cells are considered as link between innate and adaptive immune responses. In the mouse, γδ T cells primarily develop in the thymus into completely functional subsets which further secrete high levels of pro-inflammatory cytokines, such as IFN-γ or IL-17, upon activation in the periphery[30,31]. γδ T cells are abundant in the skin (dermis and epidermis), lymph node, respiratory mucosa such as nasal mucosa, bronchial mucosa, and lung[32]. Moreover, γδ T cell have been characterized in several epithelial tissues for the selective tissue homing and retention and involved in immune surveillance and immune defense. There is abundant evidence that γδ T cells are involved in allergic and inflammatory settings and suggest that they can both drive and regulate immune responses through different mechanisms. Here we will discuss the selective expression of sdc1 on innate T cells (NKT17 cell and Tgd17 cell) and its potential implications.

γδ T cells are the main source of early IL-17 in various murine models of infection, inflammation, and autoimmunity[33,34]. Tγδ17 cells develop in the thymus where a subset acquires the innate effector ability of rapidly producing IL-17. In the periphery, Tγδ17 cells localize to lymph nodes, mucosal tissues such as the intestine, skin and lung[35,36]. In human, Tγδ17 cells have been found to increase in patients with tuberculosis, bacterial meningitis, ankylosing spondylitis, and psoriasis[32,37]. These findings provide a potential explanation that IL-17-producing γδ T cells are a key component in the pathogenesis of various inflammatory and autoimmune diseases. Recently, we have found that sdc1 is selectively expressed on IL-17-producing γδ T subset, including those in the thymus, lymph nodes and skin[23]. Given selective expression of sdc1 by NKT17 cells, its specific expression of on Tγδ17 subset indicate a special relationship between sdc1 and innate-like T cells, which are major sources of IL-17 production. Therefore, sdc1 serves at least two roles on γδ T cells: (1) Acts as a surface marker for Tγδ17; and (2) A negative regulator of Tγδ17 cells.

SDC1 NEGATIVELY REGULATES HOMEOSTASIS OF Tγδ17 CELLS

Tγδ17 cells play an important role in early host defense against fungal and bacterial infections. Early reports suggested the functional involvement of Tγδ17 cells as a critical source of IL-17 that drives autoimmune disease including psoriasis[38]. Thus, identifying the factors that control homeostasis of Tγδ17 cells is important and could be useful for developing strategies to prevent pathogenic production of IL-17. Therefore, studies addressing the roles of sdc1 expressing Tγδ17 cell may provide an alternative approach to understanding its role in autoimmune diseases. Sdc1 expression on Tγδ17 might be useful for clear understanding of their biology and their physiologic role in steady state and disease condition.

In concordance and in light of our findings, that sdc1 is selectively expressed and negatively regulates homeostasis NKT17 cells[26], we thought to determine whether sdc1 is also expressed controls homeostasis of Tγδ17 cells. That turned out to be the case and as in NKT17, deletion of sdc1, significantly and selectively increased the numbers of Tγδ17 cells in thymus, lymph nodes and skin, in steady state[23]. Sdc1 deficiency significantly exacerbated imiquimod (IMQ)-induced psoriasiform dermatitis and significantly increased Tγδ17 cells, accompanied by increased skin inflammation in sdc1KO mice than wild type. Therefore, these findings suggest that targeting sdc1 could represent a novel strategy to control IL-17 production by NKT and γδ T cells.

DOES SDC1 REGULATE PRODUCTION OF IL-17 BY OTHER INNATE-LIKE T CELLS OR INNATE CELLS?

As mentioned above, the other major innate-like T cells that produce IL-17 are MAIT cells. However, whether sdc1 is also involved in regulation of IL-17 by MAIT cells is currently unknown and worthy of future investigation. Furthermore, innate-like lymphocyte 3 are major producers of IL-17[39] and need to be investigated for expression of sdc1 in future studies.

CONCLUSION

In summary, the discovery of selective expression of sdc1 on NKT17 and Tγδ17 reveals a previously unexpected role for sdc1 in regulating IL-17 by innate-like T cells. The results provide an impetus for future experiments aimed at understanding specific mechanisms by which sdc1 regulates IL-17 production by innate-like T cells. In addition, sdc1-deficient mouse strains provide new model for of the role of innate-like T cells in IL-17-mediated autoimmune diseases. Such efforts may lead to new therapeutic strategies for autoimmune diseases where IL-17 plays a central role.

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MINIREVIEWS

Guidelines and controversies in the management of diabetic ketoacidosis – A mini-review

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Abstract

Diabetic ketoacidosis (DKA) is a complication seen in patients with both type 1 and type 2 diabetes. Due to its large, growing economic impact with associated morbidity, closer look at proper management is important. Factors involved in appropriate management involves fluid resuscitation, insulin regimen, and electrolyte replacement including types of fluid and insulin treatment. The caveat with generalized protocol is application to special populations such as renal or heart failure patients the sequelae of complications due to pathophysiology of the disease processes. This leads to complications and longer length of stay in the hospital, therefore, possibly increased cost and resource utilization during the hospitalization. This review takes a closer look at current guidelines of DKA management and resource utilization, the drawbacks of current management protocols and the cost associated with it. Therefore, a need for amendment to existing protocol or initiation of a newer guideline that properly manages DKA should incorporate special populations and appropriate regimen of fluid resuscitation, insulin therapy and electrolyte management.

Key words: Diabetic ketoacidosis management; Fluid resuscitation; Insulin regimen; Electrolyte replacement

Core tip: Diabetic ketoacidosis (DKA) management in both type 1 and type 2 has been in practice for many years, yet the complications and cost associated with it is ever increasing. Treatment with proper resource utilization is the key to appropriate management of DKA, decreased complications and length of stay, therefore, decreased cost of treatment. This review aims to review previous guidelines, choice of therapy, cost

associated with it and need for amendments to existing protocols to increase efficacy of DKA treatment, decrease complications and decrease economic burden due to mismanagement of DKA.

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INTRODUCTION

Diabetic ketoacidosis (DKA) is a metabolic disorder that is common but preventable complication of diabetes often, as a result of insulin deficiency[1]. The metabolic disorder itself is hyperglycemia with increase in ketones circulating in the body leading to ketoacidosis[2]. DKA is a significant contributor of mortality and morbidity in type 1 diabetes mellitus (T1DM) patients and most common reason for hospitalization of T1DM patients and therefore contributes significantly to hospital costs. Healthcare costs in events of ketosis are high in patients with T1DM patients, adults and children alike. The number of hospital admissions secondary to DKA have steadily increased worldwide with a decrease in total length of stay[3]. The in-hospital mortality has decreased, however the cost of hospitalizations has increased significantly[4]. This literature review focuses to analyze the current practice and guidelines followed for the management of DKA.

SEARCH

MEDLINE (*via* PubMed) and EMBASE databases were searched for articles published between 1 January 2000 and 31 December 2017 (date of search execution) by a single review author. The terms used to search the databases for relevant articles were - "Management of DKA", "Guidelines for DKA" and "Cost/Burden of DKA". Only human studies published in English were included.

TREATMENT STRATEGY FOR DKA

Management of DKA includes optimizing volume status, glucose levels, ketoacidosis, electrolyte abnormalities and precipitating factors. The current protocols for DKA management calls for fluid resuscitation with goal of volume repletion within 24-36 h with 50% of resuscitation fluid administered within first 8-12 h of presentation[5]. Protocols are in place to optimize DKA management. Prior to protocol implementation, the mean intensive care unit (ICU) unit stays were 44+/-28 h, and hospital lengths of stay were 91+/73 h. After implementation of protocols, ICU stays have decreased 23% to 34+/18 h. and mean hospital lengths of stay have decreased 30% to 64+/41 h of stay[6].

Current guidelines recommend initiating volume repletion with isotonic saline (0.9% NaCl). Further volume repletion with the type of IV fluid is based on corrected serum sodium. To manage hyperglycemia, current guideline has options for insulin use either *via* intravenous or Subcutaneous/Intramuscular route. The other focus of therapy is to correct electrolytes (particularly sodium, potassium, phosphorus and magnesium) as necessary and avoid over correction. With management of DKA comes management of acid/base level as well. Bicarbonate supplementation is recommended only for pH < 6.9.

CONTROVERSIES

Although the mainstay in DKA management is regular insulin either *via* either IV continuous infusion or frequent subcutaneous or intramuscular injections, the question remains regarding the bolus dose of insulin, ideal route of insulin therapy and the cost associated with it. According to current guidelines of DKA management, an IV insulin bolus dose is recommended followed by continuous infusion. However, in a prospective observational study it was observed that administration of an initial bolus dose of insulin was not associated with significant benefit to DKA patients and was noted to be on similar efficacy and results when compared to patients who were not administered bolus dose of insulin[7]. On the other hand, for most practicing clinicians and experts, IV regular insulin after initial bolus dose of insulin still remains the preferred route due to delayed onset of action[8]. Although treating patients with IV insulin causes rapid decline in plasma glucose and ketone levels, the cost of DKA treatment with IV insulin is higher due to management of DKA requiring ICU admission or specialized care unit requiring continuous IV insulin infusion[9]. In a recent small randomized study including 3 studies in adults and 1 study including pediatric population, it was observed, patients with mild-to-moderate DKA, SC insulin lispro every 1 to 2 h conferred an alternative to continuous IV regular insulin[10].

EFFICACY OF PROTOCOL DRIVEN TREATMENT

Studies have shown that protocol driven management of DKA is safe and efficient with decreased length of stay[11]. In a retrospective study, the efficacy of protocol driven management of DKA was studied in teaching hospital in the United States based on 2009 American Diabetes Associations guidelines. Patients undergoing this protocol had resolution of DKA within approximately 10 h[12]. However, the protocol driven care of DKA differs based on different institutions. For example,

retrospective study in the United Kingdom showed that universal protocol was not adhered for reasons including patient and clinician factors[13]. Other studies revealed that low adherence was prevalent as a result of discontinuation of medical care, staffing issues[14].

SPECIAL POPULATION

Another deficit of following current protocols is the failure to address DKA management in special patient populations such as patient with chronic kidney disease or congestive heart failure or both. For example, in chronic kidney diseases (CKD) patients, osmotic diuresis due to hyperglycemia fails to occur, therefore leading to extracellular volume expansion. If according to current protocol fluid resuscitation is undertaken fatal consequences can pursue. Similarly, potassium replacement is vital in management of DKA. Potassium excretion is often impaired in patients with renal injury or failure. Therefore, potassium supplementation according to current protocol can result in life-threatening hyperkalemia. In addition, insulin is renally excreted, therefore, dose adjustment of insulin is needed in CKD patients[15]. Application of general DKA protocol to all patient populations can be dangerous to patients leading to complications and longer hospital stays. General DKA protocol in renal or heart failure patients can be detrimental due to over treatment with fluids and exacerbating fluid status and the sequelae associated in such fluid sensitive patient populations. Therefore, increasing costs of for hospitalization and treatment of DKA.

NEW GUIDELINES/RECONSIDERATION

In a review study of efficacy of DKA treatment according to Joint British Diabetes Society protocol, it was revealed that guideline adherence in DKA management is of benefit in the immediate stage of treatment. But inadequate fluid or electrolyte management, inadequate metabolic monitoring, iatrogenic hypoglycemia continues to be area of concern[16]. This often precludes to avoidable consequences that leads to longer duration of hospital course, cost, both health and economic, associated with mismanagement of DKA for patients. Therefore, it seems appropriate to highlight the need for nursing education on timely administration of fluids, hourly laboratory draws, and administration of insulin.

Due to hyperglycemia in DKA causing osmotic diuresis and severe dehydration, the mainstay of treatment is rehydration. Traditional treatment as mentioned above is "one bag protocol (1 liter/bag)" with normal saline and supplemental electrolytes *vs* "two bag protocol (1 liter/bag)" that includes two bags of fluids, one containing saline and supplemental electrolytes and another bag containing same solution with additional 10% dextrose. Closure of anion gap was noted to be earlier (10 h) with "two bag protocol" compared to "one bag protocol" (14 h). Hyperglycemia was also noted to improve faster in "two bag protocol" (7 h) compared to "one bag protocol" (9 h)[17]. Whether or not this affects length of hospital stay in the long term is difficulty to assess but should be explored in future prospective studies. Similarly, prospective studies on efficacy and cost effect on treatment of DKA with subcutaneous insulin *vs* IV insulin infusion need to be pursued.

CONCLUSION

Given the increasing cost burden on management of diabetes, with large proportion attributed to DKA management and hospitalization, it is appropriate to readdress guidelines for management of DKA. While current protocol for DKA management has been standard use, it is important to address the efficacy of it. Therefore, there is a need for new protocol where treatment with subcutaneous insulin *vs* IV insulin infusion, "one bag protocol" *vs* "two bag protocol," and management of DKA in special populations should be addressed.

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MINIREVIEWS

Effects of glucose-lowering agents on cardiorespiratory fitness

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Abstract

Exercise therapy is essential for the management of type 2 diabetes (T2D). However, patients with T2D show lower physical activity and reduced cardiorespiratory fitness than healthy individuals. It would be ideal for clinicians to co-prescribe glucose-lowering agents that improve cardiorespiratory fitness or exercise capacity in conjunction with exercise therapy. Metformin does not improve cardiorespiratory fitness and may attenuate any beneficial effect of exercise in patients with T2D. In contrast, thiazolidinediones appear to improve cardiorespiratory fitness in patients with T2D. Although evidence is limited, sodium–glucose cotransporter 2 (SGLT2) inhibitors may improve cardiorespiratory fitness in patients with heart failure, and the effect of glucagon-like peptide-1 (GLP-1) receptor agonists on cardiorespiratory fitness is controversial. Recent clinical trials have shown that both SGLT2 inhibitors and GLP-1 receptor agonists exert a favorable effect on cardiovascular disease. It becomes more important to choose drugs that have beneficial effects on the cardiovascular system beyond glucose-lowering effects. Further studies are warranted to determine an ideal glucose-lowering agent combined with exercise therapy for the treatment of T2D.

