TREATMENT OF TYPE 1 DIABETIC RATS WITH AN SGLT2 INHIBITOR: INTERACTIONS WITH EXERCISE AND HYPOGLYCEMIA

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ABSTRACT

Sodium glucose co-transporter 2 inhibitors (SGLT2i) have begun to show promise as an add-on to insulin therapy in patients with type 1 diabetes (T1D), however the effects of these drugs on plasma glucagon concentrations in T1D are not yet known. We investigated whether 8 days of SGLT2i treatment altered plasma glucagon concentrations in response to voluntary physical activity and insulin-induced hypoglycemia in male and female rats with streptozotocin-induced T1D. SGLT2 inhibition did not alter basal or post-exercise glucagon concentrations and did not affect exercise-associated changes in blood glucose levels. Additionally, SGLT2 inhibition did not affect voluntary running distance. However, in males, SGLT2 inhibition appeared to have suppressed the glucagon response to insulin-induced hypoglycemia (+40.4±48.6 pg/mL vs +22.7±30.8 pg/mL, $p=0.02$) and decreased the time to taken to reach hypoglycemia (63.8±17.2 min vs 30±13.4 min, $p<0.0001$). These results have potentially concerning implications for the use of SGLT2i in the treatment of T1D.

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1. INTRODUCTION

Sodium glucose cotransporter 2 (SGLT2) inhibitors are a new category of drug prescribed for the treatment of hyperglycemia in patients living with type 2 diabetes (T2D). These drugs are currently undergoing clinical trials examining efficacy and safety for the treatment of type 1 diabetes (T1D), but have not yet been approved by the United States Food and Drug Administration (FDA) or Health Canada for this patient population. Preliminary clinical trials have demonstrated the benefits of SGLT2 inhibitors to individuals with T1D as an adjunct to insulin therapy with respect to metabolic control (i.e. glucose lowering agent) as well as renal and cardiovascular protection through changes in kidney function and blood pressure [1]. The main metabolic benefits include reductions in hyperglycemia exposure, as measured by hemoglobin A1c (HbA1c), as well as reductions in body weight and total daily insulin intake [2]. Recently, studies have begun to investigate the effects of SGLT2 inhibitors on their potential for augmenting glucagon secretion from the alpha cells of the pancreas in healthy and diabetic animal models and humans [3–7]. Increases in glucagon secretion may make the hypoglycemic actions of the drug less effective but may also have protective effects for patients when low blood glucose (hypoglycemia) develops during exercise, or with insulin overtreatment. Emerging data also suggest that chronic SGLT2 administration in patients living with type 1 diabetes may increase the risk for euglycemic ketoacidosis, a potentially life threatening condition [8]. We are not aware of any studies, in humans with type 1 diabetes or in animal models of the disease, examining the effects of SGLT2 inhibition on blood glucose counterregulation to insulin-induced hypoglycemia or its effects during exercise. This project aimed to evaluate the interaction of empagliflozin, a commonly prescribed SGLT2 inhibitor for patients with T2D, with insulin-induced hypoglycemia and with exercise in a rat model of type 1 diabetes.

1. LITERATURE REVIEW

1.1. GLUCOSE HOMEOSTASIS

Glucose homeostasis is regulated by a complex network of various cells, tissues, hormones, neurotransmitters, and other biological factors. In healthy individuals, the concentration of glucose in the blood is maintained at a range of ~ 4-7mmol/L with slight fluctuations occurring outside of this range throughout the day. Concentrations above or below normal blood glucose levels, termed hyperglycemia and hypoglycemia respectively, can cause both short and long-term consequences to the body. Fluctuations in blood glucose concentrations, at their simplest, are a result of changes to the ratio of the glucose rate of appearance (Ra) to the glucose rate of disappearance (Rd). These fluctuations are mediated by hormonal signals and the uptake and production of glucose by various organs. It has been widely established that the endocrine cells of the pancreas, and their hormonal secretions, play a dominant role in glucose homeostasis, however there are many other organs and tissues that greatly contribute to this regulation including the liver, skeletal muscle, and kidneys.

2.1.1 Organ Contribution to Blood Glucose Regulation

2.1.1.1 The Endocrine Pancreas

The endocrine cells of the pancreas are grouped together, forming islands within the pancreatic tissue. These islands are referred to as the islets of Langerhans. Five types of cells exist within these islets: α-cells, β-cells, δ-cells, PP cells and ε-cells. Of these cells, the insulin-producing β-cells, and the glucagon-producing α-cells are central to the maintenance of blood glucose regulation. Insulin is an anabolic hormone that aids in carbohydrate metabolism by promoting the uptake, utilization, and storage of glucose molecules in insulin-

sensitive tissues including the liver, skeletal muscle, and adipose tissue. By doing so, it lowers the concentration of glucose present in the blood. When blood glucose levels begin to rise above the normal range, often occurring postprandially, insulin is secreted to lower blood glucose levels back into the target range. Glucagon, on the other hand, is a catabolic hormone that increases blood glucose levels through hepatic glycogenolysis, as well as hepatic and renal gluconeogenesis. Glucagon is often released during exercise, sleep, or other periods of fasting in order to satisfy the energy requirements of the body and prevent or treat hypoglycemia. With their opposing effects, insulin and glucagon work in tandem to tightly regulate blood glucose levels and balance the anabolic and catabolic requirements of the body.

The control of insulin and glucagon secretion is complex and has been shown to involve a variety of regulatory factors. Pancreatic islets act as functional units that modify insulin and glucagon secretions by sensing and responding to changes in blood glucose concentrations. Additionally, each pancreatic islet contains a heterogeneous population of cells at close proximity to one another, creating an ideal arrangement for paracrine interactions. There has been support for interactions between α-cells, β-cells, and the somatostatin-producing δ-cells in the normal maintenance of blood glucose levels [9], however there is still a lack of understanding into the exact relationships and mechanisms involved in these interactions.

The primary regulator of insulin secretion is glucose, and the mechanism by which pancreatic β-cells modify their secretion of insulin by sensing changes in glucose concentrations is well known. Glucose molecules freely diffuses into β-cells through glucose transporters (GLUTs) where, once metabolized, they trigger a signaling cascade that leads to the exocytosis of insulin-containing secretory vesicles [10]. Changes in glucose concentration

and insulin secretion largely parallel one another, with higher blood glucose concentrations leading to a greater amount of insulin secretion [11]. Alternatively, even minimal decreases in glucose concentrations can lower insulin secretion in healthy humans [12].

The response of α-cells to changes in glucose concentrations is more complex and there is debate over whether this is primarily a direct response to glucose or an indirect response as a result of paracrine interactions and various other factors [13–15]. Rodent α-cells express GLUT1 mRNA, which implicates a potential mechanism for direct glucose sensing [16]. However, more recently, the presence of sodium glucose co-transporters (SGLT) 1 and 2 have been discovered in human alpha cells and SGLT2 has been associated with the ability to modulate glucagon secretion from these cells [3]. On the other hand, the idea that glucagon secretion might be under paracrine regulation was originally proposed several decades ago, and has since been supported by studies examining the interactions of β-cell secretions, such as insulin, γ-aminobutyric acid (GABA), and Zinc (Zn^{2+}), with α-cell function [17–19].

Given the function of glucagon, it is intuitive to expect an inverse relationship between glucose concentrations and glucagon secretion. In line with this notion, hypoglycemia is known to induce an increase in glucagon concentrations in humans [20]. Additionally, low glucose concentrations have been shown to stimulate glucagon secretion in a perfused rat pancreas [21]. However, there has been some evidence to suggest that this interaction with glucose may be dependent on the presence of neighbouring islet cells and a decrease in insulin secretion, and that isolated α-cells may in fact interact with glucose in a similar fashion as β-cells [22,23]. Interestingly, studies have shown that glucagon secretion appears to be maximally inhibited around euglycemic glucose concentration (6-7mmol/L) in intact human islets, isolated mouse islets, and glucagon-releasing cells from hamsters [24,25]. In these same

studies, glucose was also shown to paradoxically stimulate glucagon secretion at higher concentrations (12-30mmol/L). Moreover, elevated circulating glucagon levels have long been associated with poor diabetes control (i.e. chronic hyperglycemia) [26]. It has been proposed that this observation could be due to a concentration-dependent effect of glucose on glucagon secretion, or a result of paracrine interactions with β-cell secretions [25,27], however the exact mechanisms behind the glucose-regulated secretion of glucagon is still being uncovered.

In addition to glucose signaling and paracrine interactions of α and β-cell, insulin and glucagon secretion also appear to be regulated by somatostatin, produced by the pancreatic δ-cells, which has been shown to exert inhibitory actions on both the α and β-cells [28]. Other regulatory factors include incretins hormones released from the intestines, amino acids and fatty acids, and signals from the hypothalamus via autonomic nerve innervation.

2.1.1.2 The Liver

A primary target of insulin and glucagon is the liver, which acts at the main site of glucose storage in the body and accounts for 75-80% of all endogenous glucose production [29]. Once insulin and glucagon are secreted, they travel directly to the liver via the portal vein, where these hormones can be found in their highest concentrations. The liver maintains a balance between the storage of glucose in the form of glycogen, termed glycogenesis, and the production of glucose from glycogen and non-carbohydrate sources, termed glycogenolysis and gluconeogenesis, respectively [30]. Approximately 50 years ago, it was first suggested that the relative concentrations of insulin and glucagon may be more significant to glucose homeostasis than their individual concentrations [31,32]. Later, the insulin:glucagon ratio (IGR)

was determined to be the main regulator of endogenous glucose production (EGP) from the liver [33]. After a meal, the IGR increases to promote the storage of glucose through glycogenesis and lipogenesis (the production of fatty acids). During periods of fasting, this ratio decreases in order to promote the production of glucose through glycogenolysis and gluconeogenesis. The importance of the IGR continues to be recognized in recent research as a key mechanism behind changes in blood glucose concentrations.

2.1.1.3 Skeletal Muscle

Glucose Uptake by Skeletal Muscle at Rest

Skeletal muscle is a primary location for glucose uptake. Depending on the requirements of the cell, once glucose has been transported across the membrane, it can either be oxidized and used for energy production or stored in the form of glycogen. Glucose uptake into skeletal muscle is dependent on GLUT4 transporters in the plasma membrane and transverse tubules that allow glucose to pass into the cell down its concentration gradient [34]. However, the majority of GLUT4 is stored intracellularly until a signal is given for membrane translocation [35].

At rest, skeletal muscle is dependent on insulin for glucose uptake. Insulin stimulation of insulin receptors (IR) on the cell surface triggers a signaling cascade, inducing the translocation of GLUT4 into the membrane. The ability of insulin to elicit this response determines the muscle's sensitivity or resistance to insulin.

Exercise and Glucose Homeostasis

It is well known that physical activity increases skeletal muscle uptake of glucose. Studies have revealed both insulin-dependent and insulin-independent mechanisms behind this increase in glucose uptake. Exercise has been shown to increase skeletal muscle blood flow in adults [36,37]. This increase in blood flow would expectedly result in improved glucose and insulin delivery to target tissue. Additionally, studies in rats and humans have demonstrated capillary recruitment in response to exercise, increasing the surface area available for glucose transport [38,39]. A study in dogs, however, suggested that changes in blood flow account for less than 30% of the exercise-induced increase in muscular glucose uptake [40].

The primary mechanism through which exercise increases skeletal muscle glucose uptake is currently considered to be via an insulin-independent, contraction-induced translocation of GLUT4. The ability for muscle contraction to induce an uptake of glucose without the presence of insulin has been well established [41,42]. Support for the requirement of GLUT4 in this process comes from a study where electrical stimulation of skeletal muscle in GLUT4 knock-out mice resulted in almost a complete loss of contraction-induced glucose uptake [34].

Exercise has also been shown to increase insulin sensitivity. As little as one session of physical activity is enough to elicit an improvement in insulin sensitivity, with this effect continuing to last 48 hours post exercise [43]. Furthermore, consecutive bouts of exercise have been shown to increase the amount of GLUT4 translocation in response to a standard dose of insulin, an effect that may be attributed to an increase in muscle GLUT4 content [44,45]. However, there are additional theories that may explain these adaptations, and the exact

mechanisms behind the exercise-induced increase in insulin sensitivity are still being investigated. The effects of insulin, muscle contraction, and changes in blood flow are additive, and work synergistically to increase glucose uptake into skeletal muscle during exercise [46].

In order to account for the increased energy requirements during exercise, changes in glucoregulatory hormone secretion must occur to ensure a balance between the glucose rate of appearance (Ra) and the glucose rate of disappearance (Rd) and prevent hypoglycemia. This response can vary depending on both the duration and intensity of exercise. As mentioned previously, the IGR is the main driver of changes in EGP and therefore plays an important role in glucose homeostasis during physical activity. During prolonged, moderate intensity exercise, the IGR decreases in order to stimulate EGP and counteract the increase in glucose clearance from the blood [47]. A decrease in blood glucose concentrations during exercise can cause the release of other glucoregulatory hormones as well, including catecholamines (epinephrine and norepinephrine), growth hormone, and cortisol [48]. During intense exercise, however, catecholamines have been shown to increase 14-18 fold, as opposed to the 2-4 fold increase that can be seen during moderate physical activity [49]. This response is similar to what is seen during periods of acute stress, and causes an increase in EGP and a reduction in glucose uptake. In healthy individuals, this response is followed by an increase in insulin secretion during recovery to prevent hyperglycemia [50].

2.1.1.4 The Kidneys

The kidneys are responsible for regulating the amount of glucose excreted from the body, and by extension, regulate the amount of glucose reabsorbed into systemic circulation.

This is accomplished by sodium glucose co-transporters 1 and 2 (SGLT1 and SGLT2) located in the proximal convoluted tubule of the kidney, which allows for the reabsorption of glucose from the filtrate back into the blood stream. In a healthy individual, these transporters prevent the loss of glucose in the urine by enabling the reabsorption of nearly 100% of all glucose entering the kidneys [29]. With increasing levels of glucose in the blood, renal glucose reabsorption tends to increase until it reaches its threshold at a blood glucose concentration of around 10-11mmol/L [51]. At blood glucose concentrations above this threshold, glucose begins to be excreted into the urine, causing glycosuria. Though less prominent, the kidneys also contribute to glucose homeostasis through renal gluconeogenesis [29].

2.1.2 Sex Differences in Glucose Metabolism

Humans have clear metabolic sex differences, however, these differences must be interpreted with caution as they can also be affected by factors such as age, physical fitness, nutritional status, and hormonal changes. Sex differences in glucose metabolism can be seen through distinctions in insulin sensitivity, and metabolic and hormonal changes during exercise and hypoglycemia.

2.1.2.1 Insulin Sensitivity

Research relating to sex differences in insulin sensitivity has been somewhat inconsistent, however there is a general consensus towards a greater whole body insulin sensitivity in females compared to males. Several large studies investigating sex differences in impaired fasting glucose and glucose tolerance have suggested that men may be more prone to develop insulin resistance than females [52–54]. Furthermore, some studies have utilized

a hyperinsulinemic-euglycemic clamps to estimate whole-body insulin sensitivity as well as skeletal muscle insulin sensitivity by analyzing the rate of glucose infusion per kilograms of whole body mass or lean body mass, respectively [55]. Insulin sensitivity in skeletal muscle is of interest because skeletal muscle is the primary location for insulin-induced glucose uptake [56]. Though some of these studies failed to show significant differences between sexes, several demonstrated a significantly higher estimation of whole body insulin sensitivity [57,58] and skeletal muscle insulin sensitivity [59,60] amongst females. For the most part, these results have been consistent with rodents studies. Aged male rats have been shown to be more prone to insulin resistance induced by a high fat diet [61]. Macotela and colleagues then demonstrated an increased insulin sensitivity in adipose tissue of female mice compared to male mice [62]. Furthermore, in 2011, Gorres et al. published a study which demonstrated that estrogen receptor stimulation leads to an increase in insulin stimulated skeletal muscle glucose uptake, implicating estrogens in the mechanisms behind the increased insulin sensitivity seen in female skeletal muscle. In summary, there is some evidence suggesting a greater whole body insulin sensitivity in females, possibly due to the actions of estrogens, however the lack of consistency in this area of research is likely due to the myriad of factors involved in insulin sensitivity.

2.1.2.2. Metabolic and Counterregulatory Responses to Exercise and Hypoglycemia

It is well known that women tend to have a higher body fat percentage than men [63]. Under basal conditions, women tend to store circulating free fatty acids (FFA) while men tend to oxidize them [64]. However, studies have shown that during moderate intensity endurance exercise, women have a lower respiratory exchange ratio (RER), a decreased glucose Ra and

Rd, and have greater lipolytic and ketogenic responses than men [65–67]. These results suggest that females rely more on lipid oxidation and less on carbohydrate stores in comparison to males during period of moderate endurance exercise.