Key words: Type 2 diabetes; Glucagon-like peptide I receptor agonist; Cardiorespiratory fitness; Exercise capacity; Metformin; Thiazolidinedione; Sodium-glucose cotransporter 2 inhibitors

Core tip: What is the most effective combination of drugs and exercise for the treatment of type 2 diabetes? It has become increasingly important for clinicians to prescribe drugs that reduce cardiovascular disease and mortality in addition to their glucose-lowering effects. This review summarized the current literature investigating the effect of glucose-lowering agents on cardiorespiratory fitness. Thiazolidinediones, sodium–glucose cotransporter 2 inhibitors, and glucagon-like peptide-I receptor agonists have the potential to improve cardiorespiratory fitness; however, further research will be needed to confirm.

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INTRODUCTION

More than 400 million people worldwide suffer from diabetes. Diabetes can lead to microvascular and macrovascular complications and increase the physical and psychological burden in patients[1]. Nutrition and exercise therapy are essential for the management of diabetes, and patients with type 1 and 2 diabetes are recommended to engage in regular moderate-to-vigorous intensity aerobic exercise and resistance training[2]. In addition, higher levels of physical activity are associated with reduced risk of breast cancer (14%), colon cancer (21%), ischemic heart disease (25%), and stroke (26%)[3]. Exercise is a standard component of chronic disease prevention and management[4]. However, patients with diabetes typically exhibit lower energy expenditure, physical activity duration[5], skeletal muscle mass[6], and cardiorespiratory fitness[7], and it can be challenging to effectively and safely incorporate exercise therapy in diabetes patients also presenting with vascular complications and comorbidities. Combined diet and exercise therapy is effective against diabetes; however, in more severe cases, drugs are usually required to intensively improve glycemic control. There are currently nine different groups of glucose-lowering agents available: metformin, thiazolidinediones, sulfonylureas, glinides, α-glucosidase inhibitors, dipeptidyl peptidase-4 (DPP-4) inhibitors, sodium–glucose cotransporter 2 (SGLT2) inhibitors, glucagon-like peptide-l (GLP-1) receptor agonists, and insulin. Of these, metformin[8], SGLT2 inhibitors[9,10], and a GLP-1 receptor agonists[11] have beneficial effects on cardiovascular disease (CVD) as well as glycemic control, making these the drugs of choice for of type 2 diabetes (T2D) treatment[12].

Exercise is important in the primary and secondary prevention of CVD[13] and, thus, should be an integral part of the strategy to reduce CVD risk. Individuals with low cardiorespiratory fitness (< 7.9 metabolic equivalent; MET) have a 1.70-fold and 1.56-fold increased risk of all-cause mortality and cardiovascular events, respectively, compared with those with high cardiorespiratory fitness (⩾ 10.8 MET)[14]. Ideally, clinicians should preferably prescribe drugs that improve cardiorespiratory fitness. However, the optimal combination of exercise and glucose-lowering agents remains unclear as the effects of glucose-lowering agents on exercise capacity/cardiorespiratory fitness are not well understood.

This review summarizes the current literature regarding the effects of glucose-lowering agents on cardiorespiratory fitness in humans and aims to highlight the optimum drug selection in the treatment of patients with diabetes who engage in regular exercise.

METFORMIN AND CARDIORESPIRATORY FITNESS

Metformin is the most widely used oral glucose-lowering drug with known beneficial effects on macrovascular complications in T2D[15]. While the mechanisms of action of metformin remain unclear, it is known to activate the cellular energy sensor, AMP-activated protein kinase (AMPK), suppress proinflammatory cytokine secretion, inhibit hepatic gluconeogenesis and lipogenesis, and stimulate GLP-1 secretion by modulating the gut microbiota[16]. Metformin is a complex drug with multiple mechanisms of action. While it is the first-line medication recommended by the American Diabetes Association and the European Association of the Study of Diabetes[17], clinicians usually also co-prescribe metformin with exercise therapy. It is important to understand whether metformin affects cardiorespiratory fitness/exercise capacity, and the interaction between metformin and exercise has been well studied[18-25].

Johnson *et al*[18] examined the acute effects of metformin on maximal oxygen consumption (VO_{2max}) during exercise. A cycle ergometer was used for graded maximal exercise tests. Participants cycled at 75–80 rpm with a resistance of 2.0 kp, which was increased by 0.5 kp every 3 min until volitional exhaustion. A single dose (1000 mg) of metformin increased mean VO_2 (2.9 ± 0.5 L/min *vs* 2.8 ± 0.5 L/min) during exercise but not VO_{2max} (4.00 ± 0.58 L/min *vs* 4.00 ± 0.66 L/min). Braun *et al*[19] investigated the effect of metformin on aerobic capacity in healthy individuals. Peak aerobic capacity (VO_{2peak}) was measured 7–9 d after administration of either metformin or placebo. An incremental exercise test began using a cycle ergometer at 50–150 W or a treadmill at 6.4–9.6 km/h. The cycle resistance (+25–50 W) and treadmill grade (+2%) were increased every 2 min until exhaustion. The initial dose of metformin was 500 mg/d, which was increased every second day to a maximum of 2000 mg/d. Metformin treatment reduced VO_{2peak} (3.53 ± 0.29 L/min *vs* 3.63 ± 0.9 L/min for metformin and placebo, respectively; −2.7%), and there was no significant association between the decrease in VO_{2peak} and baseline cardiorespiratory fitness. Although the effect was physiologically subtle, short-term treatment with metformin had a negative effect on cardiorespiratory fitness. The same authors also examined the effect of metformin on fat oxidation during and after exercise[20]. Fat oxidation, which was calculated from respiratory gas composition (volume of oxygen consumption (VO_2) and volume of carbon dioxide production (VCO_2), was higher with metformin compared with placebo treatment during exercise but lower during recovery. In contrast, metformin increased carbohydrate

Table 1 Effects of metformin on cardiorespiratory fitness in healthy individuals

Ref.	Study design	Subjects	Metformin dose and intervention	Results
Johnson *et al*[18], 2008	Randomized, double-blind, placebo-controlled, crossover study	11 healthy and active men Age: 29.9 ± 3.7 yr Sex: All men BMI: 25.2 ± 2.8 kg/m^2	1000 mg/d Cycle ergometer at the mean intensity of 69 ± 5.5% of VO_{2max}	VO_{2max}→, ventilator threshold→, maximal heart rate→, time to fatigue→ Lactate↓, blood glucose concentrations↓
Braun *et al*[19], 2008	Non-randomized, placebo-controlled study	18 healthy subjects Age: 27.9 ± 3.3 yr Sex: 11 men and 7 women BMI: 24.1 ± 3.6 kg/m^2	2000 mg/d Treadmill or cycle ergometer	VO_{2peak}↓, peak heart rate↓, peak ventilation ↓, peak respiratory exchange ratio↓, exercise duration↓ Rating of perceived exertion→
Malin *et al*[20], 2010	Non-randomized, double-blind, counterbalanced crossover study	15 healthy and active subjects Age: 25 ± 4.4 yr Sex: 7 men and 8 women BMI: 22.8 ± 2.7 kg/m^2	2000 mg/d Cycle exercise at 5 submaximal cycle workloads	VO_2→ During exercise: Fat oxidation↑ Postexercise: Fat oxidation↓
Learsi *et al*[21], 2015	Randomized, placebo-controlled, counterbalanced study	10 healthy men Age: 23.5 ± 3.6 yr Sex: All men BMI: No description (height: 170.4 ± 4.8 cm, weight: 66.4 ± 6.5 kg)	500 mg/d Cycle ergometer: An incremental test, 6 submaximal workload test at 40%–90% VO_{2max}, 2 supramaximal tests at 110% VO_{2max}	VO_2→, maximal accumulated oxygen deficit →, lactate concentrations→ Time to exhaustion↑, VO_2 recovery↑

BMI: Body mass index; VO_2: Oxygen consumption.

oxidation after exercise. Oxygen consumption was not different at rest or during exercise with metformin. Therefore, metformin may increase the rate of fat oxidation during exercise *via* activation of AMPK, but appears to have no effect on cardiorespiratory fitness. Learsi *et al*[21] examined the effect of metformin on high-intensity, short-duration exercise on anaerobic capacity. Exercise tests comprised a maximal incremental test to evaluate $VO_{2\ max}$, six workload tests with submaximal intensities (40%–90% of maximal power output), and two supramaximal intensity tests (110% of maximal power output). Participants took low-dose metformin (500 mg) or placebo prior to the supramaximal test. Time to exhaustion was improved with metformin (191 ± 33 s *vs* 167 ± 32 s for metformin and placebo, respectively), but VO_2 during the supramaximal test was not different between the groups. Maximum O_2 deficit and lactate concentrations did not differ between the groups. The authors concluded that metformin improves exercise performance by mediating the alactic anaerobic system. Table 1 summarizes the effects of metformin on cardiorespiratory fitness in healthy individuals. However, what is known about the interaction between metformin and cardiorespiratory fitness in patients with T2D or insulin resistance? A noteworthy study by Boulé *et al*[22] investigated the interaction between metformin and exercise on the hormonal response to a standardized meal. The authors studied 10 patients with mild T2D who took metformin or placebo for 28 d, and measured exercise capacity, glucose, lactate, non-esterified fatty acids, insulin, and glucagon levels on the last two days. Resistance and aerobic exercise tests were conducted using an isokinetic dynamometer and treadmill. After performing resistance exercise (leg extensions and flexions), the patients started three bouts of aerobic exercise comprising walking at 3.5 km/h with 0% gradient for 15 min, then increasing the speed and gradient until just below the ventilatory threshold, followed by walking at an intensity above the ventilator threshold for 5 min. The mean respiratory exchange ratio (0.96 ± 0.02 *vs* 0.98 ± 0.02) was lower, and the mean heart rate (124 ± 9 *vs* 118 ± 8 beats per min) was higher in the metformin group. Mean VO_2 was not affected. As expected, metformin improved glycemic response but glycemic response was attenuated in combination with exercise. In addition, glucagon levels were highest in the metformin plus exercise group. It is surprising that exercise has an opposing effect on the glucose-lowering effect of metformin. High-intensity exercise increases insulin counterregulatory hormones, such as epinephrine, norepinephrine, cortisol, and growth hormone, as well as glucagon, which may further deteriorate glucose response in T2D. Boulé *et al*[23] also investigated the long-term effects of metformin on glycemic control and physical fitness in participants in the Diabetes Aerobic and Resistance Exercise trial[26]. Subjects were randomly assigned to four groups, namely, aerobic exercise, resistance training, combined aerobic exercise and resistance training, and control. The exercise group performed progressive aerobic exercise, increasing to an intensity of 75% of maximum heart rate for 45 min. Resistance training included seven exercises: abdominal crunches, seated row, seated biceps curls, supine bench presses, leg presses,

Table 2 Effects of metformin on cardiorespiratory fitness in patients with type 2 diabetes and metabolic syndrome

Ref.	Study design	Subjects	Metformin dose and intervention	Results
Boulé *et al*[22], 2011	Randomized, placebo-controlled, crossover study	10 patients with type 2 diabetes Age: 58 ± 6 yr Sex: 8 men and 2 women BMI: 28.6 ± 5.3 kg/m^2 HbA1c: 6.5 ± 0.6%	2000 mg/d Exercise mode: Treadmill at three different submaximal intensities Study duration: 22 wk	VO_2→, respiratory exchange ratio↓ Heart rate↑, lactate↑, rating of perceived exertion↑
Boulé *et al*[23], 2013	Randomized controlled trial (Diabetes Aerobic and Resistance Exercise) trial	251 patients with type 2 diabetes (143 patients treated with metformin and 82 patients treated without metformin) Age: 54.9 ± 7.1 yr *vs* 53.1 ± 6.9 yr Sex (Men/Women): 100/43 *vs* 46/36 BMI: 33.3 ± 5.5 kg/m2 *vs* 33.3 ± 6.4 kg/m^2	Approximately 1600 mg/d Exercise mode: Aerobic training, resistance training, and combined aerobic and resistance training Study duration: 4 wk	VO_{2peak}→
Cadeddu *et al*[25], 2014	Non-randomized, non-controlled trial	HbA1c: 7.78 ± 0.9% *vs* 7.47 ± 0.77% 75 patients with insulin resistance Age: 46.2 ± 11 yr Sex: 35 men and 40 women BMI: 29.8 ± 4.1 kg/m^2	1000 mg/d 30–50 min of cycle exercise at the intensity of 60%–80% of the heart rate reserve Study duration: 12 wk	VO_{2peak}→
Paul *et al*[26], 2017	Prospective observational study	15 patients with metabolic syndrome Age: No description Sex: No description BMI: No description (weight: 75.4 ± 12.08 kg)	1000 mg/d No intervention Study duration: 6 wk	VO_{2max}↓

BMI: Body mass index; HbA1c: Hemoglobin A1c; VO_2: Oxygen consumption.

shoulder presses, and leg extensions. VO_{2peak} increased in the aerobic group by 0.16 L/min and in the combined exercise group by 0.11 L/min without metformin. However, VO_{2peak} did not change in any of the metformin groups. In the aerobic exercise group, HbA1c levels were reduced with metformin. In the combined exercise group, fasting glucose levels decreased with metformin. There were no significant differences in changes in HbA1c and glucose levels with or without metformin. The study concluded that metformin did not impair physical fitness or glycemic control when combined with exercise. The findings of this study are inconsistent with previous short-term studies that have shown that the addition of exercise to metformin showed a negative effect on cardiorespiratory fitness and glycemia. The authors speculated that difference in the characteristics of the study participants, such as duration of metformin treatment and glycemic control at baseline, may explain this discrepancy.