Research examining sex differences in counterregulatory responses to insulin-induced hypoglycemia and exercise suggests that men exhibit a greater counterregulatory response than women. Depending on both the degree of hypoglycemia and the time spent in hypoglycemia, studies have found sex-linked disparities in counterregulatory responses in different hormones including glucagon, epinephrine, norepinephrine, growth hormone, and cortisol [68–72]. Additionally, one study has shown that men have greater counterregulatory responses to moderate exercise than women [73]. After 90 minutes of physical activity, epinephrine, norepinephrine, and pancreatic polypeptide concentrations were significantly increased in men compared to women, while there were no differences in the levels of plasma glucagon, cortisol, or growth hormone between sexes.

2.2 TYPE 1 DIABETES

2.2.1 Pathophysiology and Etiology of Type 1 Diabetes

Type 1 diabetes (T1D) is an autoimmune disease where the β-cells of the pancreas are targeted by the body's immune system and are consequently destroyed. This destruction leads to the inability of the body to produce insulin. Therefore, individuals with T1D must take exogenous insulin in order to maintain the blood glucose concentrations necessary for survival, which can be done either through injections or with an insulin pump.

The etiology behind the development of T1D is unclear. Our current understanding is that T1D is a multifactorial disease caused by a combination of genetic predisposition as well as

environmental factors [74]. Over 50 genetic loci have been associated with the development of T1D [75]. Of these, the strongest association is to the human leukocyte antigen (HLA) gene family, a group of genes encoding the proteins of a complex involved in the regulation of the human immune system [76]. Monozygotic twin studies have identified an over 50% concordance rate for the development of T1D, supporting the idea that T1D has a strong genetic component but is triggered by an unidentified environmental factor [77]. Possible environmental factors contributing to the development of this disease include viruses, the intestinal microbiome, and dietary factors such as cow's milk and vitamin D [78].

Despite insulin treatment, individuals with T1D are still faced with the lifelong challenge of blood glucose management. Given the narrow therapeutic range of insulin and the variety of physiological processes that contribute to glucose metabolism, it is nearly impossible for individuals with T1D to avoid dysglycemia and 75% of adults with T1D fail to reach the recommended goal for glycemic control [79]. Hyperglycemia and hypoglycemia each lend unique challenges to T1D management.

2.2.2 Hyperglycemia and Diabetic Ketoacidosis

Hyperglycemia is an excess amount of glucose in the bloodstream and is the defining characteristic present at the time of diabetes diagnosis. It is generally caused by a lack of insulin relative to the requirements of the body. Symptoms of hyperglycemia include nausea, thirst, frequent urination, and weight loss. Hyperglycemia can have long-term macrovascular and microvascular complications potentially leading to the development of conditions such as cardiovascular disease, retinopathy, nephropathy, and neuropathy. In T1D, hyperglycemia

may amplify itself through positive feedback involving glucose reabsorption in the kidneys and dysregulated glucagon secretion [80–84].

Hyperglycemia influences, and is influenced by glucose reabsorption in the kidneys. As mentioned previously, in healthy individuals, renal glucose reabsorption increases with rising blood glucose concentrations until it reaches its threshold at around 10-11mmol/L. In patients with both type 1 and type 2 diabetes, the maximal capacity of renal glucose reabsorption is increased by ~20% and the threshold for glucose reabsorption has been show to increase up to ~14mmol/L [51,85,86]. This is likely due to glucose transporters in the proximal convoluted tubule, which appear to be increases in patients with T2D [87]. The increase in renal glucose reabsorption that occurs with increasing blood glucose levels further contributes to hyperglycemia in patients with diabetes. At blood glucose concentrations that exceed the maximal threshold of glucose reabsorption, glucose is then excreted in the urine causing glycosuria.

Hyperglycemia can lead to diabetic ketoacidosis (DKA), a life-threatening condition that involves an abnormally high production of ketone bodies from the liver. The two main types of ketone bodies are acetoacetate and β-hydroxybutyrate. Ketone body production commonly occurs in healthy individuals during periods of lipid oxidation, however the abnormally high levels of ketone bodies seen in DKA cause bodily fluids to become dangerously acidic. This abnormally high level of ketone bodies is a result of severe insulin deficiency, which promotes ketone production by leading to increased levels of circulation FFA and promoting gluconeogenesis in the liver. Ketone production is also known to be influenced by high glucagon levels, which may be inappropriately raised during hyperglycemia [83,88,89]. Additionally, these high levels of ketone bodies and FFA are associated

with an increase in insulin resistance, which promotes hyperglycemia even further [90]. In individuals with T1D, DKA is often seen at the time of diagnosis, before an insulin regiment has been started, but can also occur due to illness, poor compliance with insulin treatment, and malfunctioning diabetes care equipment.

2.2.3 Hypoglycemia, Exercise, and Defects in Counterregulation

Hypoglycemia is defined as a deficiency of glucose in the blood and, according to the Diabetes Canada Clinical Practice Guidelines, occurs when blood glucose concentrations fall below 4mmol/L [91]. Hypoglycemia is a severe, life threatening condition that requires immediate medical treatment. Depending on the severity of hypoglycemia, symptoms can include trembling, nausea, dizziness, weakness, and seizures, and if left untreated, can lead to loss of consciousness and death. For individuals with T1D, hypoglycemia is an unavoidable side effect of exogenous insulin intake and tightly regulated blood glucose concentrations. On average, individuals with T1D experience two episodes on symptomatic hypoglycemia per week and one episode of severe hypoglycemia per year [92]. In T1D, hypoglycemia is often caused by either an excess amount of insulin or an increase in glucose uptake during physical activity. Healthy individuals have physiological mechanisms in place to prevent hypoglycemia, however these mechanisms become defective in individuals with T1D.

The first line of defense against hypoglycemia in a healthy individual is a decrease in insulin secretion as blood glucose levels drop into the range of 4.4-4.7mmol/L, followed by a response in counterregulatory hormone secretion as blood glucose levels continue to drop [12]. With exogenous insulin treatment, individuals with T1D are unable to immediately reduce circulating levels of insulin to prevent hypoglycemia. Furthermore, research has shown that

individuals with T1D lose the ability to adequately respond to hypoglycemia through counterregulatory hormones. As blood glucose concentrations continue to fall into the range of 3.6-3.9mmol/L, glucagon is normally secreted in order to treat hypoglycemia [20]. However, in T1D, glucagon response to hypoglycemia quickly becomes blunted, beginning as early as 1 month after diagnosis and deteriorating with time [93]. The mechanism behind this lack of glucagon response is still unclear, but may involve defective α-cell glucose sensing, the absence of paracrine signalling through β-cell secretions, reduced autonomic stimulation, or elevated pancreatic somatostatin levels [94]. Without the ability to decrease circulating insulin and sufficiently increase glucagon secretion, individuals with T1D rely on a sympathoadrenal response and catecholamine secretion. Unfortunately, this response can also become defective, as patients with T1D on intensive insulin therapy were shown to have a reduced and desensitized epinephrine response to hypoglycemia [95].

These defects in counterregulation make physical activity challenging for individuals with T1D, and fear of hypoglycemia can prompt some individuals to refrain from exercise. In T1D, exercise can have varying effects on blood glucose levels depending on the type and duration as well as the circulating levels of insulin in the bloodstream at the time of activity. Moderate-intensity aerobic exercise generally promotes a drop in blood glucose levels and can often lead to hypoglycemia. As mentioned previously, exercise can also affect insulin sensitivity for up to 48 hours post-exercise. A study in children with T1D found that moderate-intensity physical activity caused blood glucose levels to decrease during, immediately after, and 7-11 hours after exercise [96]. This latent decrease in blood glucose levels can lead to nocturnal hypoglycemia, a dangerous scenario where hypoglycemia occurs during sleep. It is important to note that physical activity can also cause a rise in blood

glucose levels if accompanied by an adrenergic response, which often occurs as a result of anaerobic activity.

Strategies for hypoglycemia prevention during exercise mainly include adjusting pre-exercise insulin intake, to account for the delayed effects of exogenous insulin, and carbohydrate supplementation. However, since individuals with T1D still experience frequent episodes of hypoglycemia, current strategies aimed at preventing hypoglycemia are clearly insufficient. This population could benefit greatly from an additional therapy aimed at preventing hypoglycemia.

2.2.4 Rodent Models of Type 1 Diabetes

Rodent models of T1D have been an indispensable asset to the progression of T1D research. There are several types of rodent models of T1D; chemically induced models, spontaneous autoimmune models, genetically induced models and virus-induced models of T1D. The most popular of these models are the streptozotocin (STZ)-induced diabetic rodents, Bio-Breeding Diabetes-Resistant (BBDR) rats, and nonobese diabetic (NOD) mice.

2.2.4.1 Streptozotocin-induced Diabetes

STZ is a glucose-resembling compound synthesized by the bacteria *Streptomycetes archromogenes.* Because of its pancreatic β-cell toxicity, STZ is frequently used to produce a chemically induced model of T1D in rodents. STZ elicits its toxic effects by entering pancreatic β-cells through the Glut-2 transporter, causing DNA damage and the inhibition of insulin production. It can be administered to rodents via intraperitoneal (i.p.) or intravenous

(i.v.) injection usually as either a single high dose (100-200mg/kg in mice, 35-65mg/kg in rats) or in multiple low doses (20-40mg/kg per day).

STZ-induced diabetes provides a simple, quick, and relatively inexpensive means of inducing pancreatic β-cell loss in rodents. It is general thought to be a useful model for research involving drug trials that act independently of β-cells. Although STZ-induced diabetes can be a useful model of T1D, it does have some disadvantages. STZ may have toxic effects to other organs besides the pancreas, including the liver, kidneys, brain, lungs, intestines, and testis. Additionally, some studies have suggested that β-cell regeneration may be possible in STZ-induced diabetic rodents given insulin treatment, with better glycemic control being associated with improved recovery [97,98]. These factors must be considered when using a STZ-induced model of diabetes.

2.3 SGLT2 INHIBITION

2.3.1 History of SGLT2 Inhibitors

SGLT2 inhibitors, also known as gliflozins, are class of drug used to treat patients with type 2 diabetes. Their primary function is to induce the excretion of glucose in the urine by preventing glucose reabsorption in the kidneys. The use of this class of drug dates as far back as the year 1835, when a French chemist discovered phlorizin, a naturally occurring SGLT1 and SGLT2 inhibitor, in the bark of an apple tree [99]. Approximately 50 years after its discovery, phlorizin's ability to produce glycosuria was realized and its connection to the kidneys was elucidated [100]. After this realization, phlorizin was administered to humans in order to test kidney function, and has even been used in attempts to inhibit tumor growth [101,102]. It was later discovered that phlorizin was effective at normalizing blood sugar levels in

a diabetic rat model, however, the poor oral bioavailability and gastrointestinal side effects of the compound prevented it from being approved for human use [103]. In more recent years, these drugs have been modified to improve bioavailability and specificity, so that they can be prescribed to patients with T2D. There are now many varieties of SGLT2 inhibitors being tested and prescribed all over the world, with 4 variations approved by the US FDA and Health Canada for the treatment of T2D (dapagliflozin, canagliflozin, empagliflozin, and ertugliflozin). These drugs have not yet been approved for the treatment of T1D, however clinical trials are currently underway.

2.3.2 Mechanism of SGLT2 Inhibitor Action

SGLT2 is a transport protein found in the proximal convoluted tubule of the nephron of the kidney that allows for the symport of one glucose molecule along with one sodium molecule. SGLT2 functions in the reabsorption of glucose from the filtrate back into systemic circulation, and, in healthy individuals, prevents the loss of glucose in the urine. Nearly 100% of the glucose that is found in the filtrate is reabsorbed into circulation. SGLT2 accounts for approximately 97% of glucose reabsorption in the kidneys, with sodium glucose co-transporter 1 (SGLT1) accounting for the reabsorption of the other 2-3% of glucose that enters the filtrate [104,105]. SGLT2 inhibition blocks this reabsorption of glucose, causing glycosuria, the excretion of glucose in the urine. Consequently, SGLT2 inhibitors act as an effective, insulin-independent, treatment for hyperglycemia. It should be noted that the complete inhibition of SGLT2 causes the excretion of only 50-60% of filtered glucose, which is likely due to the compensatory role of SGLT1 [104,106,107].

2.3.3 The Effects of SGLT2 Inhibition in Type 1 and Type 2 Diabetes

2.3.3.1 Type 2 Diabetes

The treatment of T2D with SGLT2 inhibitors has demonstrated significant improvements in blood glucose control, specifically in the percentage of glycated hemoglobin (HbA1c), an approximate average of blood glucose concentrations over the last 3 months. A meta-analysis conducted by Monami et al found a maximum reduction in HbA1c of 0.6% after 24 weeks of SGLT2 inhibition in patients with T2D, with this improvement lasting up to 1 year [108]. Furthermore, SGLT2 inhibition has been shown to be effective at improving blood glucose levels along with other types of diabetes medication, including metformin, sulfonylureas, and insulin [109,110].

In addition to improvements in blood glucose levels, SGLT2 inhibition has also been shown to reduce body weight in people with T2D, a disease that is often associated with increased body weight and obesity. After 52 weeks of dapagliflozin treatment, patients with T2D had a persistent placebo-corrected reduction in body weight of over 2kg, with four times more patients having a ≥5% reduction in body weight than the placebo group [111]. SGLT2 inhibitor treatment has also been associated with reductions in blood pressure, through both weight-loss dependent and independent mechanisms. Patients treated with either 100mg or 300mg of canagliflozin both showed significant reductions in systolic blood pressure in comparison to the placebo group, where 58% of these reductions were weight-loss independent while the other 42% appeared to be associated with weight-loss [112].

Improvements in cardiovascular and renal outcomes have also been attributed to SGLT2 inhibitor use. In 2015, a large, multicenter study, called the EMPA-REG OUTCOME trial, found a 38% relative risk reduction of cardiovascular related deaths in patients on

empagliflozin in comparison to the placebo group [113]. The CANVAS program, a trial examining the cardiovascular and renal effects of canagliflozin, also showed fewer cardiovascular related deaths in the SGLT2 inhibitor treated group compared to the placebo group [114]. This study did not find any significant differences in renal outcomes, but did see possible benefits of canagliflozin with respect to albuminuria, estimated glomerular filtration rate, the need for renal-replacement therapy, and death from renal causes.

Because SGLT2 inhibitors are a blood glucose lowering drug, one of the initial concerns with its use was the possibility for the development of hypoglycemia. While some studies have shown a potential increased risk of hypoglycemia with SGLT2 inhibition [109,115], these results are likely associated with the use of sulfonylurea or insulin in these patients. The majority of studies have shown similar rates of hypoglycemia with and without SGLT2 inhibitor treatment [110,111,113,116,117]. One study even demonstrated a reduced occurrence of nocturnal hypoglycemia with SGLT2 inhibitor treatment with the use of a continuous glucose monitor [118]. The most common side effect associated with SGLT2 inhibition is genital infections. Clinical trials have shown an approximate fourfold increase in the incidence of genital infection with SGLT2 inhibition [108]. Interestingly, SGLT2 inhibition has also been associated with the occurrence of euglycemic DKA, where DKA develops without the presence of hyperglycemia [119]. The mechanism behind this may be similar to the mechanisms behind the development of DKA, with an "artificially" lowered blood glucose caused by the large increase in renal glucose clearance induced by SGLT2 inhibition.

2.3.3.2 Type 1 Diabetes

Clinical trials assessing the treatment of type 1 diabetes with SGLT2 inhibitors in addition to insulin therapy have shown similar benefits to those seen with T2D. To date, there have been three large trials examining the efficacy of SGLT2 inhibitors in T1D. In 2015, Henry et al. demonstrated that 18 weeks of treatment with canagliflozin decreased HbA1c, body weight, and total daily insulin dosage in individuals with T1D [120]. In the DEPICT-1 and 2 studies, dapagliflozin treated patients also had a significant decrease in HbA1c, body weight and total daily insulin dose compared to the placebo treated individuals after 24 weeks [121,122]. The inTandem3 trial showed that 24 weeks of treatment with sotagliflozin, an SGLT1 and SGLT2 inhibitor, caused significantly greater reductions in HbA1c, body weight, insulin dose, as well as systolic blood pressure in patients with T1D compared to placebo treated patients [123]. In earlier studies, treatment with SGLT2 inhibitors has also been shown to reduce cardiovascular risk factors and improve kidney function by decreasing hyperfiltration (an abnormally high flow rate of filtered fluid through the kidney), blood pressure, body weight, and improving plasma lipid profiles in individuals with T1D [124,125]. In all of these studies, no differences were found in the incidence of hypoglycemia between placebo treated groups and SGLT2 inhibitor treated groups, in spite of the fact that glucose levels tend to drop as more glucose is passed into the urine. This may indicate some improvement in glucose counterregulation to hypoglycemia with SGLT-2 treatment. Potential side effects of SGLT2 inhibitor use in individuals with T1D include the risk of urinary tract or genital infections, and increases in the incidence of DKA [2]. This increased risk of DKA may be linked to an increase in glucagon secretion and/or reduced renal clearance of ketones [126].