Two clinical studies have investigated metformin and cardiorespiratory fitness in individuals with insulin resistance and metabolic syndrome. Cadeddu *et al*[25] investigated the effect of metformin, exercise alone, or a combination of metformin and exercise on exercise capacity. Study participants had impaired glucose tolerance and/or impaired fasting glucose and were allocated to one of the three groups. The exercise program comprised 30–50 min cycle ergometry with an intensity of 60%–80% of heart rate reserve based on the age of the subjects. After a 12-wk intervention, the exercise only group had improved VO_{2peak}, whereas the metformin plus exercise therapy group did not. Moreover, metformin plus exercise therapy did not show an improved aerobic threshold compared with the exercise along group. The combination of metformin and exercise was not superior to exercise alone with regard to cardiorespiratory fitness. A recent study in India showed a negative effect of metformin on exercise capacity in patients with newly diagnosed metabolic syndrome[25]. This study was a simple observational study to evaluate changes in VO_2, ventilatory anaerobic threshold, and other indicators of cardiorespiratory fitness in response to metformin treatment for 6 wk, and showed that $VO_{2\ max}$ decreased from 1.10 ± 0.44 to 0.9 ± 0.39 L/min and ventilatory anaerobic threshold decreased by 1.5 mL/min per kilogram. However, these studies were non-randomized, non-controlled observational studies, and thus, the study design was suboptimal (Table 2).

Metformin improves energy metabolism in skeletal muscle and has a cardioprotective effect *via* AMPK activation[27]. Metformin also inhibits mitochondrial respiratory-chain complex 1 and decreases ATP production[27], which could potentially reduce oxygen consumption during exercise. In addition, metformin increases lactate concentrations and reduces the lactate threshold during exercise[28]; however, lactate accumulation may have a protective effective on skeletal muscle rather than cause fatigue[29]. Previous studies have suggested that the effect of metformin on cardiorespiratory fitness is clinically subtle. However, treatment with metformin does not appear to have a synergetic effect on cardiorespiratory

fitness in combination with exercise therapy.

THIAZOLIDINEDIONES AND CARDIORESPIRATORY FITNESS

The mechanism of action of thiazolidinediones is mediated by peroxisome proliferator-activated receptors (PPARs)[30]. Thiazolidinediones exert an insulin-sensitizing effect by promoting fatty acid uptake and modulation of secretion of adipokines, such as interleulin-6, tumor necrosis factor-α, and adiponectin[31]. PPAR-γ overactivation by thiazolidinediones increases body weight *via* fluid retention[30] and stimulatory effect on adipogenesis and adipose tissue accumulation[32]; thus, thiazolidinediones may be associated with increased cardiovascular risk in some patients. However, these drugs appear to improve cardiorespiratory fitness in patients with T2D.

In 2005, a randomized, double-blind, placebo-controlled study reported that rosiglitazone, a thiazolidinedione, improved exercise capacity *via* improvement in endothelial function in patients with T2D[33]. Twenty patients were divided into rosiglitazone (4 mg/d) and placebo groups. After a 4-mo intervention, $VO_{2\ max}$ increased from 1902 ± 603 mL/min (19.8 ± 5.3 mL/kg per minute) to 2074 ± 585 mL/min (21.2 ± 5.1 mL/kg per minute) in rosiglitazone-treated patients, but showed no improvement in controls. In addition, the change in $VO_{2\ max}$ negatively correlated with changes in fasting insulin and homeostasis model assessment of insulin resistance (HOMA-IR) was positively correlated with insulin sensitivity, as measured by hyperinsulinemic–euglycemic clamp. Thiazolidinediones may improve $VO_{2\ max}$ *via* multiple mechanisms. First, thiazolidinediones enhance gene transcription that promotes adipocyte differentiation and increases fatty acid transport, synthesis, and storage in the adipose tissue by binding to PPARγ. This reduces ectopic fat accumulation in muscle and liver, and improves both cellular lipotoxicity and insulin sensitivity. Second, thiazolidinediones may also activate AMPK, which leads to increased fat oxidation and PPARγ coactivator 1α expression, regulating mitochondrial biogenesis[34]. Mitochondrial dysfunction in patients with T2D is attenuated by thiazolidinediones[35], which may result in an improvement in cardiorespiratory fitness.

Another randomized controlled study investigating the effect of rosiglitazone on cardiorespiratory fitness in patients with T2D was conducted in Greece[36]. Seventy patients (28 men and 42 women) with T2D were randomly assigned to a rosiglitazone (8 mg/d) treatment group or a control group. Rosiglitazone treatment for 6 mo increased VO_{2peak} from 24.47 ± 3.98 to 26.39 ± 4.04 mL/kg per minute. Changes in adiponectin, HOMA-IR, and HbA1c levels were independent predictors of incremental increase in VO_{2peak}. Rosiglitazone, a PPARγ activator, may improve cardiorespiratory fitness *via* upregulation of adiponectin. Recently, Yokota *et al*[37] showed that pioglitazone improves cardiorespiratory fitness in Japanese patients with metabolic syndrome. Fourteen male patients with metabolic syndrome received 15 mg/d of pioglitazone for four months. Pioglitazone increased VO_{2peak} from 25.1 ± 4.9 to 27.2 ± 3.9 mL/kg per minute, and the anaerobic threshold from 12.7 ± 1.9 to 13.6 ± 0.6 mL/kg per minute. Pioglitazone also decreased the intramyocellular lipid content in resting calf muscle by 26%, with no concurrent change in the cross-sectional area of the muscle. There was an inverse correlation between the increase in anaerobic threshold and the decrease in intramyocellular lipid content. These data suggest that pioglitazone improves cardiorespiratory fitness *via* skeletal muscle fatty acid metabolism. In addition, pioglitazone decreased muscle phosphocreatinine loss during exercise, suggesting that altered mitochondrial function contributes to the improvement in skeletal muscle energy metabolism. Taken together, these studies indicate that thiazolidinediones have a beneficial effect on cardiorespiratory fitness in patients with T2D and metabolic syndrome (Table 3).

INCRETIN-RELATED DRUGS AND CARDIORESPIRATORY FITNESS

GLP-1 is secreted by the intestine and has multiple physiological effects, including brain neuroprotection, suppressing appetite, cardiovascular protection, improving cardiac function, slowing gastric emptying, decreasing glucose production in the liver, increasing glucose uptake in adipose tissue and skeletal muscle, stimulating insulin secretion, suppressing glucagon secretion, promoting pancreatic β-cell proliferation, and inhibiting pancreatic β-cell apoptosis[38]. Secretion and function of GLP-1 is severely diminished in patients with T2D, and GLP-1 receptor agonists effectively improve diabetes and obesity *via* pleiotropic effects. Additionally, there could be an interaction between exercise and GLP-1 in patients with T2D[39]. The effect of GLP-1 receptor agonists on exercise capacity/cardiorespiratory fitness remains controversial. Lepore *et al*[40] investigated whether albiglutide, a long-acting GLP-1 receptor agonist, improved cardiac function and exercise performance in patients with chronic heart failure. Eighty-one patients participated in this multicenter, randomized, placebo-controlled study, and received either 30 mg of albiglutide or placebo for 12 wk. The albiglutide group showed improved VO_{2peak} (from 16.2 ± 0.9 to 17.1 ± 1 mL/kg per minute), an increase of 1.5 mL/min per kilogram compared with the placebo group. However, no significant improvement in cardiac function, 6-min walk test, myocardial glucose, and oxygen use was observed. The authors stated that the improvement in cardiorespiratory fitness may have been mediated by a physiological effect rather

Table 3 Effects of thiazolidinediones on cardiorespiratory fitness in patients with type 2 diabetes and metabolic syndrome

Ref.	Study design	Subjects	Thiazolidinedione dose	Results
Regensteiner *et al*[33], 2005	Randomized controlled trial	20 patients with type 2 diabetes (10 patients received rosiglitazone and 10 patients received a placebo) Age: 55 ± 7 yr *vs* 56 ± 1 yr Sex (Men/Women): 5/5 *vs* 5/5 BMI: 32.2 ± 5.6 kg/m^2 *vs* 30.4 ± 5.8 kg/m^2 HbA1c: 7.2 ± 1.1% *vs* 7.2 ± 1.0%	Rosiglitazone, 4 mg/d	VO_2↑, insulin sensitivity↑, endothelial function↑
Kadoglou *et al*[36], 2008	Randomized controlled trial	70 patients with type 2 diabetes (35 patients received rosiglitazone and 35 patients received a placebo) Age: 63.8 ± 7.3 yr *vs* 66.7 ± 9.6 yr Sex (Men/Women): 14/21 *vs* 16/19 BMI: 29.5 ± 3.8 kg/m^2 *vs* 29.9 ± 4.3 kg/m^2 HbA1c: 8.2 ± 1.2% *vs* 8 ± 0.8%	Rosiglitazone, 8 mg/d	VO_{2peak}↑, duration of the exercise test↑, oxygen pulse↑ Insulin resistance↓, diastolic blood pressure↓
Yokota *et al*[37], 2017	Before-after study	14 patients with metabolic syndrome Age: 52 ± 11 yr Sex: All men BMI: 26.6 ± 3.3 kg/m^2 HbA1c: 5.7 ± 0.6%	Pioglitazone, 15 mg/d	VO_{2peak}↑, anaerobic threshold↑ Intramyocellular lipid content↓, muscle phosphocreatinine loss during exercise↓

BMI: Body mass index; HbA1c: Hemoglobin A1c; VO_2: Oxygen consumption.

than cardiac function due to the administration of albiglutide. Scalzo *et al*[41] investigated the effect of exenatide on functional exercise capacity in patients with T2D after 3-mo treatment of 10 μg twice-daily exenatide. Exenatide did not improve VO_{2peak} or endothelial function, but diastolic cardiac function and arterial stiffness improved.

The controversial results from these studies may be attributed to patient characteristics. One study was conducted using patients with chronic heart failure (without diabetes) and the other used patients with mild T2D (without heart failure). Although the underlying mechanisms are unknown, the baseline cardiac function may have influenced the change in cardiorespiratory fitness due to the GLP-1 receptor agonist treatment.

A randomized, placebo-controlled, double-blind, parallel group, phase Ⅳ trial which aims at examining the effect of liraglutide on physical performance in patients with T2D is currently underway[42], with promising results.

To the best of our knowledge, to date, no human studies have reported the effect of DPP-4 inhibitors on exercise capacity/cardiorespiratory fitness. However, one animal study suggested that exercise capacity and mitochondrial biogenesis in skeletal muscle are improved by the administration of a DPP-4 inhibitor in mice with heart failure[43]. DPP-4 inhibitors may also have the potential to improve exercise capacity/cardiorespiratory fitness in humans.

SGLT2 INHIBITORS AND CARDIORESPIRATORY FITNESS

SGLT2 inhibitors decrease glucose reabsorption at the proximal renal tubules, which increases urinary glucose excretion and improves glycemic control. SGLT2 inhibitors also exert various metabolic effects, including weight loss, insulin sensitivity improvement, blood pressure lowering, renal hemodynamic modulation, and reduction in albuminuria, which leads to cardiovascular and renal protection[44]. Treatment using empagliflozin resulted in a 35% risk reduction in hospitalization for heart failure compared with placebo[9], suggesting that SGLT2 inhibitors also have an effect on cardiorespiratory fitness in patients with T2D.

To date, two pilot studies have investigated whether empagliflozin improves cardiorespiratory fitness in patients with T2D with heart failure. Núñez *et al*[45] showed that short-term (4 wk) empagliflozin treatment increased VO_{2peak} by 1.21 mL/kg per minute (11.1%) from baseline. Conversely, Carbone *et al*[46] showed that empagliflozin treatment for 4 wk did not significantly improve VO_{2peak} (14.5 mL/kg *vs* 15.8 mL/kg per minute). Intriguingly, patients concomitantly treated with loop diuretics demonstrated improved VO_{2peak} (+0.9 mL/kg per minute), whereas those without loop diuretics demonstrated a decrease in VO_{2peak} (−0.9 mL/kg per minute). Indeed, all patients in the study by Núñez *et al*[45] received loop diuretics. The authors hypothesized that empagliflozin acts on the proximal renal tubules by interacting with sodium/hydrogen exchangers, thereby increasing sodium delivery at the distal renal tubules and enhancing the effect of loop diuretics[47,48]. Carbone *et al*[46] also speculated that empagliflozin improves cardiorespiratory fitness in patients concomitantly treated with loop diuretics by reducing the activity of the rennin–angiotensin–aldosterone system. Empagliflozin may exert cardiovascular and renal benefits *via* changes in myocardial and renal energy metabolism. Empagliflozin increases ketone oxidation instead of fat and glucose oxidation, which can improve cardiac and renal work efficiency[49]. Taken together, these studies suggest that SGLT2 inhibitors improve cardiorespiratory

Table 4 Effects of sodium–glucose cotransporter 2 inihibitors on cardiorespiratory fitness in patients with type 2 diabetes

Ref.	Study design	Subjects	SGLT2 inhibitors dose	Results
Núñez *et al*[45], 2017	Before-after study	19 patients with type 2 diabetes and heart failure Age (median): 72 yr Sex: 14 men and 5 women BMI: 30.6 ± 5.5 kg/m^2 HbA1c: No description	Empagliflozin, 10 mg/d	VO_{2peak}↑, ventilatory efficiency during exercise↑, 6-minute walking distance↑, ↓ antigen carbohydrate 125
Carbone *et al*[46], 2018	Before-after study	15 patients with type 2 diabetes and heart failure Age (median): 60 yr Sex: 7 men and 8 women BMI (median): 34 kg/m^2 HbA1c (median): 7.8%	Empagliflozin, 10 mg/d	VO_{2peak}↑ in patients using loop diuretics VO_{2peak}↓ in patients without loop diuretics

SGLT2: Sodium–glucose cotransporter 2; BMI: Body mass index; HbA1c: Hemoglobin A1c; VO_2: Oxygen consumption.