2.3.3.3 SGLT2 Inhibition and Glucagon Secretion

SGLT2 inhibition has recently been associated with changes in glucagon secretion, however the mechanisms behind this association are still being uncovered. In 2014, Merovci et al., and Ferrannini et al. were the first to show that patients with T2D had an increase in plasma glucagon concentrations with SGLT2 inhibitor treatment [4,5]. Later, researchers discovered the presence of SGLT2 on α-cells obtained from non-diabetic humans [3]. In this same study, the researchers demonstrated how treatment with the SGLT2 inhibitor dapagliflozin increased glucagon secretion in both healthy human islets exposed to glucose concentrations at or below 6 mmol/L, as well as in healthy mice. This increase in glucagon secretion was hypothesized to be caused by the inhibition of SGLT2 on the α-cells of the pancreas. Pedersen et al supported this idea with a study that created a mathematical model of glucagon secretion, which predicted SGLT2 to be involved in α-cells glucose sensing [6]. Additional studies performed in healthy and diabetic mice also showed an increase in glucagon secretion with SGLT2 inhibition [127,128]. On the other hand, in 2017, Wang et al. demonstrated how dapagliflozin did not alter plasma glucagon concentrations, but suppressed glucagon signaling and EGP from the liver in rodent models of type 2 diabetes [7]. They also found a reduction in glucose stimulated glucagon secretion in SGLT2 inhibitor treated pancreata extracted from a rat model of type 1 diabetes. Evidently, additional research is needed to uncover the true effects of SGLT2 inhibitors on glucagon secretion and action in the presence, and absence, of intact islets.

Because individuals with T2D are already afflicted with abnormally high levels of glucagon, the increase in glucagon secretions observed in SGLT2 inhibitor treated patients has been viewed as a potential concerning side effect of this drug [129]. However, for

individuals on exogenous insulin treatment, like patients with T1D, increasing glucagon secretion could also have important implications for hypoglycemia prevention. If this effect on glucagon secretion holds true for people with T1D as well, it could potentially explain the lack of an increased risk of hypoglycemia seen with this glucose-lowering drug, and be an additional benefit of SGLT2 inhibition to individuals with T1D.

3. RATIONALE AND OBJECTIVES

3.1 RATIONALE

Insulin treatment alone is not sufficient to prevent dysglycemia in patients with T1D. Tightly regulated blood glucose concentrations are crucial for maintaining health and preventing long-term complications in these patients, but this tight blood glucose control may put individuals at risk of hypoglycemia because of the narrow therapeutic range of insulin or if exercise is involved [130]. In a healthy individual, an increase in glucagon secretion normally occurs as blood glucose concentration begin to fall in order to prevent hypoglycemia [20]. However, over time, individuals with T1D lose the ability to appropriately respond to hypoglycemia with an increased glucagon response [93]. Additional strategies are clearly needed in order to prevent hypoglycemia in individuals with T1D. An ideal therapy would act to restore the normal glucagon response to hypoglycemia.

SGLT2 inhibitors have proven to be a beneficial adjuvant to insulin therapy. Currently these drugs are viewed as strictly a treatment for hyperglycemia, with the potential to improve glycemic control and kidney function while reducing insulin intake and cardiovascular risk factors in patients with T1D [2,124,125]. Clinical trials in individuals with T1D have shown no increase in the rate of hypoglycemia with SGLT2 inhibitor use despite the fact that these drugs lower blood glucose concentrations by increasing glucose output in the urine. Recently, there has been several studies indicating that SGLT2 inhibition increases glucagon secretion, specifically in patients and rodent models with T2D as well as in isolated pancreatic islets [3–5]. The mechanism behind this has been suggested to be a direct action of SGLT2 inhibitors on the α-cells of the pancreatic islets [3,6]. The *in vivo* effects of SGLT2 inhibition on glucagon secretion

in T1D remains unknown. If these drugs increase glucagon secretion in patients with T1D, they may have the potential to restore some level of glucagon response to hypoglycemia that frequently occurs with accidental insulin over administration or with exercise in these individuals [131].

This study used a streptozotocin-induced rat model of T1D to test the in vivo effects of SGLT2 inhibition on the glucagon response to insulin-induced hypoglycemia and exercise. With this approach, we aimed to get a proper understanding of the effects of SGLT2 inhibition on glucagon secretion in T1D by measuring glucagon concentration in both the systemic circulation and the portal vein, the latter of which is not possible without invasive surgery. Additionally, we chose to conduct this experiment in both male and female animals in order to account for potential sex differences that may be present regarding response to SGLT2 inhibition and physical activity. This initial rodent study is key in understanding the potential of SGLT2 inhibitors to augment glucagon secretion before it can be translated into similar investigations performed in humans with T1D.

3.2 OBJECTIVES

The primary objective of this study was to determine the effects of SGLT2 inhibition on glucagon concentrations and blood glucose concentrations during insulin-induced hypoglycemia, as well as in exercise in a rat model of type 1 diabetes. Secondary measurements included investigating the effects of SGLT2 inhibitors on voluntary running wheel activity, and exploring any sex differences that may be present in these measurements.

3.3 HYPOTHESES

We hypothesized that the SGLT2 inhibition will increase the secretion of glucagon in a streptozotocin-induced rat model of T1D. Furthermore, we anticipate that this rise in glucagon secretion will reduce the fall in blood glucose concentrations seen during both insulin overtreatment and exercise (**Figure 1**). Because SGLT2 inhibition improves blood glucose control, we also expect to see an increase in voluntary running wheel activity in the drug treated rats compared to the placebo treated animals due to an alleviation of negative symptoms. We do not expect to see any sex differences in regard to glucagon secretion.

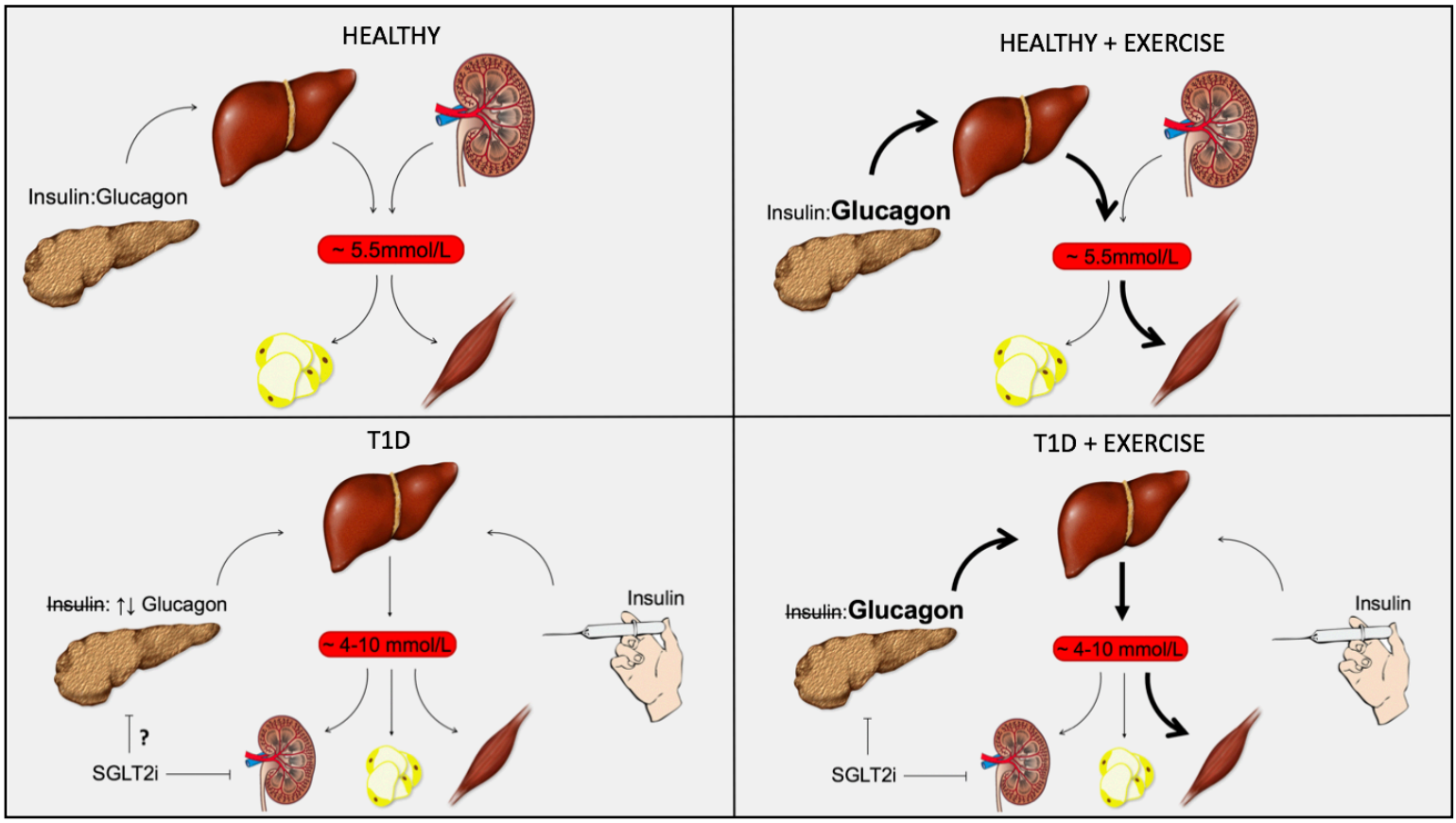

Figure 1: Glucose homeostasis in individuals with and without type 1 diabetes (T1D) at rest and during exercise. A) Glucose homeostasis in a healthy individual at rest. In healthy individuals, blood glucose concentrations are maintained at ~5.5mmol/L by maintaining a balance between the rate of glucose appearance (Ra) and disposal (Rd) in the bloodstream. The liver and kidneys have the ability contribute to the glucose Ra through endogenous glucose production (EGP). The ratio of insulin to glucagon secretion from the pancreas acts as the primary regulator of EGP from the liver. The kidneys also contribute to EGP through gluconeogenesis, and also contributes to blood glucose concentrations by preventing the loss of glucose in the urine. Glucose is removed from the blood stream and taken up into various tissues including skeletal muscle and adipose tissue. **B) Glucose homeostasis in a healthy individual during exercise.** During physical activity, skeletal muscle uptake of glucose increases. To compensate for these increased glucose requirements and maintain euglycemia, EGP often rises during exercise. Exercise causes an increase in glucagon (a decrease in the insulin to glucagon ratio) to promote EGP and maintain blood glucose levels. **C) Glucose homeostasis in an individual with T1D treated with both insulin and an SGLT2 inhibitor, at rest.** In type 1 diabetes, blood glucose concentrations become much more variable. The pancreas no longer produces insulin, and insulin must be taken exogenously through multiple daily injections, or with an insulin pump. Additionally, glucagon secretion is dysregulated in T1D. Glucagon levels appear to be inappropriately elevated postprandially, and glucagon response to hypoglycemia is suppressed. With SGLT2 inhibitors as an add-on therapy to T1D, the kidneys become a main source of glucose disposal by eliminating glucose from the blood stream and into the urine. **D) Our hypothesized model of glucose homeostasis in an individual with T1D treated with both insulin and an SGLT2 inhibitor, during exercise.** During physical activity, skeletal muscle glucose uptake is increased in T1D as well. Since glucagon secretion is dysregulated in T1D, this increased glucose uptake during exercise can often lead to hypoglycemia. We hypothesize that in T1D, SGLT2 inhibiters will act on the pancreas to increase glucagon secretion and restore some level of glucagon response to hypoglycemia.

4. MANUSCRIPT

SGLT2 INHIBITION DOES NOT ALTER BASAL OR POST-EXERCISE PLASMA GLUCAGON CONCENTRATIONS IN MALE AND FEMALE RATS WITH TYPE 1 DIABETES BUT MAY SUPPRESS GLUCAGON RESPONSE TO HYPOGLYCEMIA

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Contribution by the Authors

The protocol for this project was designed through a collaborative effort from myself, Dr. Erin Mandel, and Dr. Michael Riddell. All authors contributed to the daily animal care and testing as well as the hypoglycemic challenges. Glucagon assays were performed by myself and Mahsa Jahangiriesmaili. I analyzed all data concerning blood glucose concentrations, glucagon concentrations, and running distances. Dr. Riddell is the principle investigator and supervisor of this project.

Introduction

Type 1 diabetes (T1D) is a life-threatening disease that involves the constant self-management of blood glucose concentrations, a task which can often be both challenging and demanding. With insulin treatment alone, patients with T1D continue to experience frequent dysglycemia and face long-term health complications, including an increased risk of cardiovascular and renal disease [132]. Hypoglycemia is common in patients with T1D, who have been estimated to experience an average of two symptomatic episodes of hypoglycemia per week [92]. Since hypoglycemia is often induced by either an excess amount of insulin or physical activity, fear of hypoglycemia can be a major preventative of tight glycemic control, and has been shown to be the main barrier to exercise in people with T1D [133]. The frequency of hypoglycemia in these patients can be attributed to a combination of the inherent side effects of exogenous insulin treatment, and the lack of an appropriate counterregulatory response. Specifically, the counterregulatory response of glucagon to hypoglycemia has been shown to be blunted in individuals with T1D, deteriorating further with time [93]. Reducing the intensity of insulin treatment is currently not a feasible means of decreasing hypoglycemia occurrence since it would result in hyperglycemia. Patients with T1D would benefit from an additional therapy that reduces insulin intake and improves long-term health outcomes, making sodium glucose co-transporter 2 (SGLT2) inhibitors an attractive potential add-on to insulin treatment in this population [129].

SGLT2 inhibitors, also known as gliflozins, are a class of drugs that have already been established as an effective treatment for hyperglycemia in patients with type 2 diabetes (T2D) [134]. These drugs act on transporters in the kidneys to inhibit the reuptake of glucose from the filtrate, thereby inducing glycosuria, the excretion of glucose in the urine [135]. Clinical trials

are currently being run to examine the safety and efficacy of SGLT2 inhibition in patients with T1D, and many studies have already begun to show promise in these individuals [120–125]. A few larger trials have shown reductions in HbA1c, body weight, and insulin intake for SGLT2 inhibitor treated individuals with T1D [120–123]. Other smaller studies have also shown improvements in both cardiovascular and renal risk factors and plasma lipid profiles for these patients [124,125]. Because of their glucose excreting effects, one of the initial concerns with SGLT2 inhibitor treatment was the potential for the development of hypoglycemia. However, contrary to what was expected, given the blood glucose lowering effectiveness of these drugs, SGLT2 inhibitor treatment did not increase the frequency of hypoglycemia in patients with T1D in all of the previously mentioned studies.

Over the past few years of research, SGLT2 inhibitors have been associated with an increase in glucagon secretion, specifically in patients with T2D [4,5]. Furthermore, in 2015, Bonner et al. discovered the presence of SGLT2 on the α-cells in human islets, suggesting a possible mechanism by which these drugs could augment glucagon secretion [3]. The in vivo effects of SGLT2 inhibition on glucagon secretion in T1D remain unknown. If these drugs act to increase glucagon secretion in patients with T1D, it is possible that this rise in glucagon secretion could prevent hypoglycemia by restoring some level of glucagon counterregulation. To test this idea, we administered 8 days of treatment with empagliflozin, an SGLT2 inhibitor, to rats with streptozotocin-induced T1D and examined the effects on plasma glucose and glucagon concentrations during both insulin-induced hypoglycemia and voluntary exercise. Additionally, we used both male and female rats to test for any sex-related differences in the effects of SGLT2 inhibition. We hypothesized that there would be an

increase in plasma glucagon concentrations following SGLT2 inhibition during both insulin-induced hypoglycemia and exercise for males and females alike.

Methods

Ethics Statement

This study was carried out in accordance with the recommendations of the Canadian Council for Animal Care guidelines and was approved by the York University Animal Care Committee (Protocol 2017-16).

Animals

Thirty-three male and thirty-one female Wistar rats (bred in the York University vivarium, six weeks old) were housed in individual running wheel cages (Harvard Apparatus) with access to standard chow (Purina chow #5012) and water *ad libitum*. All animals were exposed to a 12h:12h light-dark cycle, 50-60% humidity, and temperatures between 22-23°C from birth. Prior to the start of the study, all rats were habituated to the running wheels with a full 7 days of continuous, voluntary running wheel access.

Experimental Design

A complete timeline of the experimental protocol is shown in **Figure 2.**

Diabetes Induction and Maintenance

Diabetes was induced in all animals using an intraperitoneal injection of 65mg/kg streptozotocin (STZ) (CAS #18883-88-4, Sigma-Aldrich, St Louis, MO). To promote

diabetes induction, rats were fasted the night before injection and were given a 10% sucrose solution in their drinking water for 2 days following the injection. All running wheels were locked during this time to further promote diabetes. Tail capillary blood glucose levels were monitored daily to confirm diabetes using a sterile needle poke (30 gauge) and hand held glucose meter (CONTOUR® NEXT Meter). One week after diabetes induction, each rat received individualized insulin treatment to manage their blood glucose levels at a target range of 15-25mmol/L. A fraction (1/8-1) of a sustained release insulin pellet (Linplant pellet, ~2U/24 hour/implant, LinShin Canada Inc.) was implanted subcutaneously under the dorsal skin while the animal was under isoflurane anesthetic. Linplant insulin pellets are designed to release a standard dosage of insulin in order to maintain blood glucose levels at a tolerable range and limit ketosis and glucosuria. Because potential β-cell regeneration can occur with STZ use in rodents [97,98], animals were excluded from this study if they reached a consistent basal morning blood glucose level of 11mmol/L or below at any point during this protocol.