Table 5 Effect of glucose-lowering agents on cardiorespiratory fitness

Glucose-lowering agents	Cardiorespiratory fitness
Metformin	→ or ↓
Thiazolidinediones	↑
DPP-4 inhibitors	Unknown (↑ in mice with heart failure)
GLP-1 receptor agonists	↑ in patients with heart failure → in patients with type 2 diabetes
SGLT2 inhibitors	↑ in patients treated with loop diuretics

DPP-4: Dipeptidyl peptidase-4; GLP-1: Glucagon-like peptide-l; SGLT2: Sodium–glucose cotransporter 2.

fitness in patients with T2D with heart failure (Table 4).

CONCLUSION

Metformin does not improve cardiorespiratory fitness and may attenuate a beneficial effect of exercise on cardiorespiratory fitness in patients with T2D. In contrast, thiazolidinediones appear to improve cardiorespiratory fitness in patients with T2D. The effect of GLP-1 receptor agonists on cardiorespiratory fitness remains controversial and is not fully understood. Notably, SGLT2 inhibitors may improve cardiorespiratory fitness in patients with heart failure by modulating cardiac energy metabolism or *via* a synergetic effect with loop diuretics. Unfortunately, no human studies have examined the effect of DPP-4 inhibitors, sulfonylureas, glinides, or α-glucosidase inhibitors on cardiorespiratory fitness (Table 5). This review cannot recommend the optimal combination of exercise and glucose-lowering agents with regard to cardiorespiratory fitness in patients with T2D; however, thiazolidinediones, GLP-1 receptor agonists, and SGLT2 inhibitors have the potential to improve both glycemic control and cardiorespiratory fitness without interfering with exercise therapy. Further studies are warranted to demonstrate the clinical benefits of glucose-lowering agents for cardiorespiratory fitness, and to elucidate the underlying mechanisms of action.

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ORIGINAL ARTICLE

Basic Study

Pathological changes in the cellular structures of retina and choroidea in the early stages of alloxan-induced diabetes

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Abstract

AIM

To investigate the temporal sequence of pathological changes in the cellular structures of retina and choroidea in the early stages of diabetes in laboratory animals.

METHODS

Experimental type 1 diabetes was modeled by three intraperitoneal injections of an alloxan solution into 30 male nonlinear rats at 16 wk of age. The 30th and 60th days from the final alloxan injection were chosen as the endpoints. Light and electron microscopy and morphometric and immunohistochemical studies were performed on histological slices of eyeballs from experimental animals.

RESULTS

Diabetic disturbances progressed to 60 d of the experiment. Thus, in the retina, a partial destruction of photoreceptors accompanied by interstitial edema was observed. The morphometric analysis revealed a reduction in the thickness of the retina. A reduction in the number of blood vessels of the choroid with disturbances of the endothelial cells and the vascular walls and a persistent reduction in the number of melanocytes were observed. The number of proliferating Ki-67 positive cells decreased, and the number of macrophages increased with diabetes development.

CONCLUSION

The starting point in the development of destructive changes involves early reduction in the number of melanocytes of the choroidea and alterations in the retinal pigment epithelium.

Key words: Alloxan; Diabetes; Diabetic retinopathy; Early stage; Morphology; Histological changes

Core tip: Diabetic retinopathy is the most frequent microvascular complication of diabetes. However, most of therapeutic approaches being developed do not address the early and potentially reversible failure of retinal perfusion. Thus, we examined pathological changes in the cellular structures of retina and choroidea in the early stages of diabetes in laboratory animals. According to the obtained results, the starting point in the development of destructive changes involves the early reduction in the number of melanocytes of the choroidea and the destruction of the retinal pigment epithelium, accompanied by an inflammatory process, which may represent a potential therapeutic target.

Danilova I, Medvedeva S, Shmakova S, Chereshneva M, Sarapultsev A, Sarapultsev P. Pathological changes in the cellular structures of retina and choroidea in the early stages of alloxan-induced diabetes. *World J Diabetes* 2018; 9(12): 239-251
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INTRODUCTION

Diabetic retinopathy (DR) is one of the major complications associated with diabetes, and has equally been implicated as one of the leading causes of visual impairment and blindness globally. Because of this, DR is in the limelight of most clinical studies[1-4]. Hyperglycemia, hypertension, renal disease, and dyslipidemia, which are typical conditions in the manifestation of diabetes, have all been linked to the pathogenesis of DR[5,6]. According to the prevailing point of view, the leading causes of DR development include metabolic disturbances and vascular bed abnormalities, which accompany diabetes development[7-11]. In diabetes, hyperglycemia and associated oxidative stress trigger the pathological cascade underlying the vascular injury (micro- and macroangiopathy development)[12-14]. Due to the subsequent disturbances of vessel walls, the permeability of the hematoretinal barrier breaks down, and hypoxia appears, leading to trophic retinal degeneration and photoreceptor cell death[15-17]. The subsequent progression of the developed retinopathy leads to retinal neovascularization, vitreous hemorrhages, and the formation of fibrous tissue in the foci of preretinal hemorrhages, which forms the pathogenomic picture of diabetic complications[18-20].

However, despite the seeming transparency of DR pathogenesis and the progress in its treatment observed in recent years, a number of issues remain that warrant further study[6,21-23]. One of them is the temporal sequence of pathological changes in DR development[19-22]. Studies in rodents have highlighted that biomarkers of inflammation, such as leukostasis, overexpression of adhesion molecules in retinal vascular endothelial cells and leukocytes, vascular permeability alteration, and aggrevated production of nitric oxide, prostaglandins, cytokines, and other inflammatory mediators appears in the retina during 1-6 mo of diabetes crisis[5]. Most developed therapies for DR, have primarily focused on the terminal stage of this disease, and as thus, failed to address the early potentially reversible stage of this disease. In addition, most of

Table 1 Level of glucose and glycosylated hemoglobin in the blood of experimental animals (mmol/L)

Biochemical parameters	Control animals (Group 1)	Diabetes, 30 d (2nd group)	Diabetes, 60 d (2nd group)
Glucose (mmol/L)	5.99 ± 0.33	25.98 ± 1.84[a]	32.60 ± 0.80[a]
Hb A1c (%)	5.12 ± 0.24	7.10 ± 0.60[a]	6.45 ± 0.29[a]
Insulin (μg/L)	1.28 ± 0.19	0.47 ± 0.05[a]	0.36 ± 0.04[a]

[a]Differences to control animals were significant at $P < 0.05$.

these therapies have been associated with severe sight-threatening side effects[6].

With that, understanding of the temporal sequence and stages of pathological disturbances of DR development is of great prognostic and scientific value, as it might contribute to improvements to current methods or even the development of new methods of diagnosis and treatment of such a serious complication of diabetes. Thus, this work investigated the temporal sequence of pathological changes in the cellular structures of retina and choroidea in the early stages of diabetes in laboratory animals.

MATERIALS AND METHODS

Animal preparation

Healthy, sexually matured male Wister rats were used for the purpose of this experiment. The animals employed in this study were quarantined in the vivarium of the Institute of Immunology and Physiology of the Ural Division of RAS (Ekaterinburg, Russia). Only animals showing no symptoms of any disease were selected. All experimental animals were housed in similar conditions, and fed according to a customary schedule. All the experimental procedures conducted on the animals were approved by the Institute of Animal Care and Use Committee at the Institute of Immunology and Physiology of the Ural Division of RAS (diab-1-04-2016), and implemented in compliance with the principles formulated in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, France, 18.03.1986), APS's Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training, and the Laboratory Practice Regulations of Russia Federation (Ministry of Public Health Order No. 267 from 19.06.2003).

Experimental model of type 1 diabetes

Experimental type 1 diabetes was modeled by three intraperitoneal injections (10 mg/100 g of weight) of an alloxan solution (Sigma-Aldrich, St. Louis, MO, United States) dissolved in physiological saline at 1 d intervals (total dose of alloxan 30 mg/100 g) according to a modified version of the standard model of diabetes in rats[24,25]. Alloxan is a toxic glucose analogue that has been employed to induce experimental diabetes. This compound accumulates in pancreatic cells and selectively destroys the insulin producing beta-cells[26,27].

Experimental protocol

The experiments were conducted on 30 male nonlinear rats of the same age (16-wk-old). The 30th and 60th days from the final alloxan injection were chosen as the endpoints of the experiment. This duration of diabetes in rats corresponds to a duration of diabetes in humans approximately equal to 4.25 and 8.5 years, which is a sufficient time for the development of diabetes complications, including neurodegenerative complications[17,28]. Thirty rats with body weight of 190-220 g were randomly divided into three groups (n = 10 in each group): control (group 1), diabetes 30 d (group 2), and diabetes 60 d (group 3). The control animals (group 1) received *i.p.* saline injections at day 1 and between 30-60 d (20 injections in total). The diabetes 30 d animals(group 2), weighing approximately 207 ± 10 g, were rendered diabetic after 16 h fasting conditions, by a single *i.p.* administration of alloxan monohydrate (Sigma-Aldrich, St. Louis, MO, United States) at a dose of 300 mg/kg of body weight, dissolved in 10 mmol/L of sodium citrate (pH 4.5). Afterwards, the animals were housed in standard conditions until the end of the 30 d experimental duration of the group. The diabetes 60 d animals (group 3), weighing 207 ± 10 g, received a single *i.p.* dose of 300 mg/kg alloxan monohydrate and were housed in similar conditions for 60 d. Peripheral blood glucose from the tail vein was obtained to determine glycemia in all experimental groups (Table 1).

On the respective sacrifice dates of each animal, they were first anaesthetized with 40 mg/kg pentobarbital sodium administered intraperitoneally. Blood samples (approximately 3 mL) were collected by heart puncture for biochemical and enzyme immunoassay investigations. Histological, immunohistochemical, and light and electron microscopy methods were used to study the rat's eye slices.

Laboratory blood tests

Plasma glucose levels were determined with a standard glucose oxidase test kit (Novogluk-R, "VektorBest", Russia)[29,30]. The plasma insulin level was determined using a standard ELISA Rat assay (Insulin ELISA, Mercodia AB, Switzerland). Biochemical testing was carried out with a DU-800 spectrophotometer (Beckman Coulter Int S.A., Switzerland).

HbA1c measurement was performed by affinity chromatography ("Diabetes-test", (HbA1c) TOR 9398240-16404416-01, Fosfosorb OJSC, Russian Federation), according to the manufacturer's instructions ("Fosfosorb" OJSC, Russia)[31].

Histological studies

A neutral buffered solution of 10% formalin was used to preserve the eye samples for 24 h, then paraffinized through a series of solutions[30]. The standard dehydration procedure was performed. The tissue was processed and embedded in paraffin wax using the autoprocessor Leica EG 1160. Hematoxylin and eosin (HE) staining of the 3-5 micron thick sections were performed for morphological and morphometric studies. The remaining sections were placed in a buffer for antigen unmasking and further immunohistochemical studies.

Immunohistochemical studies

For immunohistochemical evaluation, tissues were first fixed in formalin, then embedded in paraffin, and sectioned at 3 μm. The antibody staining of the tissues was performed with the Autostainer DAKO, according to a standard protocol. High-temperature treatment in a citrate buffer (pH = 6) using Pascal DAKO[32-34], was employed for the unmasking procedure of antigens. The visualization of antigen-reactive cells was performed using the NovolinkTM Polymer Detection System (Novocastra Lab., Ltd), with its buffer solution consisting of a chromogenic agent 3.3-diaminobenzidine (DAB). Macrophages were visualized with anti-CD68 antibodies (clone KP1, Thermo Scientific). The assessment of proliferation was performed with mouse anti-rat monoclonal antibodies to the Ki-67 marker (clone MM1, Leica Microsystems).

Morphometric analysis

Using sections of eyeballs stained with HE, the number of vessels and melanocytes per unit area (0.01 mm^2 tissue of choroid) (N/0.01 mm^2) was estimated in the choroidea, whereas the total thickness and the thickness of separate layers (in μm) were estimated in the retina.

The number of proliferating cells in the ganglionic and internal nuclear retinal layers was estimated on sections stained with the Ki-67 proliferation marker, the ratio of the total proliferating cells to total number of cells in the retina layer was subsequently calculated. Using sections stained with CD68 marker, the number of CD68 positive cells per unit area (1 mm^2 tissue) (N/mm^2) was determined in the choroidea and the retina.

Optical-microscopic examination

Optical-microscopic examination was conducted with the microscope (Leica DM 2500), and the analysis of the image was done using Video TesT "Morphology" 5.0 program (VideoTesT, St. Petersburg, Russia).

Electron microscopy examination

For ultramicroscopic examination after enucleation of the eyeball, the lens of the eye and the posterior wall of the eyeball containing the retina and the choroid were fixed in a 2.5% solution of glutaraldehyde followed by postfixation in a 1% solution of osmium tetroxide (OsO4). After thorough washing, dehydration in alcohols of increasing concentrations (50%, 70%, 96% and 100%) was performed followed polymerization in an araldite resin at a temperature of 60 ℃[35]. Slices were created using ultramicrotome (Leica EM UC6), contrasted with lead citrate, and examined with the aid of a digital transmission electron microscope (Morgagni™ 268).

Statistical analysis

Analysis of data was performed using Statistica 6.0 software (StatSoft, United States), variables showing results with a heterogeneous distribution were analyzed using the nonparametric (*U*) Mann-Whitney test. All analysis was carried out at 0.05% significance level of probability.

RESULTS

Confirmation of diabetes development

The development of diabetes in experimental animals was confirmed by biochemical study. According to the results, a significant increase in the levels of glucose and glycosylated hemoglobin (HbA1c) and a decrease in the level of insulin were detected after alloxan administration in the animals of experimental groups 2 and 3 compared to the control group (Table 1).

Experimental diabetes: Thirty days

Retina: Histological examination of the retina and choroid of animals in the control group exhibited no structural disturbances (Figures 1 and 2A). However, in experimental group 2, moderately pronounced interstitial edema and fullness of dome capillaries in the ganglionic and inner nuclear layers of the retina were observed (Figure 3A).