Animal Grouping and Running Wheel Re-habituation

Rats were randomized into either an active group or an inactive group and then further separated into drug (10mg/kg empagliflozin [Jardiance™] dissolved in H_2O) and placebo groups (H_2O) (**Figure 3)**. Rats in the inactive groups were prohibited from running by locking their wheels for the entirety of the study. The rats in the active groups were given voluntary running wheel access at various, predetermined times throughout the protocol. After diabetes induction, rats in the active groups were given 7 days of controlled but voluntary running wheel access daily from 5-9PM in order to habituate the animals to the running wheel exposure that they would be receiving in the following portion of the experiment.

SGLT2 Inhibitor Treatment and Voluntary Running Wheel Activity

Animals were given 8 days of treatment (drug or placebo) via oral gavage, twice a day at 9AM and 5PM. Tail blood glucose measurements were taken daily at 9AM, 10AM, 5PM, 6PM and 9PM. These time points were chosen because of the t_{max} (1 hour) and half-life (1-6 hours) of empagliflozin in rats [136].

Throughout this treatment period, animals in the active groups were allowed 4 hours/day of voluntary running wheel access from 5PM to 9PM. This time restriction in running wheel activity was given to allow us to better monitor the effects of voluntary running wheel activity on blood glucose levels under the time window with the most prominent effects of SGLT2 inhibition. This limited running wheel exposure also allowed us to determine if SGLT2 inhibition influences voluntary wheel running behavior. Wheel revolutions were tracked with magnetic revolution counters and running distance was calculated using the running wheel circumference (106cm). During these 4 hours, food availability was limited to 10g in order to reduce variability in food intake and to promote voluntary wheel running [137]. For the purpose of measuring plasma glucagon concentrations, blood samples were taken via saphenous vein bleed (using a sterile needle puncture of the exposed vein after hair was trimmed) on the first and last day of treatment at 9PM (post-exercise), and on the fifth day of treatment at 9AM and 10AM (pre and post treatment).

Insulin-Induced Hypoglycemic 'Challenge'

An insulin-induced hypoglycemic 'challenge' was administered on the day following the last day of SGLT2i treatment. Food was removed from all animal cages thirty minutes prior to the start of the hypoglycemic challenge, and animals remained fasted for the entirety

of this challenge. Thirty minutes after drug or placebo administration, all animals were injected with a subcutaneous bolus of insulin aspart (10U/kg, NovoRapid®, Novo Nordisk) in order to induce hypoglycemia (≤3.5mmol/L). Blood glucose concentrations were monitored throughout the challenge every 5-10 minutes via tail bleed. In order to investigate any changes in glucagon concentrations, blood samples were collected from the saphenous vein at set points prior to and during the challenge (**Figure 4)**. When blood glucose reached ≤3.5 mmol/L, rats were immediately anesthetized with inhaled isoflurane and additional blood samples were taken from the saphenous vein and from the portal vein. The animals were then killed via exsanguination.

Plasma Analysis

All blood samples were collected in potassium-EDTA coated microvette capillary tubes (Sarstedt, Des Grandes Prairies, Montreal, Québec, Canada, Cat #16.444.100) and immediately centrifuged (12,000 rpm for 5 minutes). The plasma was then extracted and stored in polyethylene tubes at -80°C for further analysis. Plasma glucagon concentrations were batch measured using a glucagon ELISA kit (Cat# 10-1271-01, Mercodia, Uppsala, Sweden).

Statistical Analysis

Data are reported as means ± standard deviations (SD). The ROUT method (GraphPad Prism 8.0) was used for all outlier identification. Data were analyzed using a two-way ANOVA, three-way ANOVA, three-way ANOVA with repeated measures, or a mixed effect model

(GraphPad Prism 8.0). Where appropriate, simple effects were examined and Tukey's post hoc tests were conducted. The criteria for significance was set at $p<0.05$.

Results

A total of fourteen animals were excluded from this study – eleven animals were excluded because of presumed β-cell regeneration (blood glucose levels reached a consistent morning measurement of 11mmol/L or below) and three animals died before completing the protocol. Causes of death are unknown. Data was analyzed from a total of twenty-seven male and twenty-three female animals.

SGLT2 inhibition improved overall blood glucose control

In order to confirm the effects of SGLT2 inhibition on overall blood glucose control, blood glucose measurements were taken daily at 9AM, 10AM, 5PM, 6PM, and 9PM, and averaged at each time point to obtain the mean daily blood glucose trend for each animal. Overall blood glucose data are displayed for males (**Figure 5a**) and females (**Figure 5b**), separately. In inactive, placebo treated animals, blood glucose concentrations remained hyperglycemic, with small but significant increases observed throughout the day. For placebo-treated males, blood glucose concentration was higher at 6PM (27.7±4.1 mmol/L) and 9PM (26.7±4.1 mmol/L) in comparison to their 9AM glucose level (24.8±3.6 mmol/L) ($p=0.0009$, $p=0.03$, respectively). For placebo-treated, inactive females, blood glucose concentration was higher at 10AM (26.1±3.6 mmol/L), 6PM (26.5±3.1 mmol/L) and 9PM (27.9±2.8 mmol/L) in comparison to their 9AM glucose level (24.1±2.7 mmol/L) ($p=0.01$, $p=0.046$, $p=0.008$, respectively).

SGLT2 inhibition caused a significant decrease in overall blood glucose concentrations for both males (26.3±4.0 mmol/L vs 13.4±4.9 mmol/L, $p<0.0001$) and females (23.5±4.3 mmol/L vs 14.7±5.6 mmol/L, $p<0.0001$), with no obvious sex-related differences observed. No episodes of hypoglycemia were observed in any animal during these daily blood glucose checks. The effects of SGLT2 inhibition varied with time, depending on the time of day the treatment was given, as well as the amount of time that had passed since administration ($p<0.0001$). In comparison to the placebo treated group, SGLT2 inhibitor treatment caused a significant drop in blood glucose concentrations from 9AM to 10AM (+1.4±1.9 mmol/L vs -9.2±1.9 mmol/L, $p<0.0001$) (**Figure 6**). These effects continued to last until 5PM (8 hours post-gavage) in males specifically, where blood glucose levels remained significantly lower than 9AM measurements (19.6±3.6 mmol/L vs 14.0±3.9 mmol/L, $p<0.0001$) (**Figure 5a**). After a second treatment at 5PM, SGLT2 inhibition again caused a significant decrease in blood glucose concentrations, however by 9PM (4 hours post-gavage), blood glucose levels were no longer different than 5PM measurements in either males or females (**Figures 5a and 5b)**.

SGLT2 inhibition did not alter exercise-associated changes in blood glucose concentrations

To investigate the effects of SGLT2 inhibition on blood glucose concentrations during exercise, blood glucose measurements were taken at 5PM, 6PM, and 9PM, while animals had voluntary access to running wheel activity. When examining the change in blood glucose during the four hours of habitual exercise (5PM-9PM), we found no effect of SGLT2 inhibitor treatment in comparison to vehicle treatment (**Figure 7**). However, we did find a significant

interaction of activity and sex (p=0.008). More specifically, four hours of voluntary wheel running caused a significant decrease in the blood glucose concentrations of female rats (-2.9±2.6 mmol/L) in comparison to control (i.e. inactive) female rats (+1.7±1.8 mmol/L) (p=0.0003). This glucose lowering effect of exercise was not seen in male rats. Furthermore, in female animals alone, there was also a significant interaction of activity and time ($p<0.05$). Exercise lowered blood glucose concentrations specifically at 6PM (18.8±3.1 mmol/L vs 14.8±2.9 mmol/L, p=0.01) and 9PM (22.9±2.8 mmol/L vs 15.9±3.9 mmol/L, p=0.001), during the time period where the animals had access to running wheels (**Figure 5b**). There was no correlation between average running distance and change in blood glucose levels from 5PM-9PM (r^2=0.02) (**Figure 9)**.

Voluntary running wheel activity was unaltered by SGLT2 inhibition

The mean running distances are displayed in **Figure 8**, with individual data points indicating the average daily running distance of each animal from 5-9PM. Outliers were excluded from all data analysis. According to our analysis, SGLT2 inhibition did not have a significant effect on voluntary running wheel activity. However, voluntary running wheel activity was affected by sex. On average, female animals ran significantly further than male animals over the four hours of voluntary wheel access (539.2±199.4 m vs 308.4±215.6 m, for female and male rats respectively, p=0.02).

Basal and post-exercise plasma glucagon concentrations were unaffected by SGLT2 inhibition

Plasma glucagon concentrations were measured at 9AM and 10AM on the 5th day of treatment in order to examine baseline effect of SGLT2 inhibition on glucagon secretion (**Figure 10**). Basal 9AM glucagon concentrations were unaffected by five days of SGLT2 inhibition. However, there was a trend towards a significant interaction of sex and activity ($p=0.06$). Although this interaction did not quite meet our pre-selected threshold for significance, upon further investigation, we found that in physically active animals, basal 9AM glucagon concentrations were significantly higher in males (65.0±32.2 pg/mL) than in females (27.1±9.8 pg/mL) ($p=0.0009$). Furthermore, physical activity tended to increase basal glucagon levels significantly in males (36.7±16.6 pg/mL vs 65.0±32.2 pg/mL, $p=0.06$) but not in females. SGLT2 inhibition also did not appear to affect the change in plasma glucagon concentrations from 9AM to 10AM in either sex (**Figure 11**). However, there was a significant main effect of previous physical activity on the change in glucagon levels during this time, which, on average, appeared to reduce the increase in glucagon that was seen in the inactive groups (+4.1±32.6 pg/mL vs +32.6±41.5 pg/mL, $p=0.04$).

Plasma glucagon concentrations were also measured post-exercise (9PM) on the first and last days of treatment, in order to examine the effects of SGLT2 inhibition on any exercise-associated changes in glucagon secretion, and to investigate any changes in these effects with prolonged SGLT2 inhibitor treatment. On the first day of treatment, there was no effect of SGLT2 inhibition on glucagon levels (**Figure 12**). We did, however, see a trend towards a significant interaction between activity and sex ($p=0.05$). Upon further investigation of this interaction, in female animals, exercise led to significantly lower plasma

glucagon concentrations in comparison to the glucagon concentrations of inactive animals at this time (117.9±86.0 pg/mL vs 37.3±47.9 pg/mL, p=0.01). There were no significant differences between 9PM glucagon levels on the first and last day of treatment (**Figure 13**).

SGLT-2 inhibition did not prevent insulin-induced hypoglycemia

Blood glucose concentrations were measured throughout the insulin-induced hypoglycemic challenge in both males (**Supplementary Figure 1)** and females **(Supplementary Figure 2)**. Any animal with an initial blood glucose concentration of 10mmol/L or below at the start of this challenge was excluded from all analyses. We first investigated the effects of SGLT2 inhibition at -30 minutes and at 0 minutes (with time 0 representing insulin administration). In both male **(Figure 14a)** and female **(Figure 14b)** animals, there was a main effect of SGLT2 inhibition, which produced significantly lower overall blood glucose levels at the beginning of the challenge (Males: 23.5±5.3mmol/L vs 13.1±6.3 mmol/L, p=0.0001) (Females: 23.1±5.9 mmol/L vs 16.1±6.1 mmol/L, p=0.03). In male animals, a drop in blood glucose levels was seen from -30 minutes to 0 minutes in SGLT2 inhibitor-treated animals (17.8±5.3 mmol/L vs 8.5±2.7 mmol/L, p<0.0001) but not in placebo-treated animals. Alternatively, in female animals, a drop in blood glucose levels from -30 minutes to 0 minutes was seen overall (22.6±6.2 mmol/L vs 17.1±6.5 mmol/L, p<0.0001).

Time to hypoglycemia was calculated as the amount of time taken to reach a blood glucose concentration of ≤3.5 mmol/L after insulin injection (t=0 min) (**Figure 15**). After analysis using a three-way ANOVA, we found a significant interaction between treatment and sex (p=0.02). In males, SGLT2 inhibition significantly decreased time to hypoglycemia in comparison to the placebo group (63.8±17.2 min vs 30±13.4 min, p<0.0001). SGLT2

inhibition did not have a significant effect on time to hypoglycemia in female rats. However, for placebo-treated animals, time to hypoglycemia was significantly shorter in females compared to males (63.8±17.2 min vs 41.2±14.5 min, p=0.002). Activity did not appear to affect blood glucose levels during the insulin-induce hypoglycemic challenge or the time taken to reach hypoglycemia.

SGLT2 inhibition may lower plasma glucagon response to insulin-induced hypoglycemia

To investigate the effects of SGLT2 inhibition on glucagon secretion during hypoglycemia, plasma glucagon concentrations were measured from the saphenous vein at set time points throughout the insulin-induced hypoglycemic challenge. Glucagon concentrations followed a similar trend in all groups for both males (**Figure 16a**) and females (**Figure 16b**). Baseline glucagon concentrations were defined as the concentrations at 0 minutes, immediately before insulin injection **(Table 1)**. There were no significant differences in basal glucagon concentrations between groups. Glucagon concentrations at baseline were subtracted from all glucagon values during this challenge in order to analyze changes in these concentrations **(Figure 16)**. Plasma glucagon concentrations significantly increased from baseline at euglycemia (4-6mmol/L) and hypoglycemia (≤3.5mmol/L) for both males (Euglycemia: +63.8±57 pg/mL, p=0.0005; Hypoglycemia: +57.3±36.9 pg/mL, p<0.0001) and females (Euglycemia: +48.3±35.8 pg/mL, p<0.0001; Hypoglycemia: +30.7±47.7 pg/mL, p=0.04). Additionally, male animals showed significantly higher glucagon levels at -30 minutes than at baseline (+14.8±12.9 pg/mL, p<0.0001).

A main effect of SGLT2 inhibition was seen in male animals but not in female animals. In males, SGLT inhibitor-treated groups has significantly smaller increases in

glucagon levels than placebo-treated groups (+40.4±48.6 pg/mL vs +22.7±30.8 pg/mL, p=0.02). There was no effect of SGLT2 inhibition on portal vein glucagon concentrations during hypoglycemia (**Figure 17**). However, a previous bout of physical activity appeared to increase portal vein glucagon concentrations during hypoglycemia in comparison to the inactive groups, with a trend towards statistical significance (102.1±80.2 pg/mL vs 152.4±150 pg/mL, p=0.05).

Discussion

We used a rat model of T1D to investigate the ability of SGLT2 inhibition to oppose the hypoglycemic effects of both physical activity and excess exogenous insulin, two scenarios that frequently cause hypoglycemia in individuals with T1D. Because SGLT2 inhibition has been shown to increase the secretion of glucagon in both humans and mice with T2D, we hypothesized that SGLT2 inhibition would increase glucagon secretion in T1D as well. However, here we showed that these previously demonstrated effects of SGLT2 inhibition on glucagon secretion may not extend to T1D and that SGLT2 inhibition alone did not help prevent or delay hypoglycemia. On the contrary, we found that, in male rats specifically, SGLT2 inhibition reduced the time to hypoglycemia, perhaps because baseline glucose levels were effectively lowered by the drug, and blunted the glucagon response to insulin-induced hypoglycemia. These findings do not support the notion that SGLT2 inhibition helps protect against hypoglycemia in diabetes by increasing glucagon counterregulation.

According to previous research, SGLT2 inhibition has been shown to increase fasting plasma glucagon concentrations in healthy mice one hour after a single dose of an SGLT2

inhibitor, and after 4 days of treatment [3]. Additionally, both a single dose and a 4 week dose of SGLT2 inhibition increases post-meal plasma glucagon concentrations in humans with T2D for up to 5 hours [4]. Contrary to what has been presented in the literature, we report here that SGLT2 inhibition does not appear to impact basal glucagon levels in rats with T1D. We demonstrated this lack of impact both acutely and chronically; 30 minutes post drug administration (during the insulin-induced hypoglycemic challenge), 1 hour post drug administration (at 10AM on the fifth day of treatment), 4 hours post drug administration (at 9PM on the first and last days of treatment), and basal measurements after 4 full days of treatment (9AM on the fifth day of treatment). Our findings here appear to challenge the idea that SGLT2 inhibitors act directly on α-cells to augment glucagon secretion [3,6]. Previous studies that have shown an SGLT2 inhibitor-induced increase in glucagon secretion were performed in circumstances where β-cells were present [3–5,127,128]. In comparison, we used a high dose of STZ to induce severe β-cell loss in our animals to model what occurs in T1D. Some studies have suggested that β-cell secretions may play a central role in the regulation of glucagon secretion [138]. Therefore, a possible explanation for the lack of effect of SGLT2 inhibition on glucagon secretion presented in our results may be related to some level of β-cell interaction that is not present in T1D. However, in a study examining the effects of dapagliflozin in rodent models of T2D, Wang et al proposed that the lack of a significant change in plasma glucagon concentrations that they observed may be due to a combination of a decrease in glucagon secretion and a reduced sequestering of glucagon by the liver [7]. These researchers also found decreases in glucagon production from dapagliflozin-treated perfused pancreata that originated from STZ-treated type 1 diabetic rats, and glucagon-producing cells

treated with siRNA that targets SGLT2. According to this one study, intact islets may not be necessary for augmentations in glucagon secretion in response to SGLT2 inhibition.