Electron microscopic examination confirmed the presence of edema in the form of an expansion of the spaces between the layers of rods and cones and their partial deformation and disorganization of the outer and inner segments of the photoreceptors (Figures 3B and 4). In the outer nuclear layer, round-shaped nuclei with irregular intervals between them were observed. This feature was attributed to the developing interstitial edema. The contours of the nuclei were even. The chromatin was osmiophilic in the center of the nucleus and bright on the periphery. The monolayer of cells

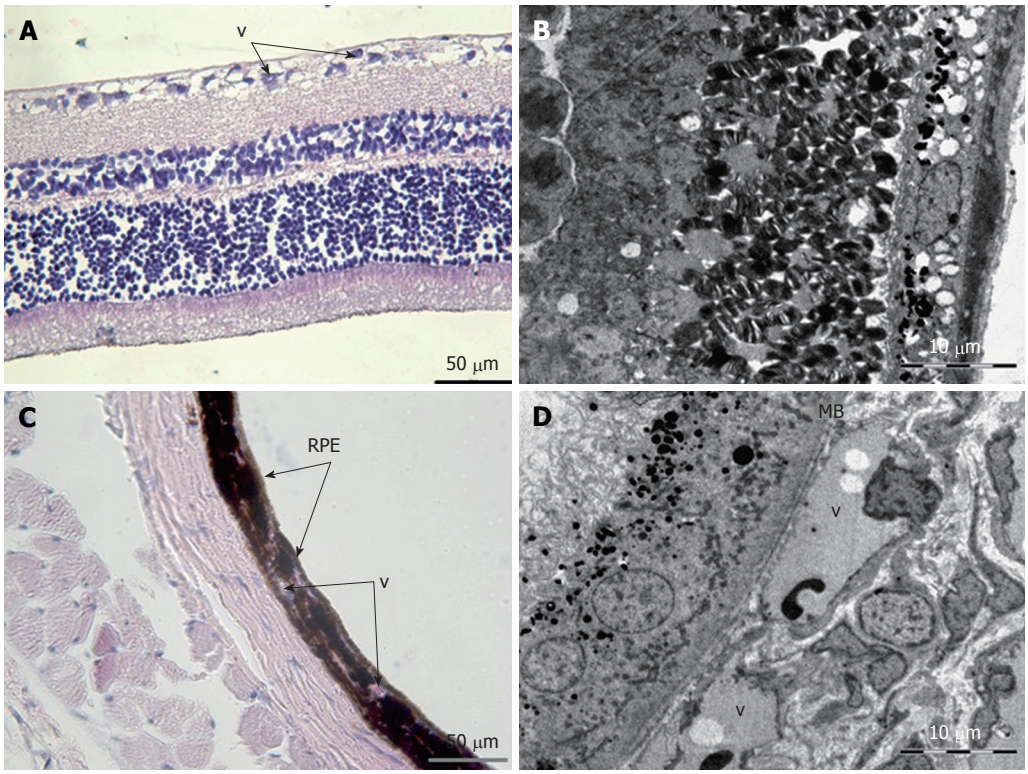

Figure 1 Back of the eye of a control animal. A: Light microscopy visualization of the retina. v: blood vessels; B: Electron microscopy of the outer layers of the retina; C: Light microscopy of the choroid and sclera of the eye. v: choroid vessels; RPE: pigment epithelium of the retina; D: Electron microscopy of the retinal pigment epithelium and choroid. v: choroid vessels; Light microscopy: staining with hematoxylin and eosin, magnification × 400, bar 50 μm; Electron microscopy: bar 10 μm.

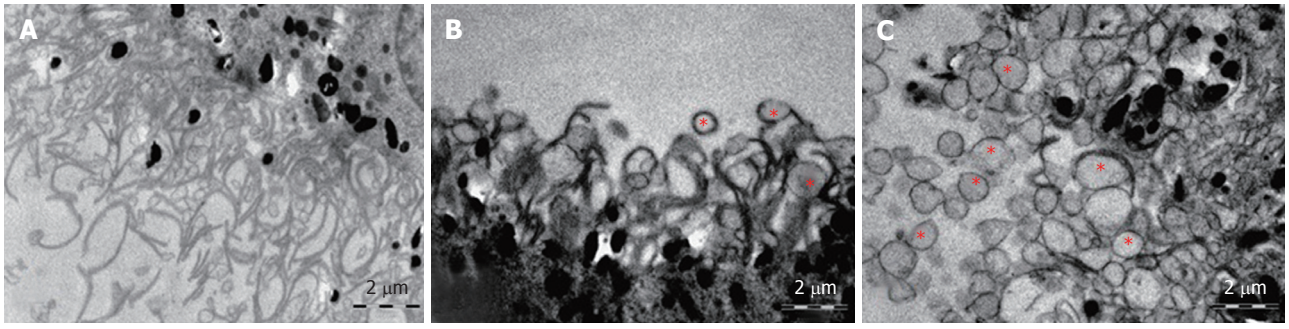

Figure 2 Cell processes of retinal pigment epithelium. A: Control animals (group 1); B: Diabetes at 30 d (group 2); C: Diabetes at 60 d (group 3); Bar 2 μm. *: vacuolation of cell processes.

of retinal pigment epithelium adhered to the Bruch's membrane. In the cytoplasm of the pigment epithelium, an uneven distribution with a quantitative decrease of pigment granules was detected (Figure 2B). Electron microscopy revealed loosening of the membranes of the pigment epithelium nuclei, mitochondrial swelling, the destruction of the crista, and the enlightenment of the mitochondrial matrix (Figure 3D).

Morphometric examination of the retina revealed changes in the thickness of different layers. Thus, a decrease in the total thickness of the retina and in the rods and cones, outer nuclear and ganglionic layers was revealed, indicating the development of dystrophic processes during the time course of diabetes (Table 2).

Choroidea: Morphometric analysis of the choroidea revealed a decrease in the number of blood vessels per unit area in group 2 (1.79 ± 0.07) compared to the control animals (2.62 ± 0.33) (Table 3, Figures 5 and 6).

According to the results of optical microscopic examination, alterations of the microcirculatory vessels in the choroidea were detected accompanied by desquamation and swelling of endothelial cells. These features led to the occlusion of small capillaries, the expansion of their limen, and the development of edema (Figure 2C).

Electron microscopic examination revealed a pro-

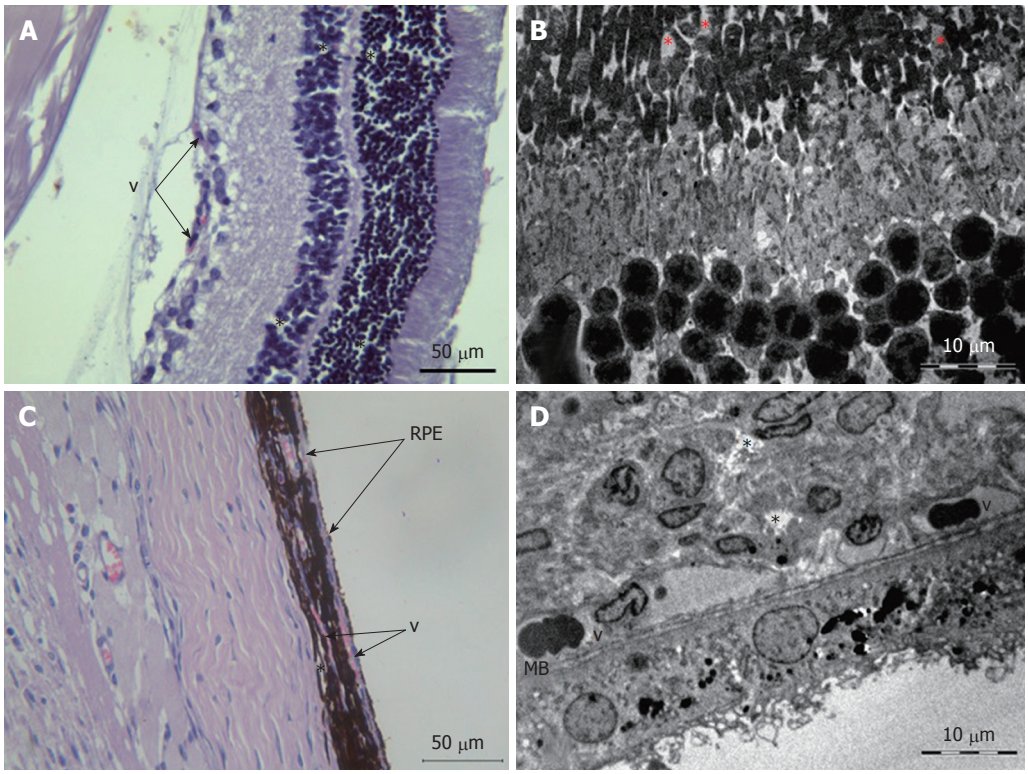

Figure 3 Posterior wall of the eye of an animal with diabetes at 30 d. A: Light microscopy of the retina; *: interstitial edema; v: full blood vessels; B: Electron microscopy of the outer layers of the retina; *: destroying rods and cones; C: Light microscopy of the choroid and sclera of the eye; *: interstitial edema; v: full blood vessels; RPE: destructive changes in retinal pigment epithelial cells; D: Electron microscopy of the retinal pigment epithelium and choroid; *: interstitial edema; v: choroid vessels with sludge complexes; MB: unevenly thickened Bruch's membrane; Light microscopy: staining with hematoxylin and eosin, magnification × 400, bar 50 μm; Electron microscopy: bar 10 μm.

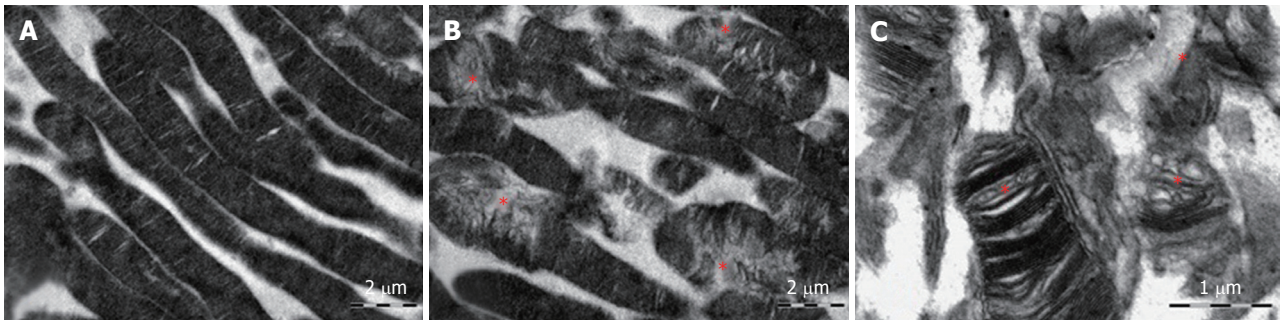

Figure 4 Photoreceptors of the retina. A: control animals (group 1), bar 2 μm; B: diabetes at 30 d (group 2), bar 2 μm; C: diabetes at 60 d (group 3), bar 1 μm. *: destruction of photoreceptors.

nounced loosening of the connective tissue with the formation of edema foci in the perivascular zone. The choroid was hypovascularized, and only a small number of vessels that were generally small in diameter were detected. In vessels, various alterations of the integrity of basal membranes as well as endothelial cell swelling and their partial destruction were clearly defined. The sluggish erythrocytes were visible in the lumen of capillaries (Figure 3D).

Based on light microscopy, the pigmented layer of the choroid after 30 d of experimental diabetes was characterized by pronounced dystrophic changes in melanocytes with the destruction of their cytoplasmic membrane and the release of pigment granules into the intercellular space.

According to the results of optical microscopic examination, the layer of melanocytes in the choroid was characterized by pronounced dystrophic changes in melanocytes with the destruction of their cytoplasmic membranes and signs of pigment granule release into the intercellular space. Melanocytes located perivascularly were characterized by the presence of pronounced dystrophic changes in their ultrastructure: the destruction of mitochondria and endoplasmic reticulum and the output of secretory granules to the extracellular space. The number of choroidal melanocytes was significantly

Table 2 Characteristics of the thickness of the retina and its individual layers (μm, M ± m)

Group	Retinal layers						Total thickness of the retina
	Layer rods and cones	Outer nuclear layer	Outer plexiform layer	Inner nuclear layer	Inner plexiform layer	Ganglion cell layer	
Control (group 1)	36.31 ± 5.11	56.43 ± 1.72	9.85 ± 1.68	25.82 ± 0.76	38.93 ± 4.79	17.82 ± 0.72	185.16 ± 9.42
Diabetes at 30 d (group 2)	28.65 ± 3.44[a]	51.62 ± 6.51[a]	11.46 ± 1.59	26.1 ± 1.55	40.21 ± 7.14	15.98 ± 1.37[a]	174.00 ± 2.93[a]
Diabetes at 60 d (group 3)	28.38 ± 1.43[a]	56.87 ± 5.30	9.69 ± 1.04[c]	26.24 ± 0.95[a]	39.94 ± 7.10	14.65 ± 2.05[a]	175.77 ± 5.22[a]

[a]Differences compared to control animals were significant at $P < 0.05$; [c]Differences compared to animals with diabetes at 30 d were significant at $P < 0.05$.

reduced per unit area (20.5 ± 0.39) compared to the control animals (10.1 ± 2.42) (Table 4).

Immunohistochemical study results: Proliferating cells are localized in the inner nuclear and ganglionic layers or retina, where glia cells capable of proliferating are present. Ki-67 positive cells were reduced in the inner nuclear and ganglionic layers of the retina in both the absolute and relative indices, and the decrease was more pronounced in the ganglionic layer (Table 4, Figures 7 and 8).

Immunohistochemical staining of the choroid and retina with anti-CD68 antibodies revealed a decrease in the number of macrophages in the retina, both in the ganglionic and inner nuclear layers compared to control animals. No significant changes were observed in the choroidea (Table 5).