Another possible explanation for the observed lack of effect of SGLT2 inhibition on plasma glucagon concentrations may be related to our specific model of T1D. One of the limitations of our study was the chronic state of hyperglycemia experienced by all animals. We maintained hyperglycemic blood glucose levels in our animals because previous research has shown that improved glycemic control in STZ-induced diabetes can induce β-cell regeneration [97,98]. In a study published in 2007, Abdul-Ghani and Defronzo proposed a glucotoxic effect of chronic hyperglycemia on the glucose-mediated suppression of glucagon secretion from α-cells [139]. If this is indeed the case, it may explain the lack of effect of SGLT2 inhibition on plasma glucagon concentrations that we observed in our study, since the effects of SGLT2 inhibition on α-cells would be dependent on the response of these cells to glucose.

We demonstrated that SGLT2 inhibition did not affect plasma glucagon concentrations post-exercise. Furthermore, SGLT2 inhibition did not significantly alter exercise-associated changes in blood glucose concentrations. According to these results, it appears as though the effects of exercise and the effects of SGLT2 inhibition were independent of one another. Because the blood glucose levels of our animals were maintained at hyperglycemia, voluntary exercise did not cause a large enough decrease in these levels to induce hypoglycemia and we could not analyze the effects of SGLT2 inhibition on exercise-induced hypoglycemia directly, as we have done with other new therapeutic options for hypoglycemia protection in diabetes [140]. However, we did measure the effects of SGLT2 inhibition on insulin-induced hypoglycemia and again, we found no obvious improvement on

glucagon counterregulation with the therapeutic agent. During the insulin-induced hypoglycemic challenge, we showed that SGLT2 inhibition alone, without appropriate adjustments to exogenous insulin intake, did not prevent insulin-induced hypoglycemia. Furthermore, male animals treated with an SGLT2 inhibiter reached hypoglycemia faster, and had a decrease in overall glucagon levels during the challenge. Unfortunately, some of our sample sizes were too small to confidently determine if this effect extends to females as well. Given the independent blood glucose lowering mechanisms of SGLT2 inhibitors and insulin, it would be logical to expect a compounded effect on glucose clearance from the blood stream. However, the decrease in plasma glucagon concentrations that we observed may represent a direct action of SGLT2 inhibition on α-cells during hypoglycemia. These results are in line with a study performed by Pedersen et al, which demonstrated a trend towards a decreased glucagon secretion in human pancreatic islets exposed to a glucose concentration of 1mmol/L [6]. Additionally, in 2017, Wang et al demonstrated that SGLT2 inhibition suppressed glucagon secretion in perfused pancreata from rats with STZ-induced T1D, at a glucose concentration of 2.5mmol/L [7]. Although the research is limited, our results appear to support the idea that SGLT2 inhibition decreases glucagon response to hypoglycemia, contrary to our initial hypothesis. This could represent a concerning side effect of SGLT2 inhibitors to patients with T1D as well as T2D patients treated with insulin or sulfonylureas, who are already at risk of hypoglycemia. Further research is needed to determine the effects of SGLT2 inhibition on glucagon counterregulation to hypoglycemia in humans with T1D.

In addition to our primary outcome, we demonstrated some other interesting and unanticipated findings. A common theme throughout this study was an interaction between sex and activity. Consistent with previous research which has demonstrated increased

voluntary locomotor activity in female Wistar rats compared to males [141], we showed larger voluntary running distances in females. Most notably, we also found that voluntary running wheel activity decreased blood glucose levels in female rats at 6PM and 9PM, whereas this effect of physical activity was not seen in male rats. However, since we showed no correlation between running distance and changes in blood glucose concentrations during exercise for either sex, we cannot attribute the sex differences we observed in exercise-associated blood glucose changes to differences in running wheel activity. We reason that there must be an alternative sex-specific mechanism behind these differences in blood glucose response to voluntary physical activity.

At their simplest, changes in blood glucose concentrations are a result of changes to the ratio of the rate of glucose appearance (Ra) to the rate of glucose disappearance (Rd). Because we did not observe any significant effects of exercise on blood glucose concentrations in males, the ratio of Ra to Rd must not have been affected by exercise in these animals. Following this idea, either exercise did not significantly increase Rd in these animals, or there was a sufficient amount of counterregulation to balance out an increased glucose uptake. In our protocol, glucagon was not affected by a bout of exercise in the male animals. However, past research has shown significant differences in the counterregulatory abilities of males and females to moderate physical activity, in counterregulatory hormones other than glucagon. This counterregulatory response has generally been shown to be higher in males than in females, in hormones such as epinephrine, norepinephrine, growth hormone, and cortisol, both in healthy individuals [68–70] and individuals with T1D [142,143]. These counterregulatory hormones were not measured in our study, but may play a role in the sex differences that we observed.

Contrary to what we observed in the males rats, a bout of voluntary physical activity caused a significant decrease in the blood glucose levels of female rats, indicating that this exercise must have been associated with an increased Rd, a decreased Ra, or both. An increased Rd in female rats could be related to estrogen signalling, which has been implicated in skeletal muscle adaptations to exercise [144]. Activation of estrogen receptors in rats has also been shown to lead to an increased skeletal muscle insulin-induced glucose uptake [145]. On the other hand, we showed that physical activity lowered post-exercise glucagon concentrations in female rats, which would be expected to lead to a decreased Ra. The sex differences that we observed in post-exercise glucagon levels are intriguing. Previous research in non-diabetic individuals has shown an increased glucagon counterregulatory response to insulin-induced hypoglycemia in males compared to females [71,72]. Research on sex differences related to glucagon secretion in T1D, however, is lacking. Because glucagon response to hypoglycemia is known to be blunted in T1D, this may overshadow any potential sex differences in glucagon secretion that still exist in these patients. [143]. One study examined sex differences in glucagon secretion during euglycemic exercise in patients with T1D, but did not find any significant differences [142]. As far as we know, this is the first study to examine sex differences in exercise-associated changes to blood glucose and glucagon concentrations in T1D. This gap in knowledge has recently been acknowledges in the literature, where it has been suggested that females may have a greater defense in blood glucose control after exercise than men [146,147]. Possible explanations for this suggestion come from studies showing that healthy females appear to have an increased sensitivity to the lipolytic actions of counterregulatory hormones [148], and are able to regain control over post-exercise glycaemia more quickly than men [149]. However, our study suggests that females with T1D may instead have a weaker

defense to exercise-induced decreases in blood glucose concentrations. These results may have important implications relating to sex differences in exercise-associated changes in blood glucose concentrations that do not lead to hypoglycemia for individuals with T1D.

We also feel that it is also important to acknowledge that in females, an exercise-associated drop in blood glucose concentrations was associated with a change in glucagon levels, whereas an SGLT2 inhibitor associated drop in blood glucose levels was not. This observation suggests that, in our protocol, blood glucose concentrations themselves were not the primary regulator of glucagon secretion. We propose that the reduction in post-exercise glucagon concentrations that we observed may instead reflect an increased carbohydrate uptake and a decrease in the fasted state of the animals.

In conclusion, we demonstrated that SGLT2 inhibition does not appear to alter basal glucagon concentrations in T1D, but may reduce the glucagon response to hypoglycemia. However, because of the chronic state of hypoglycemia in our animals, and our limited sample sizes, these results should be interpreted cautiously. Since rates of hypoglycemia are already high in individuals with T1D, our results may represent a concerning side effect of SGLT2 inhibition. Further research into the effects of SGLT2 inhibitor treatment on the glucagon response to hypoglycemia in individuals with T1D is warranted. Furthermore, we also demonstrated novel sex differences in exercise-associated changes in blood glucose and glucagon concentrations in rodents with T1D. Additional research is needed to determine the mechanisms behind these observed differences and to confirm whether or not these results translate to human with T1D.

5. TABLES AND FIGURES

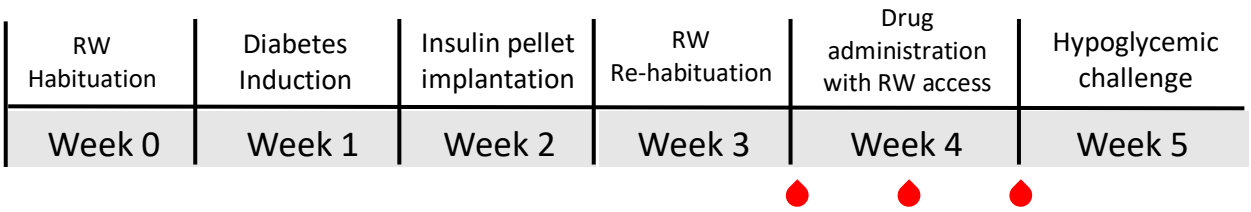


Figure 2: Experimental design. Running wheel is abbreviated as RW. After running wheel habituation, the experimental protocol consisted of 4 main weeks followed by an insulin-induced hypoglycemic challenge at the beginning of week 5. Red droplets indicate saphenous vein blood sample collection on the first and last day of drug treatment (9PM) and midway through the treatment (9AM and 10AM).

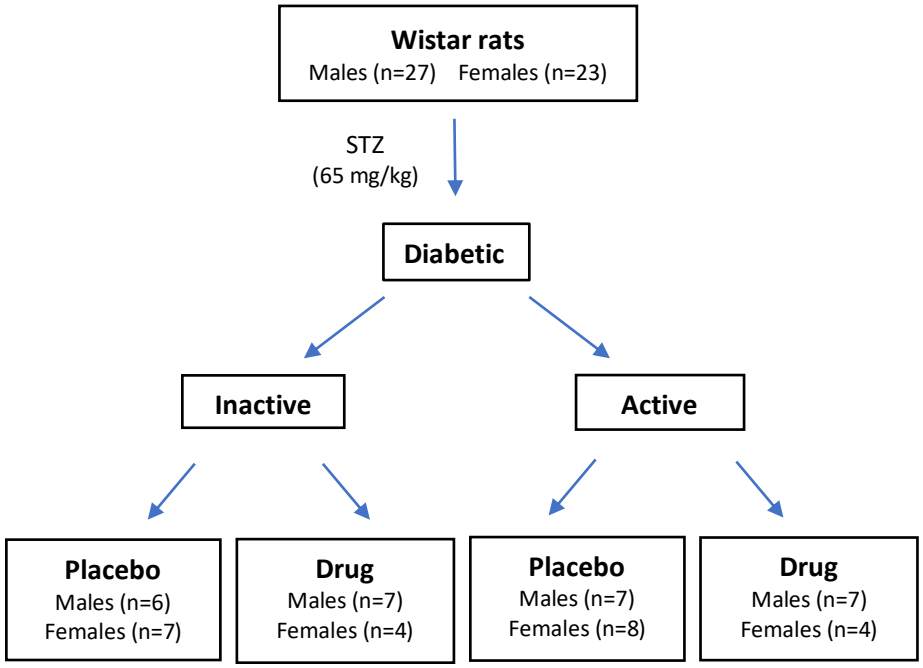


Figure 3: Animal grouping. All Wistar rats were made diabetic using an intraperitoneal injection of streptozotocin (STZ) at a dose of 65mg/kg. Animals were then randomly divided into four groups: Inactive Placebo [Male (n=6), Female (n=7)], Inactive with Drug [Male (n=7), Female (n=4)], Active Placebo [Male (n=7), Female (n=8)], and Active with Drug [Male (n=7), Female (n=4)].

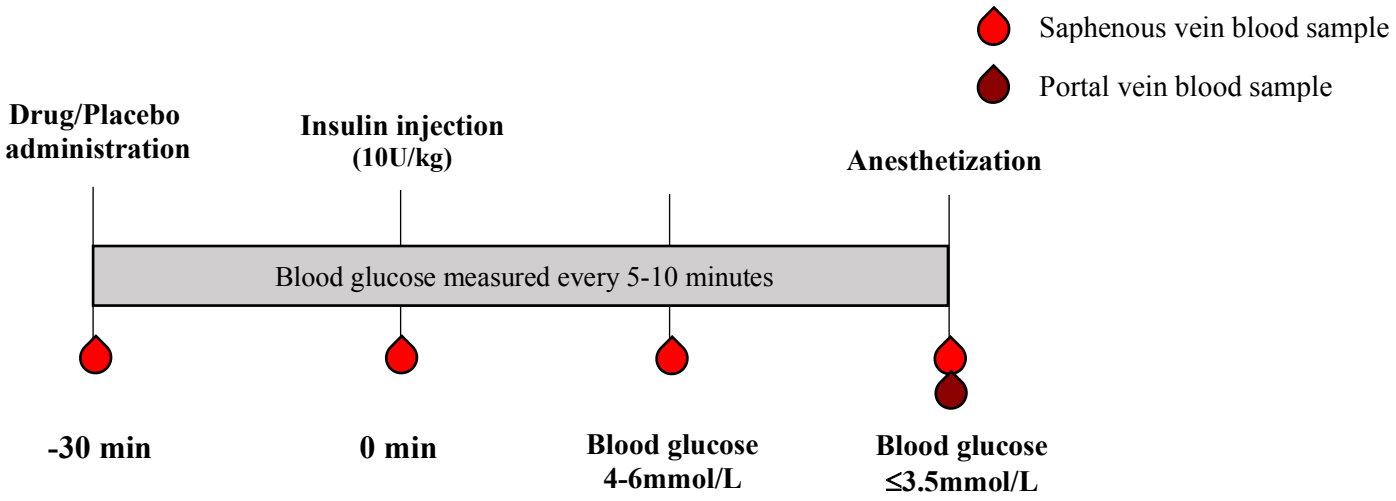


Figure 4: Insulin-induced hypoglycemic challenge protocol. A subcutaneous injection of insulin aspart (10U/kg) was administered 30 minutes after regular morning drug administration. Bright and dark red droplets indicate blood samples taken for glucagon analysis from the saphenous vein and portal vein, respectively. Blood samples were taken immediately before drug/placebo administration (-30 minutes), before insulin injection (0 minutes), when blood glucose levels reached euglycemia (4-6mmol/L), and when blood glucose levels reached hypoglycemia upon anesthetization (≤3.5mmol/L). Blood glucose concentrations were measured every 5-10 minutes via tail vein bleed.

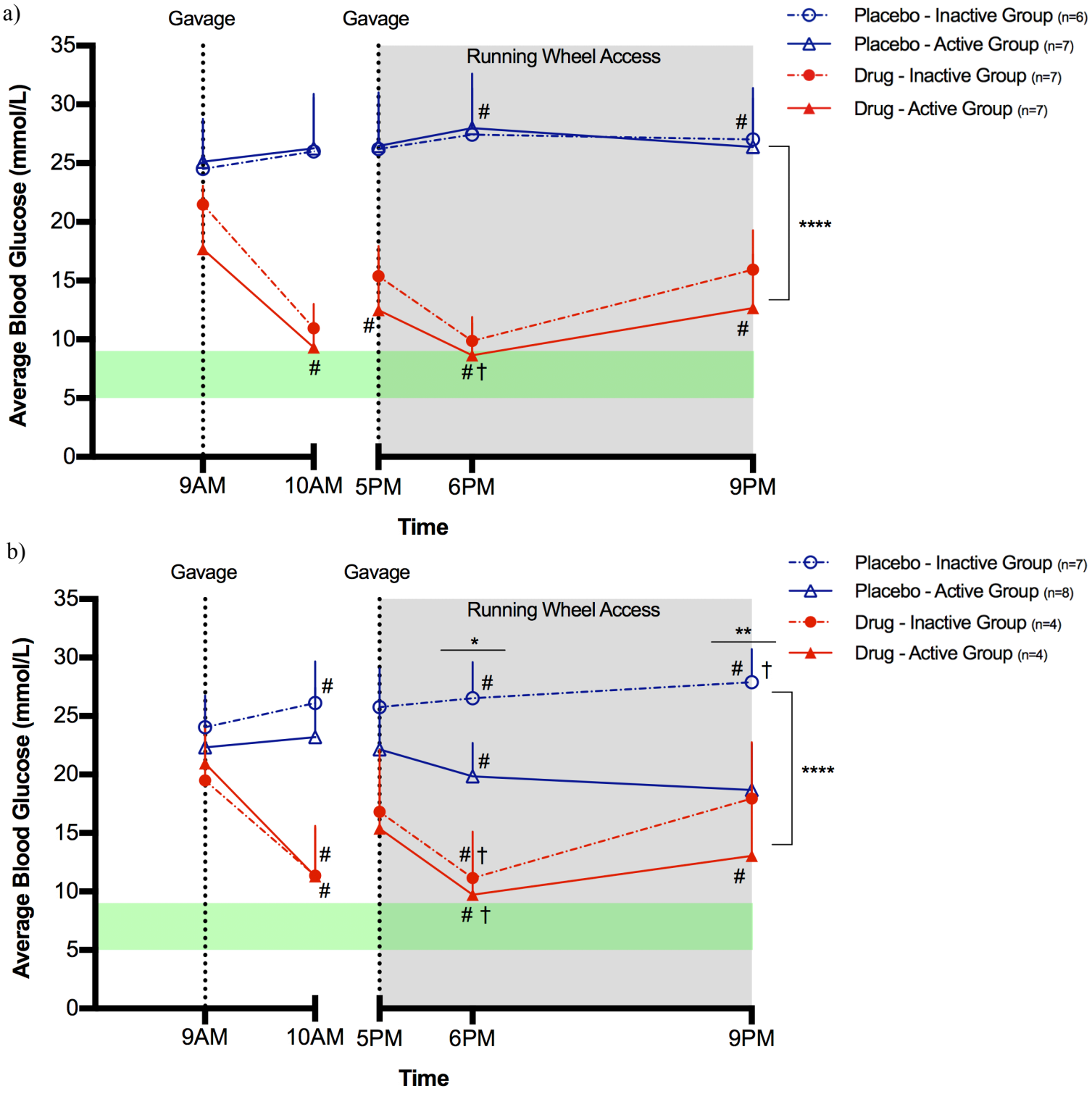


Figure 5: Average daily blood glucose levels were improved with SGLT2 inhibition in males (a) and females (b). Treatment was given by oral gavage twice a day at 9AM and 5PM (vertical dotted lines). Animals in active groups had daily access to voluntary wheel running from 5PM-9PM (grey shaded area). Optimal blood glucose levels were defined as 5-8mmol/L (shaded green area). All data are expressed as mean ± SD. **** $p<0.0001$ for main effect of treatment, # $p<0.05$ compared to 9AM, † $p<0.05$ compared to 5PM, * $p<0.05$ for simple effect of activity at 6PM, ** $p<0.01$ for simple effect of activity at 9PM.