Experimental diabetes: Sixty days

Retina: Histological examination of the retina of experimental animals from group 3 revealed an increase in dystrophic changes of photoreceptor and pigment epithelium layers compared to the histological features of group 2 animals (Figure 4). A plethora of capillaries of the retinal ganglionic layer and foci of angiomatosis in the inner nuclear layer were also observed (Figure 9).

Morphometric examination of the retina revealed changes in the thickness of different layers. Thus, a decrease in the thickness of the photoreceptor layer, internal nuclear, ganglionic, and outer reticular layers was revealed, indicating the dynamics of the development of dystrophic processes during the time course of diabetes (Table 2 and Figure 6).

Electron microscopic examination revealed signs of partial destruction of the layer of rods and cones. The remains of the membrane discs were observed, some of which were clearly visualized. In the inner nuclear layer, small diameter vessels of the sinusoidal type were observed (Figure 9B). Cells of the pigment epithelium of the retina were arranged on Bruch's membrane, exhibiting a folded, uneven shape with invagination sites (Figure 2C). The nuclei of the pigment cells and pigment granules were determined extracellularly, and cell outgrowths were in a state of destruction (Figure 9D).

Choroidea: Melanocyte dystrophy (a redistribution of melanin granules with a decrease in the total number of cells), which was described in group 2, was preserved (Table 3 and Figure 9C).

In the connective tissue layer, focal vascular fullness with the formation of sludge complexes was revealed and accompanied by the occlusion of some vessels, endothelial cell swelling, and the destruction of the basal membrane. The number of vessels per unit area corresponded to the values obtained at 30 d (Table 3).

Electron microscopy examination revealed loosening of connective tissue and massive perivascular edema. Most of the observed vessels were characterized by an enlarged lumen with swollen endothelial cells. The cytoplasmic membrane of the endothelial cells and their nuclei were uneven and folded. Swollen mitochondria with a visible matrix and the remnants of crista were detected inside the cells.

Results of immunohistochemical study: The immunohistochemical study of Ki-67 positive cells revealed that their quantity did not decrease and were similar to group 2 (Table 4, Figures 7 and 8).

Table 3 Average number of blood vessels and pigment cells in the choroid of the eyes of experimental animals (per unit area, S = 0.01 mm^2)

	Control (group 1)	Diabetes at 30 d (group 2)	Diabetes at 60 d (group 3)
No. of blood vessels	2.62 ± 0.33	1.79 ± 0.07[a]	1.59 ± 0.22[a]
No. of pigment cells	35.23 ± 5.69	20.5 ± 0.39[a]	10.1 ± 2.42[a]

[a]Differences compared to control animals were significant at $P < 0.05$.

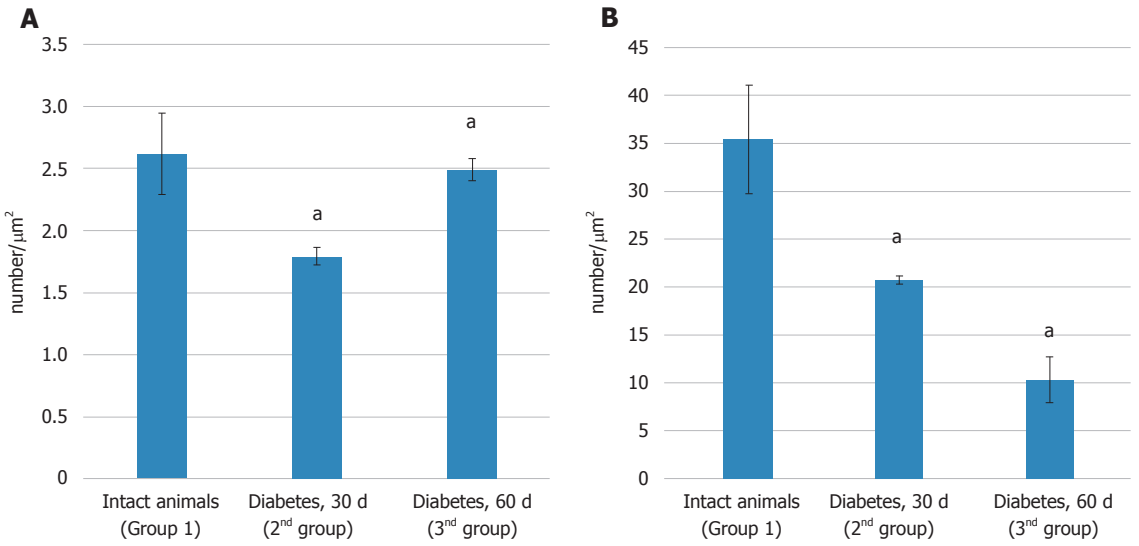

Figure 5 Morphometric examination of the choroid of the eyes of animals in experimental groups. A: The average number of choroidal vessels per unit area (0.01 mm^2); B: Average number of pigment cells of the choroid per unit area (0.01 mm^2). [a]Differences compared to the control animals were significant at $P < 0.05$.

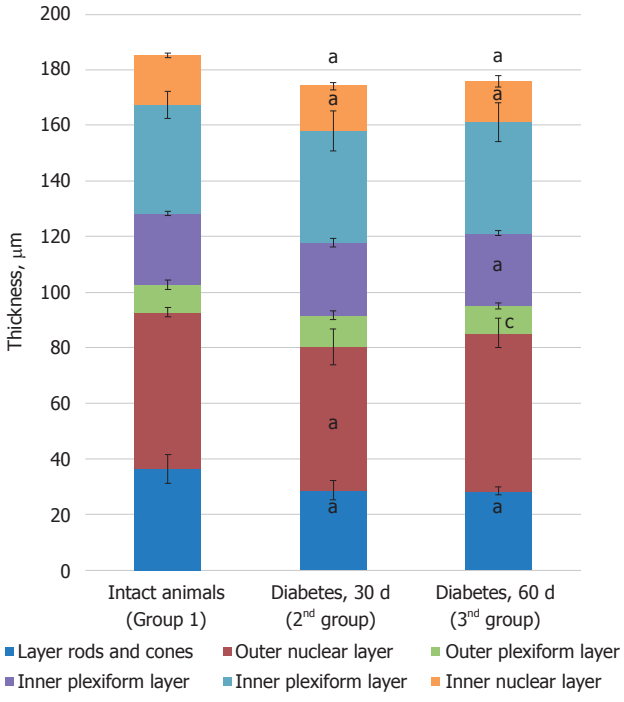

Figure 6 Morphometric examination of retinal layers of experimental groups. [a]Differences compared to control animals were significant at $P < 0.05$; [c]Differences to animals with diabetes for 30 d were significant at $P < 0.05$.

Immunohistochemical staining of the choroidea and retina with anti-CD68 antibodies revealed an increase in the number of macrophages in choroidea compared to group 1 and group 2. The quantity of macrophages in the inner layer of the retina was similar to group 2. In the ganglionic layer, an increase in the number of macrophage was equal to the control group (Table 5).

DISCUSSION

A plethora of evidence obtained over the past 20 years based on different clinical studies and experimental data have shed more light on the development and pathogenesis of DR and how it develops[6,10,11,36,37]. However, the complexity of pathogenic pathways that lead to the development of DR is beyond the scope of this article and are reviewed elsewhere[5,6,10,11,36]. The typical histological picture of diabetes characterized by the destruction of stroma and cell elements was also described in a number of studies[37].

The aim of the present study was to supplement this picture with the use of immunohistochemical and morphometric methods of investigation to estimate the numbers and proliferation status of individual cellular elements (melanocytes), thus providing information about the time course of destructive processes with the focus on the early stages of diabetes development.

In the present study, the alloxan-induced diabetes model demonstrated that in the early stages of the

Table 4 Number of Ki-67 positive cells in the layers of the retina (M ± m)

Group	Layers of the retina					
	Inner nuclear layer			Ganglion cell layer		
	1000 at 1 mm^2		% of Ki-67 positive cells	1000 at 1 mm^2		% of Ki-67 positive cells
	All cells	Ki-67 positive cells		All cells	Ki-67 positive cells	
Control (group 1)	28.60 ± 2.11	7.25 ± 0.93	25.46 ± 3.53	7.71 ± 1.01	0.99 ± 0.3	12.98 ± 3.24
Diabetes at 30 d (group 2)	27.94 ± 1.14	4.92 ± 0.92[a]	17.82 ± 3.79[a]	5.45 ± 0.78[a]	0.42 ± 0.18[a]	7.83 ± 3.11[a]
Diabetes at 60 d (group 3)	29.24 ± 2.56	4.55 ± 1.5[a]	15.4 ± 4.76[a]	6.19 ± 0.79[a]	0.59 ± 0.3[a]	9.95 ± 5.12

[a]Differences compared to control animals were significant at $P < 0.05$.

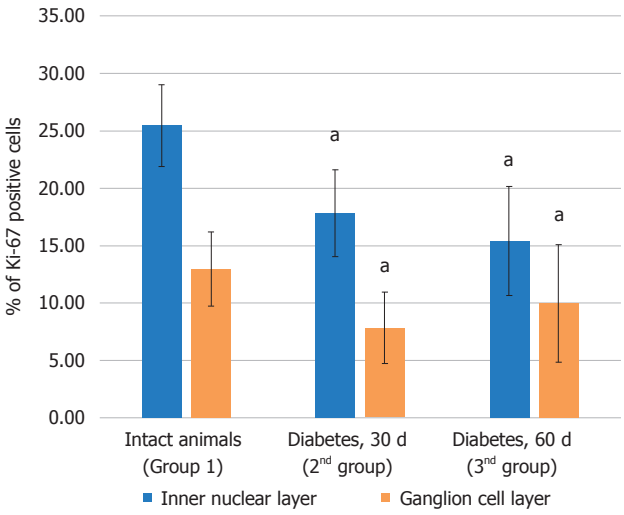

Figure 7 Relative content of Ki-67 positive cells from the total number of cells in the inner nuclear and ganglionic layers of the retina. [a]Differences compared to control animals were significant at $P < 0.05$.

disease (30 d), diabetic alterations in the structures of the retina and choroid are present, and these alterations progress slightly after 60 d.

In the retina, these disorders manifest themselves as a partial destruction of the structural-functional elements, namely, photoreceptors and are accompanied by a stromal reaction in the form of the development of interstitial edema, which was confirmed by the histological and electron microscope images of the examined structures[38]. In addition, morphometric analysis revealed a reduction in the thickness of the retina due to photoreceptor destruction. Moreover, in retinal layers that are capable of proliferation (the inner nuclear layer and ganglionic layer), the number of Ki-67 positive cells decreased with the development of diabetes.

The choroidea consists of a network of chorio-capillaries and stroma. Similar to other types of connective tissue, mast cells, macrophages, and lymphocytes are present in the stroma[39]. It is believed that the vascular membrane fulfills the function of supplying the outer layers of the retina with oxygen and nutrients. Thus, disruption of the choriocapillary structure causes degenerative changes in the latter and its neovascularization[39-41]. However, the precise cellular mechanisms leading to retinal dysfunction under high glucose levels remain unclear.

According to these results, a reduction in the number of blood vessels of the choroid with the pathological alterations of endothelial cells and vascular walls were observed. Moreover, the described changes develop during early stages of the disease (30 d) and generally do not change as time progresses.

Pathological changes in the number and state of cellular elements of the stroma of choroidea (melanocytes and macrophages) complete the picture of DR. Thus, the persistent reduction in the number of melanocytes in the choroidea (1.5-fold at 30 d and 3-fold at 60 d) was observed. Moreover, the pigment epithelium of the retina exhibited signs of dystrophic changes in the ultrastructure of cells accompanied by a reduction in the amount and redistribution of melatonin granules in these cells. Moreover, given that melanocytes release the key factors of angiogenesis, such as fibromodulin, a reduction in melanocytes may be one of the factors that leads to the above described reduction in the number of capillaries in the choroidea[42].

Macrophages are present in the choroidea under normal conditions, performing homeostatic functions[42]. However, in DR macrophages play a key role in the development of the inflammatory response, releasing pro-inflammatory cytokines that lead to capillary degeneration[43]. Moreover, according to Aveleira *et al*[44], the proapoptotic effect of inflammatory cytokines is significantly increased with hyperglycemia. According to our results, an increase in the number of macrophages (3.5-fold) in the choroidea was observed in diabetes[44]. Apparently, such a pronounced macrophage infiltration was caused by the recruitment of cells of the monocyte-macrophage lineage from the blood stream, as evidenced by their perivascular localization. The initiating factor of the observed migration of macrophages into the choroid was the development of destructive disorders (inflammation) in the latter[45].

Finally, a significant reduction (3.5-fold) in the number of pigment cells was also observed, which corresponds to findings reported in the literature[46]. This feature characterized the progression of pathological changes in the choroidea and led to further disruption of the integrity of the hematoretinal barrier[47].

In general, based on the results of our study, it can be assumed that the starting point in the development

Table 5 Quantitative distribution of macrophages in the eyes based on the structures (M ± m / 1 mm^2)

Structure of the eye		Control (group 1)	Diabetes at 30 d (group 2)	Diabetes at 60 d (group 3)
Choroidea		4.16 ± 3.31	4.99 ± 2.84	14.4 ± 6.69[a,c]
Retina	Ganglionic layer	18.46 ± 2.66	9.42 ± 1.00[a]	16.25 ± 5.30[c]
	Inner nuclear layer	11.25 ± 3.71	6.6 ± 2.59[a]	7.87 ± 1.71[a]

[a]Differences compared to control animals were significant at $P < 0.05$; [c]Differences compared to animals with diabetes at 30 d were significant at $P < 0.05$.

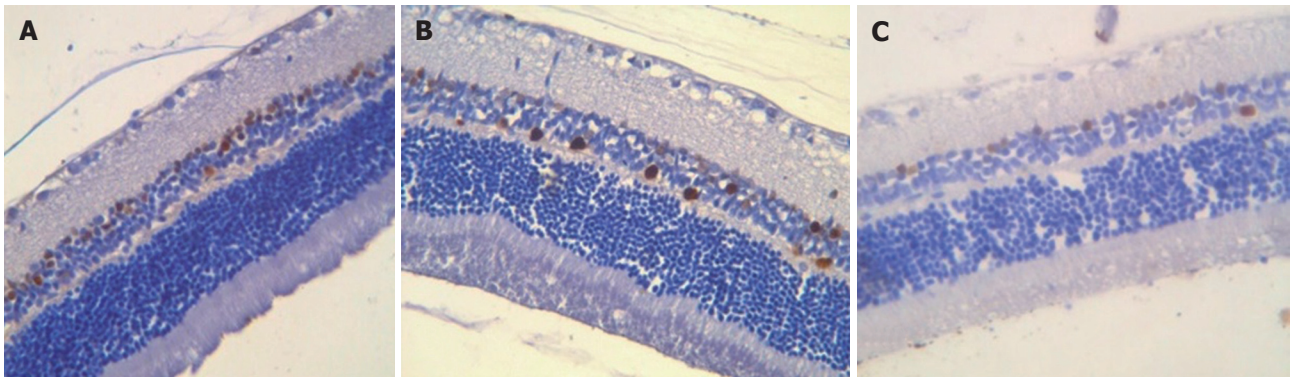

Figure 8 Ki-67 staining of the retina. A: Control animals; B: Diabetes at 30 d; C: Diabetes at 60 d.

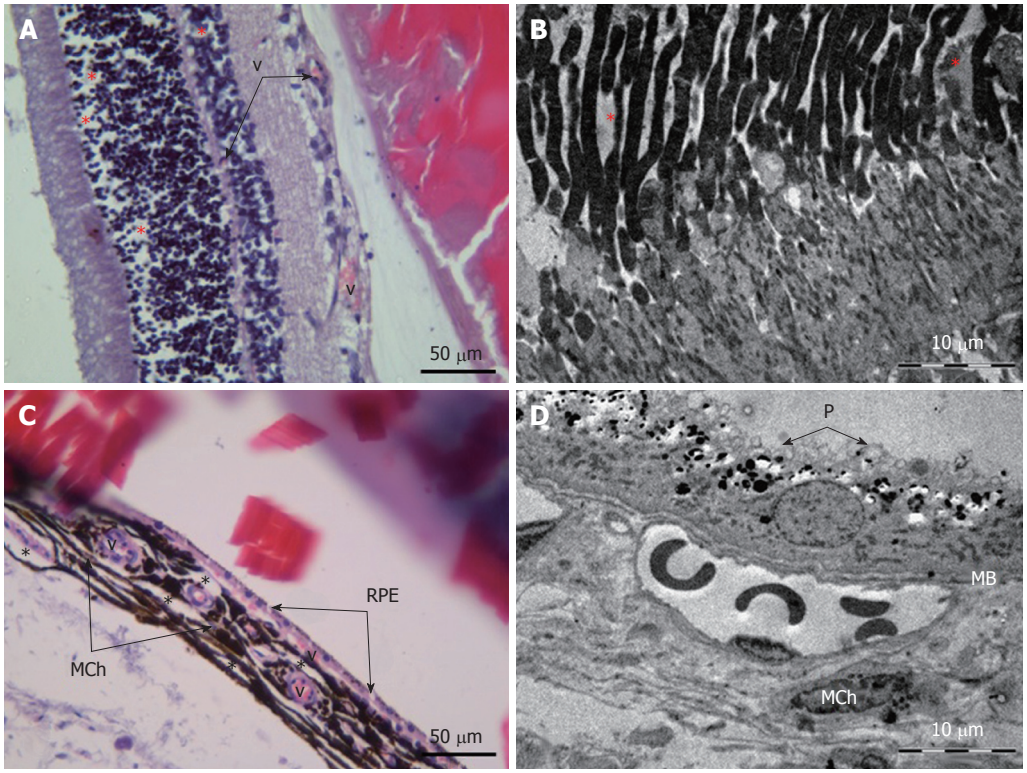

Figure 9 Posterior wall of the eye of an animal with diabetes at 60 d. A: Light microscopy of the retina; *: interstitial edema; v: full blood vessels; B: Electron microscopy of the outer layers of the retina; *: destruction of rods and cones; C: Light microscopy of the choroid of the eye; *: interstitial edema; v: full blood vessels with swollen endothelial cells; RPE: destructive changes in retinal pigment epithelial cells; MCh: destruction of pigment cells of the choroid; D: Electron microscopy of the retinal pigment epithelium and choroid; MB: uneven Bruch's membrane with invagination sites; MCh: choroidal melanocyte with reduced amount of pigment granules; P: destructive changes in the outgrowths of retinal pigment epithelial cells; Light microscopy: staining with hematoxylin and eosin, magnification × 400, bar 50 μm; Electron microscopy: bar 10 μm.

of destructive changes in DR involves the early reduction in the number of melanocytes of the choroidea and the destruction of the retinal pigment epithelium, which are the primary components of the hematoretinal barrier.

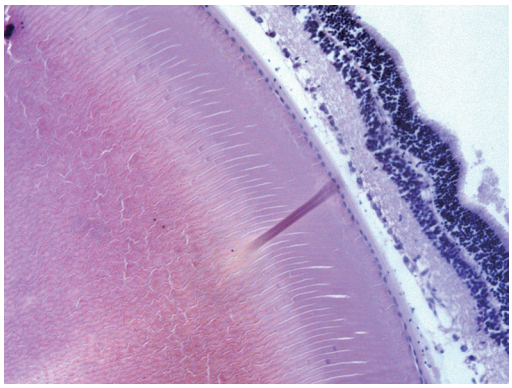

Figure 10 Fragment of the eye of an animal after alloxan administration at the dose of 30 mg/kg at 14 d. No visible structural changes are detected. Light microscopy: staining with hematoxylin and eosin, magnification × 400.

Limitations of the study

According to the literature, the direct toxic effects of alloxan on the retina, rather than secondary changes from diabetes, have been described[48-50]. Some teratogenic effects of alloxan in mice have been observed, including abnormalities of the lens and iris[49]. However, according to our results, the injection of alloxan in the total dose of 30 mg/100 g did not cause any disturbances at 14 d that could be observed via optical microscopy (Figure 10).

ARTICLE HIGHLIGHTS

Research background

Diabetic retinopathy (DR) is a disease commonly associated with diabetes complications. It is known as one of the primary causes of visual impairment and blindness globally. More recent discoveries have shown that indicators of inflammation, altered vascular permeability, and increased production of inflammatory mediators occurs in the retina after 1-6 mo of the presence of diabetes. However, most of therapeutic approaches being developed do not address the early and potentially reversible failure of retinal perfusion.

Research motivation

Better understanding of the temporal sequence and stages of pathological disturbances of DR development is of scientific value, as it might contribute to improvements to current methods or even the development of new methods of diagnosis and treatment of the early and potentially reversible failure of retinal perfusion.

Research objectives

We have investigated the temporal sequence of pathological changes in the cellular structures of retina and choroidea in a rat model of alloxan-induced diabetes in the early stages of disease.

Research methods

Alloxan accumulates in pancreatic cells, resulting in selective β-cell necrosis and diabetes. Experimental diabetes was modeled by three intraperitoneal injections (10 mg/100 g of weight) of an alloxan solution dissolved in physiological saline at 1-d intervals (total dose of alloxan 30 mg/100 g). The 30th and 60th days from the final alloxan injection were chosen as the endpoints of the experiment. Biochemical and enzyme immunoassay were performed. Furthermore, histological, immunohistochemical, and electron microscopy methods were employed to evaluate the rat's eye slices. Similarly, light microscopy and morphometric analyses of slides were also conducted.

Research results

In the present study, the alloxan-induced diabetes model demonstrated that in the early stages of the disease, diabetic alterations in the structures of the retina and choroid are present, and these alterations progress with time. In the retina, DR manifest itself as a partial destruction of the structural-functional elements, namely, photoreceptors and are accompanied by a stromal reaction in the form of the development of interstitial edema and a reduction in the thickness of the retina due to photoreceptor destruction. The reduction in the number of blood vessels of the choroid, melanocytes, and pigment cells along with an increase in the number of macrophages were also observed at early stages of the disease.

Research conclusions

The results of this study provide evidence that DR manifests itself at the early stages of diabetes. The starting point in the development of DR involves the early reduction in the number of melanocytes of the choroidea and the destruction of the retinal pigment epithelium, which are the primary components of the hematoretinal barrier.

Research perspectives

Further studies that estimated vascular endothelial growth factor, prostate-derived Ets transcription factor, cytokines, NO, and antioxidants and correlated them with blood glucose levels and changes in the retina in various experimental models and at different time periods will contribute to the improvements and the development of new methods of diagnosis and treatment of DR.

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ORIGINAL ARTICLE

Observational Study

Using real world data to assess cardiovascular outcomes of two antidiabetic treatment classes

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Author contributions: Stapff MP developed the scientific concept, literature search, study design, applied the data querying, result interpretation, scientific discussion, and prepared the manuscript.

Institutional review board statement: As a federated network TriNetX received a waiver from Western IRB since only aggregated counts, statistical summaries of de-identified information, but no protected health information is received, and no study specific activities are performed in retrospective analyses.

Informed consent statement: This was an observational study based on analyses of anonymized electronic medical records describing real world treatment. No intervention or any study specific activity was done. Therefore, no informed consent was necessary and would even have been not feasible considering the anonymized and retrospective character of the analysis.

Conflict-of-interest statement: The author is employee of TriNetX Inc., the data network and analytics platform used for this publication. TriNetX as a company was not involved in the design of the study; the collection, analysis, and interpretation of data; writing the report; or the decision to submit the report for publication. The author does not declare conflicting interests (including but not limited to commercial, personal, political, intellectual, or religious interests).

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Abstract

AIM

To evaluate the effect on cardiovascular outcomes of sodium-glucose co-transporter-2 (SGLT2) inhibitors in a real world setting by analyzing electronic medical records.

METHODS

We used TriNetX, a global federated research network providing statistics on electronic health records (EHR). The analytics subset contained EHR from approximately 38 Million patients in 35 Health Care Organizations in the United States. The records of 46,909 patients who had taken SGLT2 inhibitors were compared to 189,120 patients with dipeptidyl peptidase (DPP) 4 inhibitors. We identified five potential confounding factors and built respective strata: elderly, hypertension, chronic kidney disease (CKD), and co-medication with either insulin or metformin. Cardiovascular events were counted

as stroke (ICD10 code: I63) or myocardial infarction (ICD10: I21) occurring within three years after the first instance of the respective medication in the patients' records.

RESULTS

Of the 46909 patients with SGLT2 inhibitors in their EHR, 1667 patients (3.6%) had an ICD code for stroke or for myocardial infarction within the first three years after the first instance of the medication. In the control group, there were 10680 events of 189120 patients (5.6%), which represents a risk ratio of 0.63 (95%CI: 0.60-0.66). The overall incidence of stroke or myocardial infarction in the strata with a potential confounding risk factor reached from 4.9% in patients taking metformin to 12.5% in the stratum with the highest risk (concomitant CKD). In all strata, the difference in risk of experiencing a cardiovascular event was similarly in favor of SGLT2 *vs* control, with Risk Ratio ranging from 0.62 to 0.81.

CONCLUSION

Real world data replicated the results from randomized clinical trials, confirmed the cardiovascular advantages of SGLT2 inhibitors, and showed its applicability to the US population.

Key words: Sodium-glucose co-transporter-2 inhibitors; Cardiovascular events; Clinical trials; Electronic medical records; Dipeptidyl peptidase 4 inhibitors; Real world evidence; Diabetes

Core tip: Cardiovascular advantages of sodium-glucose co-transporter-2 (SGLT2) inhibitors were shown in complex clinical trials or in countries with large registries. However, it was unclear whether these findings could be applied to routine medical practice in the US. This real world analysis from 46909 patients with SGLT2 inhibitors revealed a 0.63 (95%CI: 0.60-0.66) risk ratio of SGLT2 inhibitors compared to 189120 patients with dipeptidyl peptidase 4 inhibitors. This analysis of electronic health records could replicate the results of randomized clinical trials, which supports the usefulness of such real world studies (*e.g.,* for long-term outcome or safety observations).

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INTRODUCTION

An estimated 30.3 million people of all ages (or 9.4% of the United States population) had diabetes in 2015[1]. It is expected that the world prevalence of diabetes among adults will increase to 7.7%, or 439 million adults, by 2030. Between 2010 and 2030, there will be a 69% increase in the number of adults with diabetes in developing countries, and a 20% increase in developed countries[2].

While short-term treatment targets focus on the normalization of values for glucose and hemoglobin A1c, the long-term objective is to avoid late-stage complications of diabetes and end-organ damage. Up to 70% of patients with diabetes type Ⅱ (T2DM) also have arterial hypertension[1] and are thus exposed to an increased risk of experiencing a stroke or heart attack. It is therefore important that treatment paradigms for T2DM consider the long-term cardiovascular risk.

In 2015, the EMPA-REG OUTCOME trial found a significant mortality benefit of sodium-glucose co-transporter-2 (SGLT2) inhibitors *vs* placebo[3]. Because the findings were unexpected, unprecedented and not linked to obvious mechanistic pathways, it was suggested that the results be replicated in future investigations[4]. Recently, CVD-REAL Nordic, a multinational observational study, analyzed the cardiovascular mortality and morbidity in patients with T2DM following initiation SGLT2 inhibitors[5]. CVD-REAL Nordic was an observational analysis of individual patient-level data from national registries in three Scandinavian countries, showing that SGLT2 inhibitor use was associated with reduced cardiovascular disease and cardiovascular mortality.

The objective of the following analysis was to support or contradict the results of EMPA-REG OUTCOME and CVD-REAL Nordic by using electronic medical records (EMR) from a predominately United States-based research network, thus evaluating the representativity of these results outside the experimental setting of a randomized clinical trial and beyond a European population, respectively.