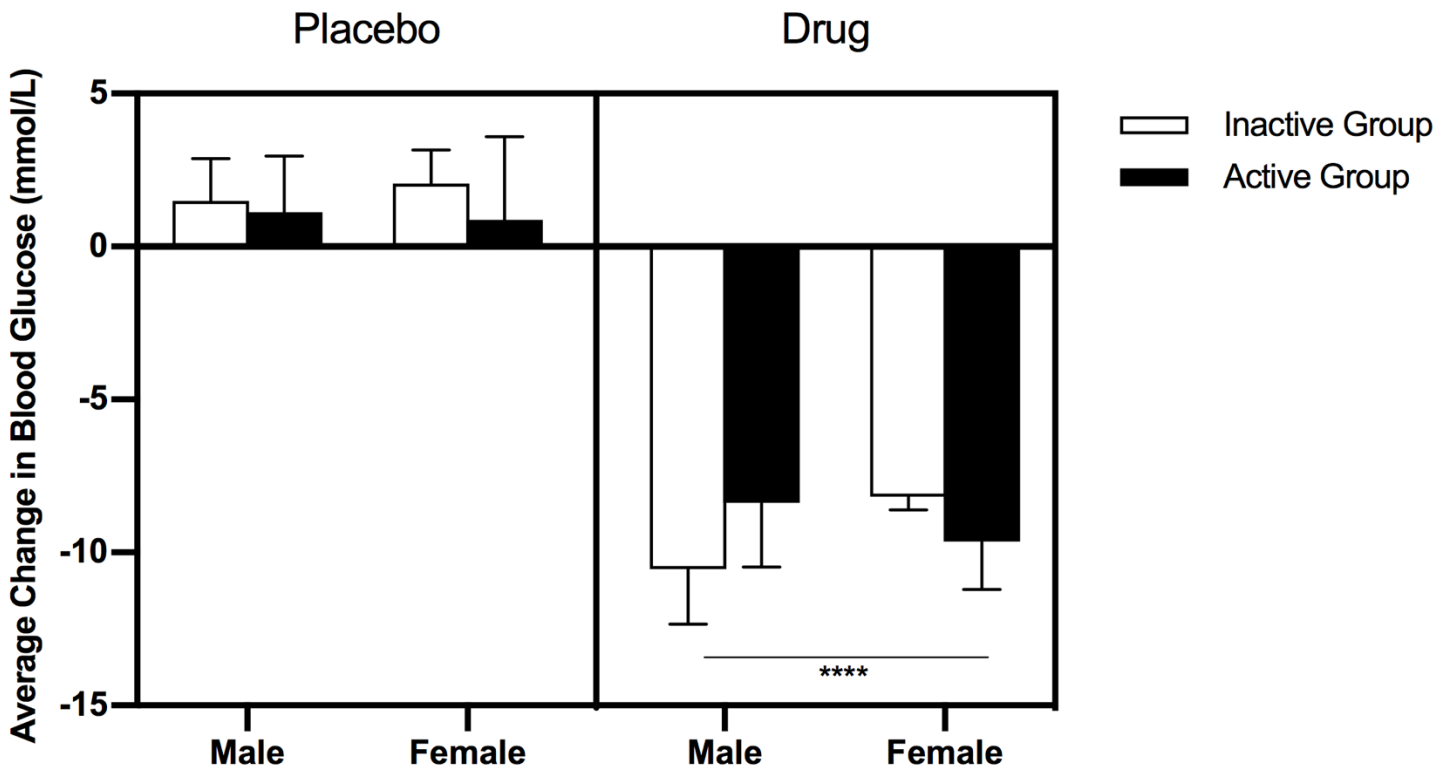


Figure 6: SGLT2 inhibition lowered blood glucose concentrations from 9AM to 10AM. Blood glucose concentrations were measured immediately before treatment was administered (9AM) and 1 hour post-treatment (10AM). Data are expressed as mean ± SD. **** $p<0.0001$ compared to placebo.

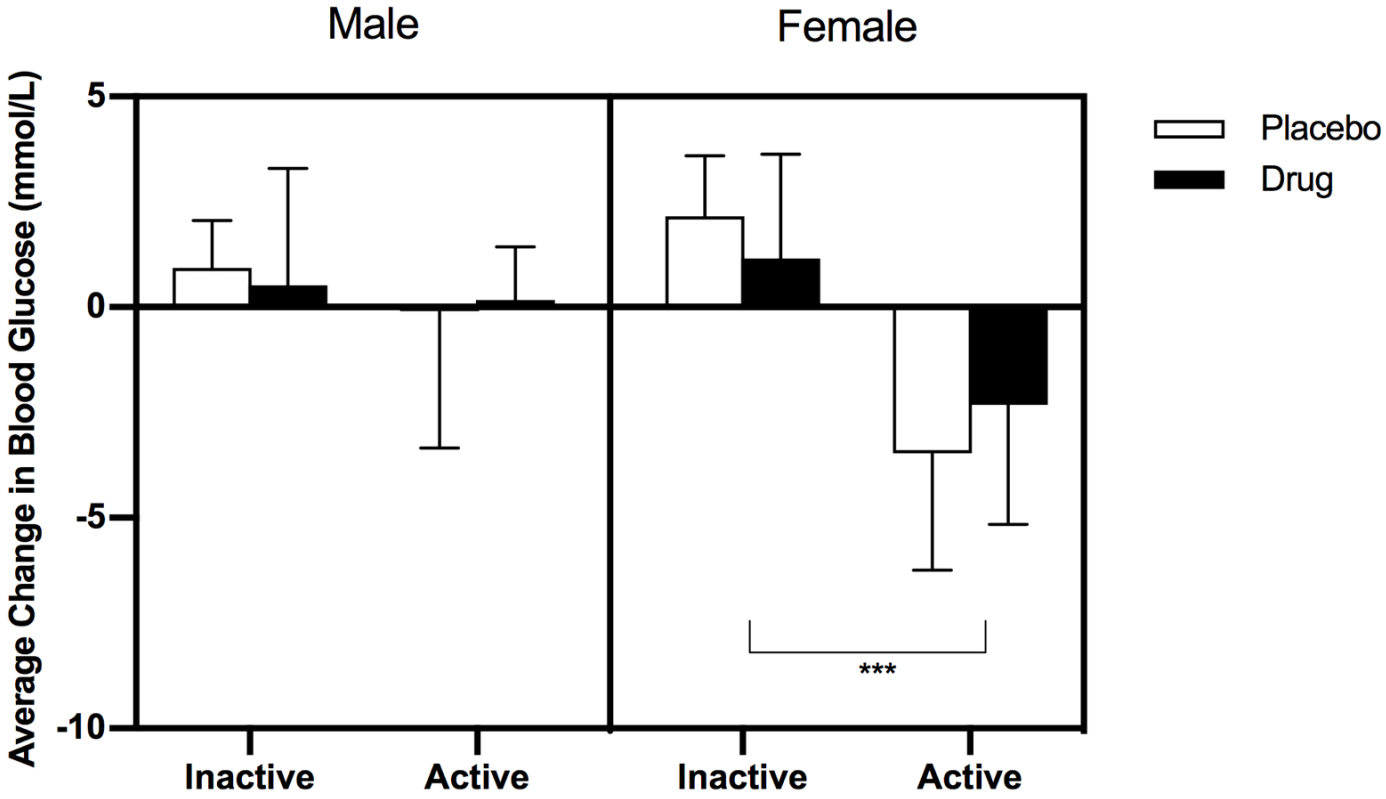


Figure 7: Physical activity lowered blood glucose concentrations from 5PM-9PM in females but not males. Animals in active groups were given 4 hours of voluntary wheel running from 5PM to 9PM, daily. Treatment was administered at 5PM immediately before running wheel access was given. SGLT2 inhibition did not alter change in blood glucose concentrations during exercise. Data are expressed as mean ± SD. *** $p<0.001$ for simple effect of activity.

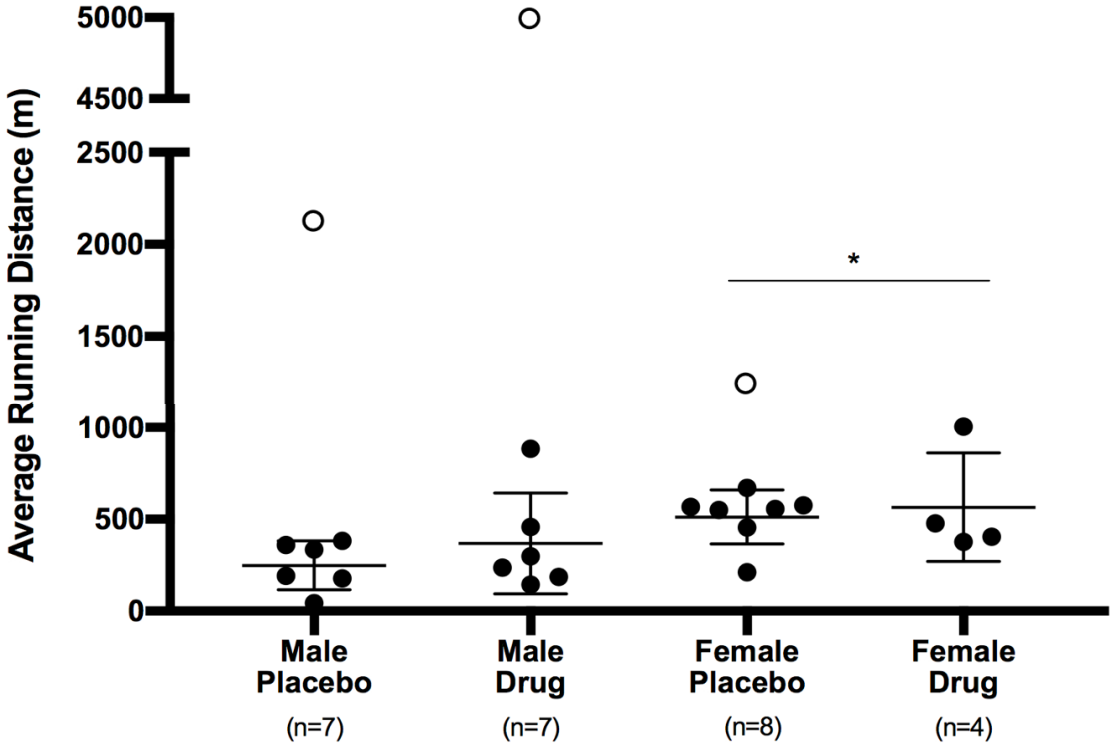


Figure 8: Voluntary running wheel activity was unaltered by SGLT2 inhibition but greater in females than males. Average daily running distance from 5PM-9PM. Solid dots represent the daily averages of each individual animal. Open dots represent outliers, which were excluded from all data analyses. Data are expressed as mean ± SD
* $p<0.05$ compared to males.

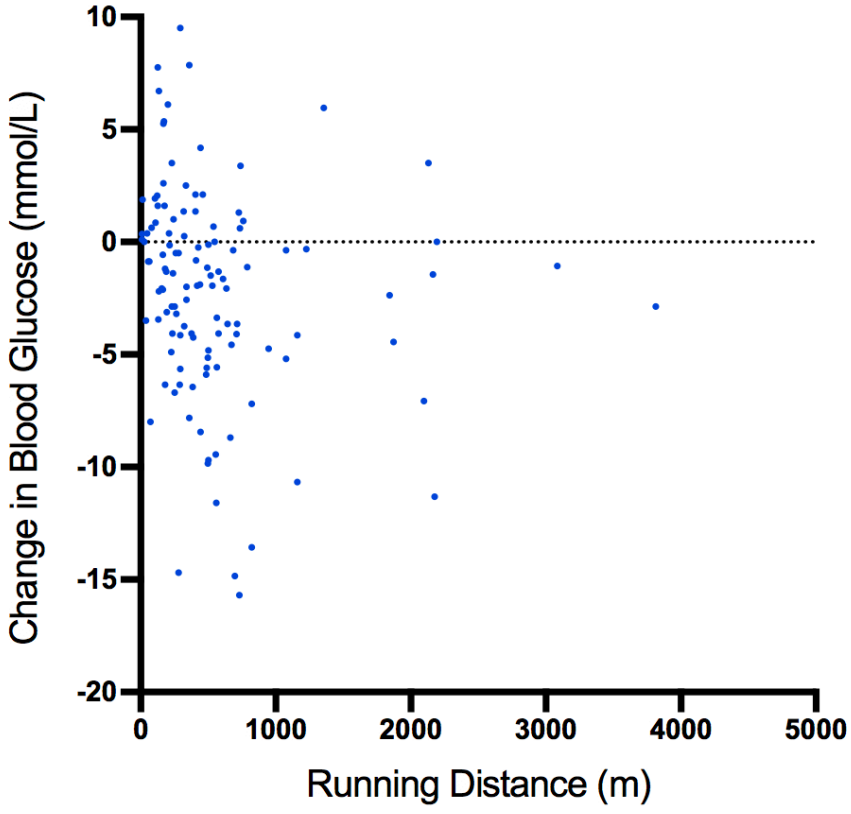


Figure 9: Voluntary running wheel activity was not correlated with change in blood glucose concentration during exercise. Data was analyzed using the daily running distances and changes in blood glucose concentrations of animals from 5PM-9PM for active placebo groups alone (r^2=0.02). Blue dots represent measurements taken from a single animal on a single day.

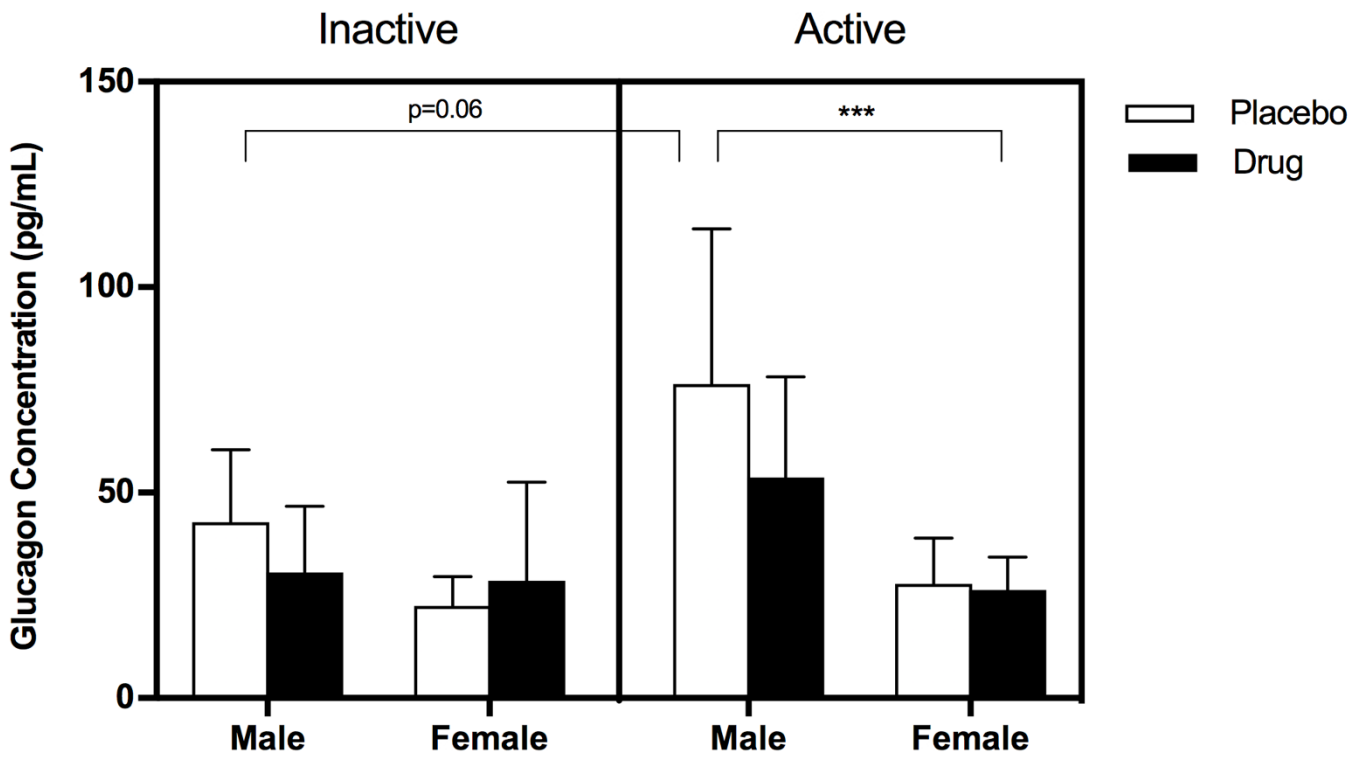


Figure 10: Basal glucagon concentrations were higher in physically active males than physically active females. Basal glucagon measurements were taken at 9AM on day 5 of treatment, immediately before treatment (after 4 full days of treatment). SGLT2 inhibition did not affect basal glucagon levels. Data are expressed as mean ± SD. *** $p<0.001$ for simple effect of sex in active animals, $p=0.06$ for simple effect of activity in males.