MATERIALS AND METHODS

We used TriNetX, a global federated research network providing access to statistics on EMR (diagnoses, procedures, medications, laboratory values, genomic information). The analytics subset allowed the analysis of approximately 38 million patients in 35 large Health Care Organizations predominately in the United States. As a federated network, TriNetX received a waiver from Western IRB, since only aggregated counts, statistical summaries of de-identified information, and no protected health information is received. In addition, no study-specific activities are performed in retrospective analyses. Details of the network have been described elsewhere[6-8]. All analyses were done in the TriNetX "Analytics" network using the browser-based real-time analytics features. At the time of the analysis in June 2018, we analyzed the EMR of 46909 patients in the network who had an instance of any SGLT2 inhibitor

Table 1 Patient characteristics and results before correcting for potential confounding factors

	SGLT2	Control
n	46909	189120
Mean age	59	66
SD age	11	13
Percent male	53%	52%
Comorbidities		
Hypertension (I10)	45%	41%
CKD (N18)	4%	8%
Co-medication		
On insulin	32%	19%
On metformin	52%	33%
LDL cholesterol (mg/dL)	91.6	93.1
HDL cholesterol (mg/dL)	43.6	43.2
After index event		
Total stroke (I63) or MI (I21)	12347 (5.2%)	
n in group	1667	10680
Percent in group	3.60%	5.60%
RR SGLT2 *vs* control	0.63	

SGLT2: Sodium-glucose co-transporter-2; RR: Risk ratio; SD: Standard deviation; CKD: Chronic kidney disease; LDL: Low density lipoprotein; HDL: High density lipoprotein; MI: Myocardial infarction.

(empagliflozin, dapagliflozin or canagliflozin) any time within the past ten years in their electronic medical record. As a comparison group, we chose patients who had taken dipeptidyl peptidase (DPP) 4 inhibitors (linagliptin, alogliptin, sitagliptin or saxagliptin) during the same time, and found 189120 patients. Using a Bayesian statistical approach[9] on demographics and pre-existing (baseline) comorbidities of the two groups, we identified five potential confounding factors and built strata with the following criteria: age ⩾ 60 years, presence of hypertension [International Classification of Diseases (ICD)10 code I10], presence of CKD (ICD10 code N18), co-medication with insulin, and co-medication with metformin. Separately analyzing strata allowed us to address potential bias in the federated data model without direct access to the individual data sets on the patient level.

Cardiovascular events were counted by selecting any stroke (ICD10 code I63) or myocardial infarction (ICD10 code I21) occurring during a three-year observation period after the first instance of the above mentioned medications in the patients' records.

The risks of experiencing an event in each stratum were calculated by dividing the number of patients with an event (numerator) by the total number of patients with the respective medication in each stratum (denominator). The risk ratios for SGLT2 inhibitors *vs* the comparison group were calculated by dividing the risk for each SGLT2 stratum by the risk in each corresponding DPP4 stratum.

RESULTS

Of the 46909 patients taking SGLT2 inhibitors, 1667 patients (3.6%) had an ICD code for stroke or myocardial infarction during their three-year observation period, compared to 10680 of 189120 (5.6%) in the control group (Table 1). This translates into a risk ratio of 0.63 without any correction for potential bias ($P < 0.001$; 95%CI: 0.60-0.66).

SGLT2 inhibitors carry a contra-indication for renal insufficiency[10]. Indeed, the percentage of patients with CKD was only 4% in the SGLT2 group, compared to 8% in the control group. While the groups were similar in gender distribution (53% and 52% male, respectively) and low density lipoprotein, as well as high density lipoprotein levels, the SGLT2 group was younger than the control group (mean age 59 *vs* 66) and had more patients with concomitant hypertension (45% *vs* 41%). There were also differences in the use of insulin (32% *vs* 19%) and metformin (52% *vs* 33%). To balance for these potential confounding factors, strata were built for age ⩾ 60 years, CKD, hypertension, and antidiabetic co-medication (insulin and metformin). The overall incidence of stroke or myocardial infarction in each stratum reached from 4.9% to 12.5%. In all strata, the difference in the risk of experiencing a cardiovascular event in the SGLT2 group *vs* control was similarly in favor of SGLT2, with risk ratios ranging from 0.62 (co-medication insulin) to 0.81 (patients with CKD) (Table 2).

DISCUSSION

Drug therapy of type Ⅱ diabetes mellitus should both bring glucose and hemoglobin A1c values into an acceptable and stable range, and reduce the likelihood of end organ damage or cardiovascular events.

Several studies and meta-analyses have suggested a positive effect on cardiovascular outcomes by the SGLT2 inhibitor class[11,12]. EMPA-REG OUTCOME and CANVAS were randomized placebo controlled prospective trials that used empagliflozin[4] and canagliflozin[13], respectively.

A recent observational cohort study observed protective effects of SGLT2 inhibitors compared to sulfonylureas by a database analysis[14]. Another study, CVD-REAL Nordic, was the first large observational analysis performed in real world settings in three Scandinavian countries that evaluated the cardiovascular benefits of this class, which also showed that SGLT2 inhibitor use was associated with reduced cardiovascular disease and cardiovascular mortality compared with the use of other glucose-lowering drugs[4]. Such real-world studies are less complicated and significantly less costly than traditional prospective randomized clinical outcomes trials. In addition, the reduced number of eligibility criteria ensures that the study results are representative and applicable to a much wider population. Recently, another study confirmed that real-world data analyses of patients receiving routine care provide findings similar to those found in a randomized clinical trial, and may even support (supplemental) regulatory applications[15]. Real world evidence can sometimes complement or

Table 2 Results from the patient subgroups (strata) with potential confounding factors

	Stratum 1 > 60 yr		Stratum 2 hypertension		Stratum 3 CKD		Stratum 4 insulin		Stratum 5 metformin	
	SGLT2	control	SGLT2	control	SGLT2	control	SGLT2	control	SGLT2	control
n	23594	131219	27499	115703	3786	34388	24395	90978	37762	136569
patients with stroke or MI	9784 (6.3%)		10827 (7.6%)		4755 (12.5%)		8976 (7.8%)		8629 (4.9%)	
n in group	1077	8707	1452	9375	391	4364	1275	7701	1394	7235
percent in group	4.60%	6.60%	5.30%	8.10%	10.30%	12.70%	5.20%	8.50%	3.70%	5.30%
RR SGLT2 *vs* control	0.69		0.65		0.81		0.62		0.7	

SGLT2: Sodium-glucose co-transporter-2; MI: Myocardial infarction; RR: Risk ratio; CKD: Chronic kidney disease.

even replace randomized controlled trials, but prejudices and reservations so far have limited their acceptance[16].

Therefore, the underlying data sources must be reliable, and the methods used have to be defined in advance to avoid "data dredging" based on the findings[17]. Furthermore, the data usually come from non-consented patients and therefore the highest standards of data privacy must be ensured.

The present study was undertaken to evaluate whether the results of the EMPA-REG OUTCOME and CVD-REAL Nordic studies can be replicated in a federated network of EHR, and if they can be applied to a predominantly United States American population. As controls, we chose DPP4 inhibitors that represent another homogeneous and relatively new non-metformin class. We found a significantly lower incidence of stroke or myocardial infarction in the SGLT2 group within the three-year observational period compared with the control group.

In a federated data network, individual data sets never leave the source (*i.e.*, the data warehouse of a healthcare organization). Instead, the analyses are done based on aggregated statistical counts. At the time of this analysis, our platform limited the methods that could be applied to correct for potential confounding factors, such as pair matching or propensity score matching (PSM). While PSM is a popular method of preprocessing data for causal inference, it is controversial since it may accomplish the opposite of its intended goal, such as increasing imbalance or bias[18]. In addition, the censoring by PSM that excludes certain patients from the analysis, reduces the sample size and the representation of a diverse patient population, thus reintroducing the criticism often applied to randomized clinical trials regarding their very restrictive eligibility criteria.

We therefore chose to build subgroups of the study population according to the presence of potentially confounding factors, and to test these strata individually. SGLT2 inhibitors have a contraindication for renal insufficiency and are a relatively new class of antidiabetics with less long-term experience than comparator classes, such as metformin or DPP4 inhibitors. One can therefore assume that the treatment decision by prescribing physicians may be driven by a patient's renal function, patient age, and other potential risk factors. Indeed, we found a lower mean age in the SGLT2 group, similar to CVD-REAL Nordic before matching. Furthermore, the SGLT2 group had fewer patients with CKD than the comparison group. In prospective randomized clinical trials, such factors usually get balanced by randomization, which must be corrected for when a retrospective analysis is done. We therefore created five strata, based on age $\geqslant$ 60 years, hypertension, CKD, insulin therapy or metformin therapy, and tested the event rates individually in each of these subgroups. The fact that the overall highest event rate was found in the higher risk stratum (patients with CKD) provides internal validation for the selection of the strata.

All strata showed very similar hazard ratios for cardiovascular events (according to our definition using ICD10 codes for myocardial infarction or stroke), which were consistently in favor of the SGLT2 inhibitor group, *i.e.*, between 0.62 and 0.81. This generally confirms the findings of the CVD-REAL Nordic study, where the risk ratio for cardiovascular mortality and for major cardiovascular events was in a similar range of 0.53 and 0.78, respectively.

Limitations

Due to the nature of the design (retrospective, non-randomized) and data analysis (federated, aggregated strata), this study could be done very quickly, simplistically and with minimal cost, but may have several limitations. Non-randomized comparisons bear the risk that patients' disease state influence the treatment decision and thus introduce imbalances. We limited balancing for confounders to five major factors and did not further correct for residual, potentially confounding factors like other co-morbidities, duration of diabetes, glucose or HBa1c values, concomitant medications or length of exposure to concomitant treatment. Our outcome criteria were simply the ICD10 codes for myocardial infarction or stroke, relying on correct coding at the source without differentiation between morbidity and mortality. Despite the fact that one specific compound numerically dominated in each group (SGLT2: canagliflozin 78%, DPP4: sitagliptin 69%), we consider the results as representative of a class but not robust enough for a comparison of two individual compounds.

Real world studies depend on the prescribing and documentation behavior of the data-providing institutions. We used EHR in structured form rather than

Table 3 Data density in the two comparator cohorts

	SGLT2	Control
Total facts	54852092	261813664
Avg facts per patient	1143	1325
Avg diagnosis facts per patient	231	262
Eastern United States, patients (%)	68	69
Western United States, patients (%)	32	31

SGLT2: Sodium-glucose co-transporter-2; Avg: Average.

claims data. This has the advantage of complete medical information coming from the respective Health Care Organization, but data may be lacking if a patient visits another institution. This especially applies to medication and prescription refills. While we defined an observational period of three years, we could not validate whether the patients actually stayed with their medication for the whole period, as we defined the treatment group based on one documentation of SGLT2 or DPP4 in their records. Insofar as a difference in compliance or persistence between the groups could introduce a potential imbalance, the approach would be similar to the intent-to treat principle, which is applied to randomized clinical trials.

Furthermore, differences in the completeness of medical records between comparison groups need to be taken into consideration as well. In searching for a potential documentation bias, we found similar data density in the SGLT2 cohort compared to control (Table 3).

Theoretically, one could assume that more events had been found in the control group simply because this patient cohort was better documented. In real world studies, consideration of different therapeutic settings and documentation completeness is important, *e.g.,* when comparing oral *vs* injectable medication, or inpatient *vs* outpatient procedures. However, SGLT2 inhibitors and DPP4 inhibitors are both taken orally and prescribed in similar settings. In addition, our data overall found about 20% more events in the DPP4 group, but the density of facts per patient in the documentation of this group was only 6% higher. Therefore, a documentation bias as an explanation for the difference in CV events in this study is very unlikely.

In conclusion, this study was conducted by analyzing EHR of approximately 38 million patients from 35 healthcare organizations, mainly from the United States. This real world clinical setting allows the analysis of data from patients with a much broader cardiovascular risk profile than the highly selective population in randomized clinical trials. The federated structure of this network ensures the highest level of data privacy standards, but poses some restrictions on the possible analytics, such as matching by propensity scores. Despite these limitations: (1) this analysis could replicate the results from much more complex and costly studies on the same topic, which validates our methods and the quality of data in the network; (2) our analysis shows that the cardiovascular advantages of SGLT2 inhibitors found in the Scandinavian CVD-REAL Nordic study can be applied to the United States American population.

ARTICLE HIGHLIGHTS

Research background

Therapy for diabetes mellitus intends to control blood glucose values, to prevent or delay diabetic complications such as chronic kidney disease or retinopathy, and to reduce the likelihood of cardiovascular events like myocardial infarction or stroke. Several randomized clinical trials and sophisticated European registries have suggested that sodium-glucose co-transporter-2 (SGLT2) inhibitors may have an advantage in preventing cardiovascular events.

Research motivation

Randomized clinical trials are conducted on highly selected patient populations and follow very artificial treatment protocols. This makes it sometimes questionable whether the results are representative and can be applied to routine medical practice.

Research objectives

To determine whether positive results from randomized clinical trials with SGLT2 inhibitors can be confirmed by real world data from actual routine medical practice in the United States.

Research methods

A federated research network was used, allowing analyses of electronic medical records (EMR) from 38 million patients in 35 large Health Care Organizations predominately in the United States. Cardiovascular events occurring during a three-year observation period after start of a therapy with an SGLT2 inhibitor were counted and compared to a control group starting dipeptidyl peptidase 4 inhibitors. Comorbidity strata were created to address potential confounders.

Research results

In the overall cohort and in all comorbidity strata, the risk of experiencing a cardiovascular event was similarly in favor of SGLT2, with risk ratios ranging from 0.62 to 0.81.

Research conclusions

The analysis of data from patients with a much broader cardiovascular risk profile than the selected population in randomized clinical trials could replicate the results of such trials. This validates the methods and quality of data in the network, and allows extrapolation of the trial results to the general patient population.

Research perspectives

Sophisticated analyses of high quality EMR can complement costly, complex and lengthy randomized clinical trials, can assess their representativity for actual medical practice in the real world, and may even, in certain instances, be able to replace them.

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