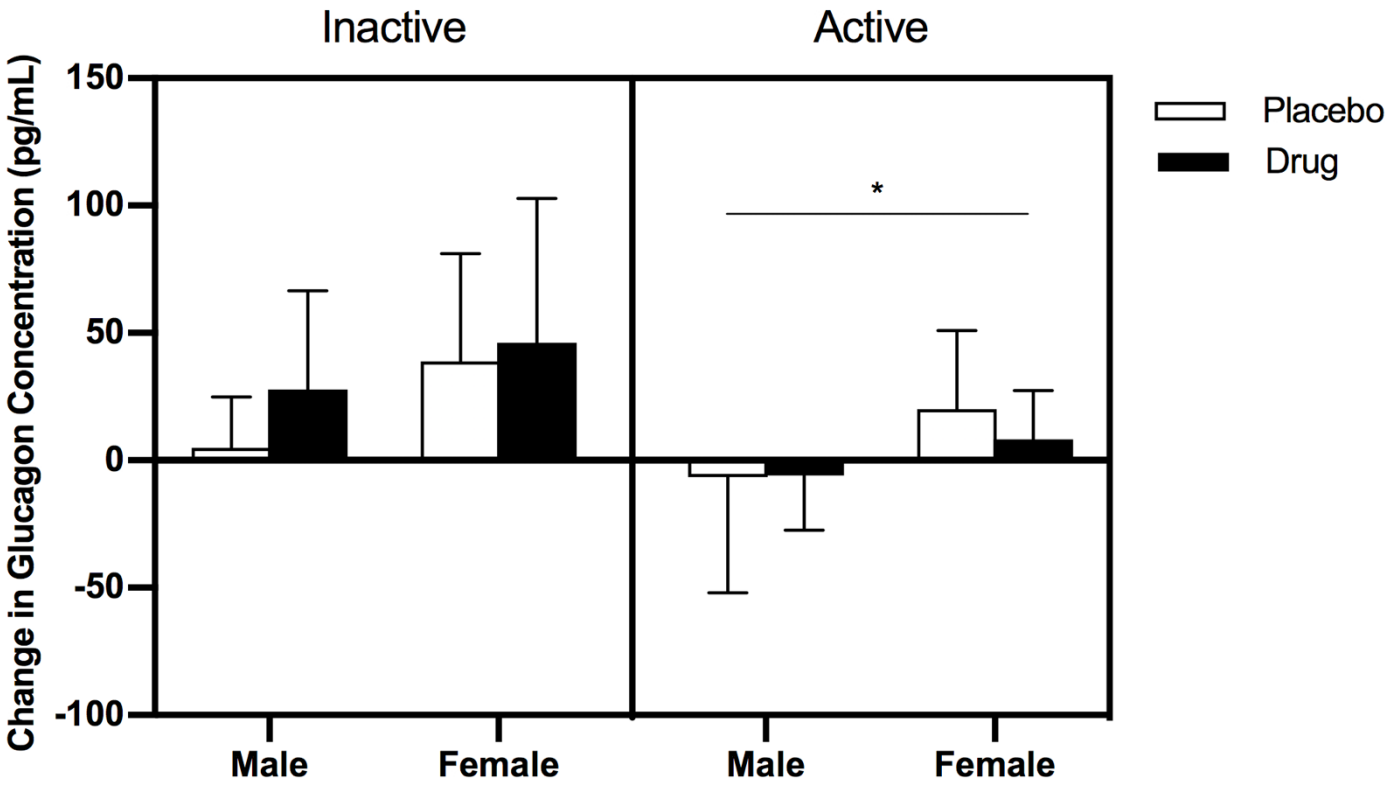


Figure 11: Changes in morning glucagon concentrations were reduced in physically active animals. Glucagon measurements were taken on day 5 of treatment at 9AM, immediately before treatment, and at 10AM, 1 hour after treatment. SGLT2 inhibition did not cause a change glucagon levels. Data are expressed as mean ± SD. * $p<0.05$ compared to inactive animals.

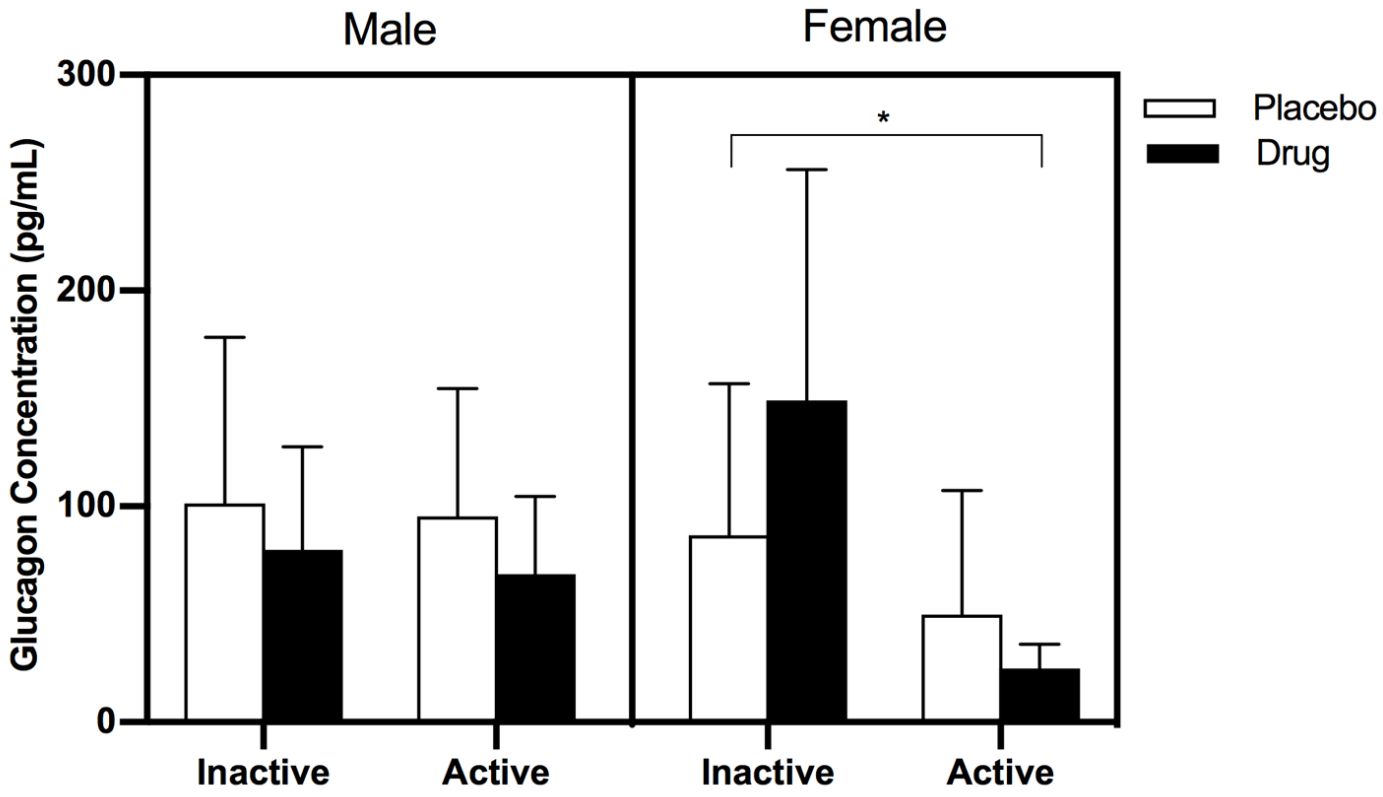


Figure 12: Post-exercise glucagon concentrations were reduced in physically active females but not physically active males. Glucagon measurements were taken on the first day of treatment at 9PM following 4 hours of voluntary wheel running. SGLT2 inhibition did not affect post-exercise glucagon levels. Data are expressed as mean ± SD.
* $p<0.05$ for simple effect of activity.

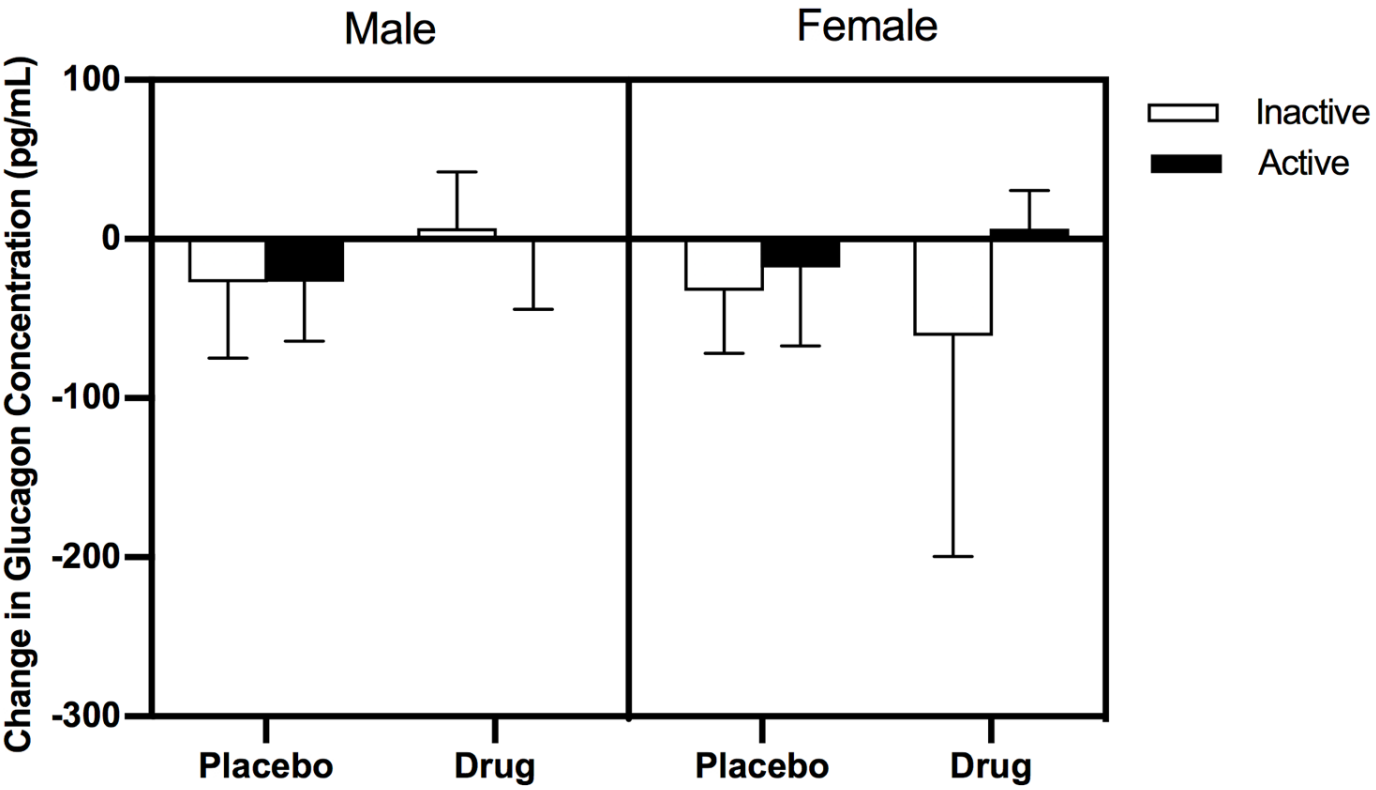
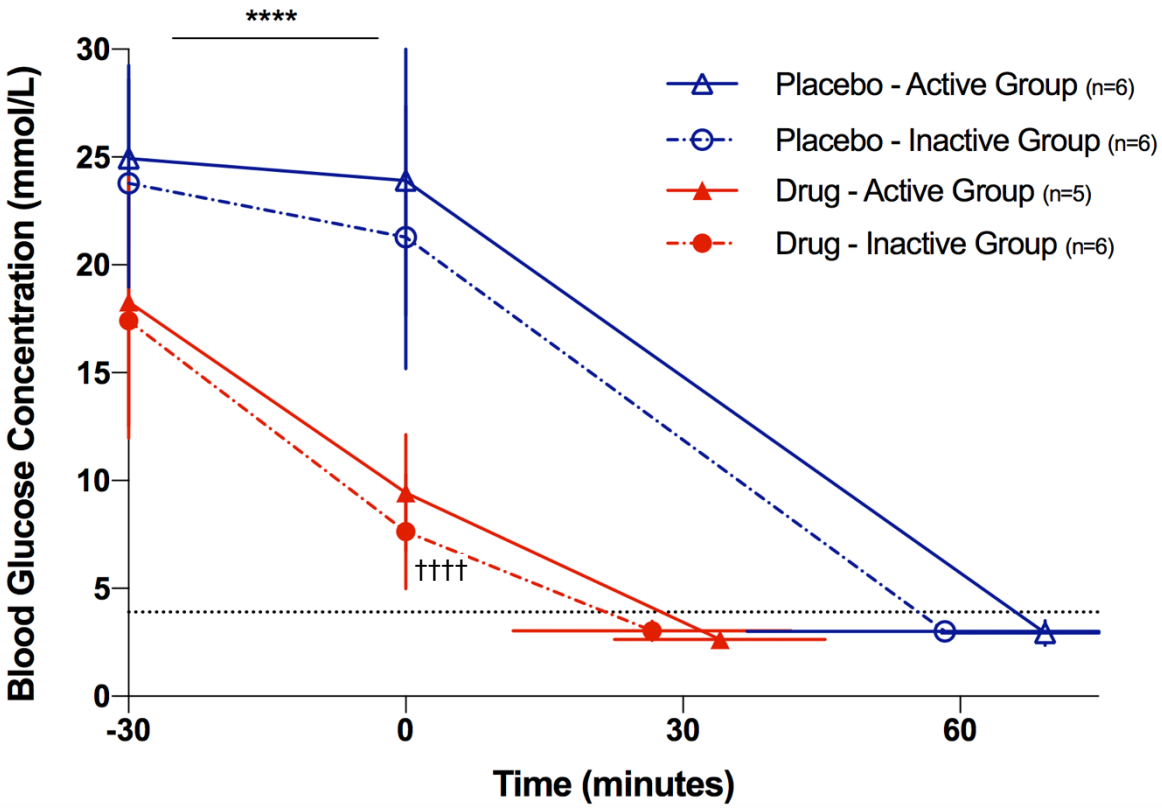


Figure 13: Prolonged SGLT2 inhibitor treatment did not alter glucagon concentrations. Glucagon measurements were taken on the first and last day of treatment at 9PM, 4 hours after treatment administration. Data are expressed as mean ± SD.

a)



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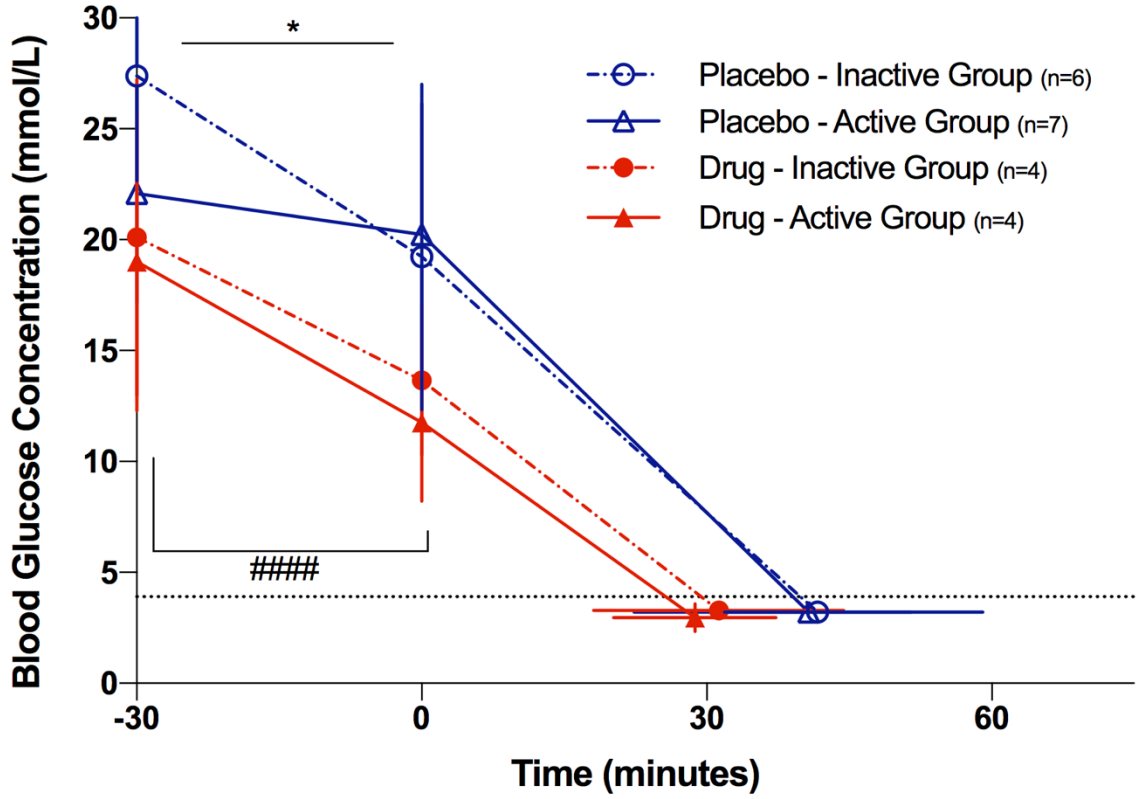

Figure 14: Blood glucose concentrations during the insulin-induced hypoglycemic challenge for males (a) and females (b). Regular morning treatment was given at -30 minutes and insulin aspart (10U/kg) was injected subcutaneously at 0 minutes. Animals were anesthetized once hypoglycemic. The horizontal black dotted line indicates hypoglycemia at ≤3.5 mmol/L. Horizontal error bars indicate SD for mean time to hypoglycemia. A three-way ANOVA with repeated measures was performed using only the -30 and 0 time points. Data are expressed as mean ± SD. **** $p<0.0001$ for main effect of treatment, †††† $p<0.0001$ compared to -30 minutes, * $p<0.05$ for main effect of treatment, #### $p<0.0001$ for main effect of time.

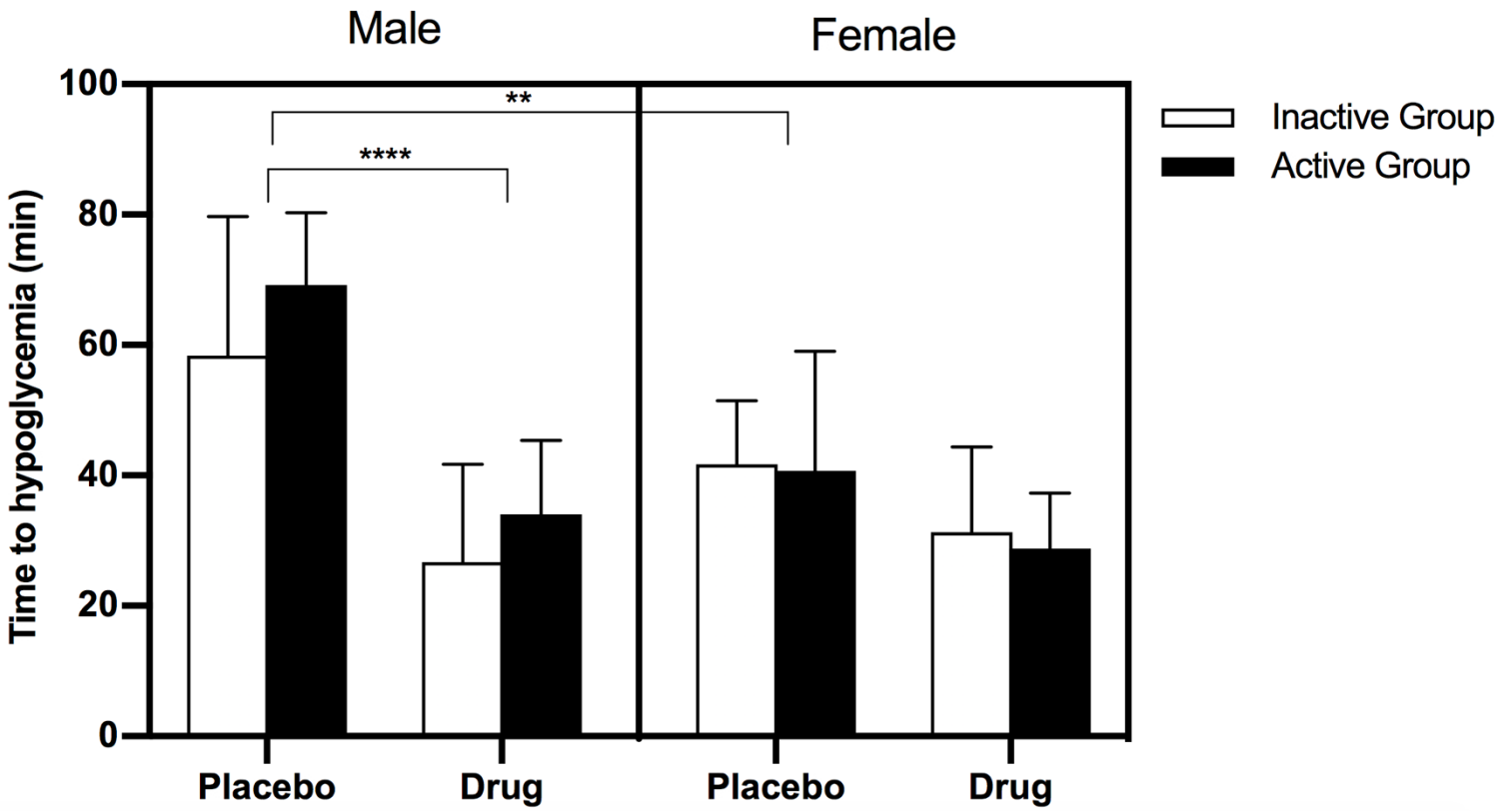

Figure 15: Time to hypoglycemia was decreased in males treated with an SGLT2 inhibitor. Time to hypoglycemia was measured as the time taken to reach a blood glucose concentration of ≤3.5 mmol/L after insulin injection (t=0). Data are expressed as mean ± SD. **** $p<0.0001$ for simple effect of treatment, ** $p<0.01$ for simple effect of sex.

	PLACEBO		DRUG	
	Inactive	**Active**	**Inactive**	**Active**
MALE	11.0±6.0	22.8±18.7	33.8±23.9	11.7±6.5
FEMALE	16.4±27.5	31.6±58.3	10.2±3.7	20.5±17.4

Table 1: Basal glucagon concentrations during the insulin-induced hypoglycemic challenge. Plasma glucagon samples were taken from the saphenous vein immediately before insulin injection (0 minutes). Data are expressed as mean ± SD (pg/mL).

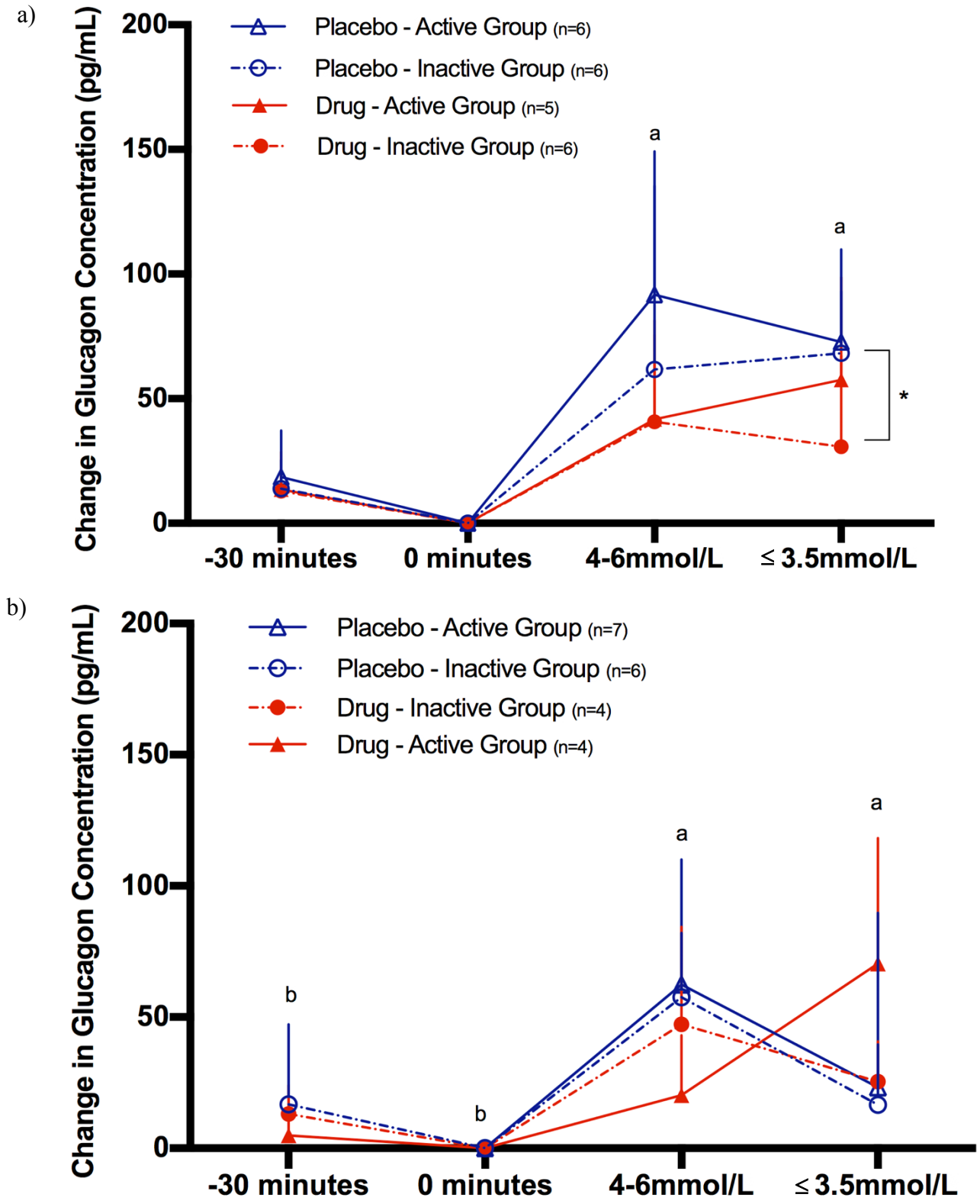

Figure 16: SGLT2 inhibition lowered changes in glucagon concentration in males (a) during the insulin-induced hypoglycemic challenge, but not in females (b). Treatment was administered at -30 minutes and insulin aspart (10U/kg) was injected subcutaneously at 0 minutes. Plasma glucagon samples were taken from the saphenous vein immediately before treatment (-30 minutes) and before insulin injection (0 minutes), as well as at euglycemia (4-6mmol/L) and hypoglycemia (≤3.5mmol/L) Data was calculated as change from baseline (0 minutes) and are expressed as mean ± SD. * $p<0.05$ for main effect of treatment. Matched letters indicate insignificant differences between time points. All time points without matched letters are significantly different from one another ($p<0.05$).

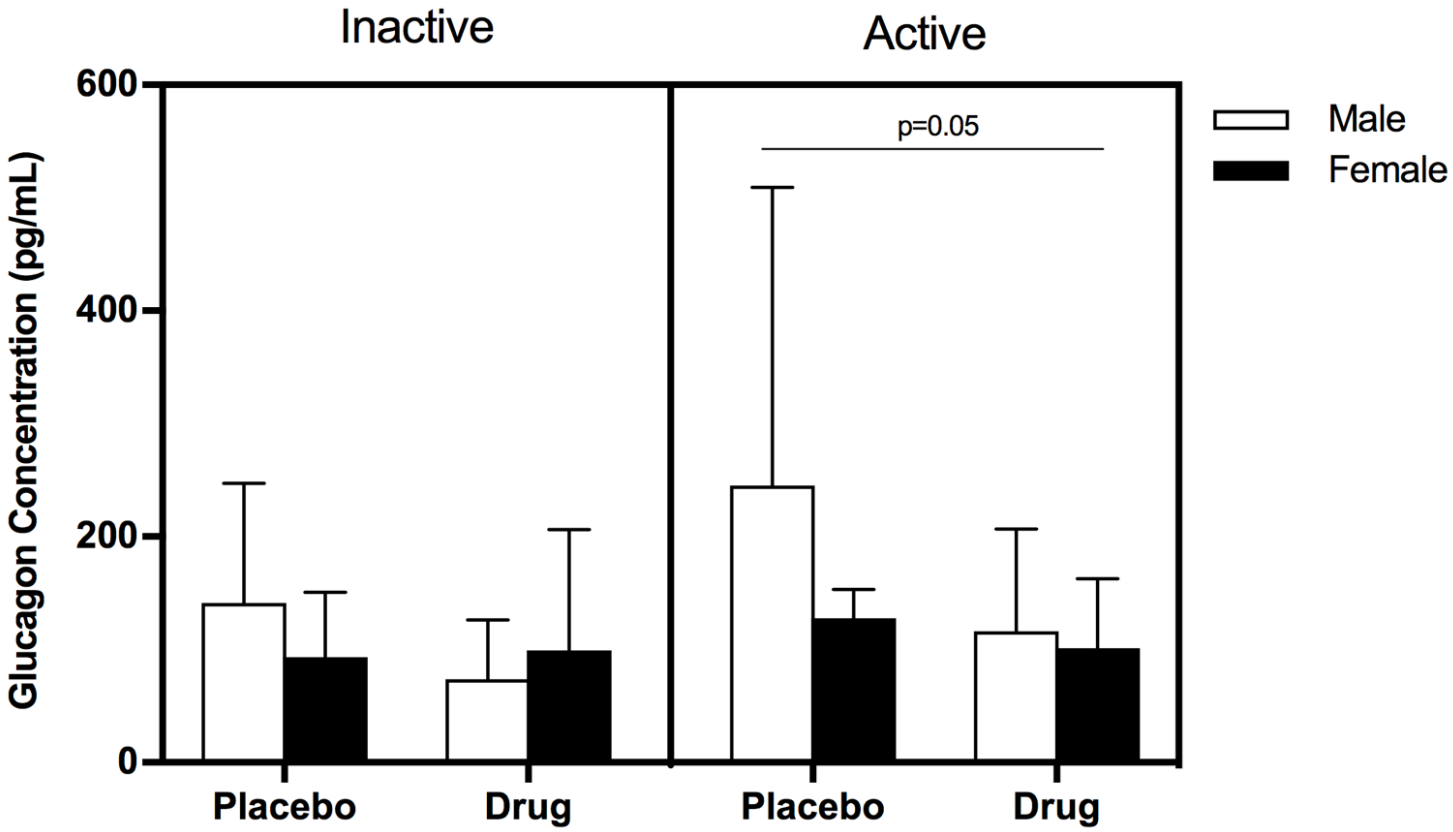

Figure 17: Portal vein glucagon concentrations at hypoglycemia were not affected by SGLT2 inhibition. Portal vein blood samples were taken while animals were under anesthesia, immediately after the insulin-induced hypoglycemic challenge when blood glucose concentrations were ≤3.5 mmol/L. Data are expressed as mean ± SD. p=0.05 for main effect of activity.

6. SUMMARY OF FINDINGS AND FUTURE DIRECTIONS

We investigated the effects of SGLT2 inhibition on glucagon secretion and on changes in blood glucose concentrations during physical activity and insulin-induced hypoglycemia, in a rodent model of T1D. Our findings demonstrate that SGLT2 inhibition does not affect basal glucagon concentrations or post-exercise glucagon levels in type 1 diabetic rats. However, one of our limitations was the chronic hyperglycemia state of all animals, which could have potentially hidden the effect of this drug on glucagon secretion. Furthermore, the hyperglycemic state of our animals prevented us from being able to examine the effects of SGLT2 inhibition during exercise-induced hypoglycemia. Because the STZ-induced diabetic model that we chose to use may not be ideal for studies involving chronic euglycemia, it may be wise to repeat our study in another rodent model of T1D.

Additionally, we found that, in male rats with T1D, SGLT2 inhibition reduces the glucagon counterregulatory response to insulin-induced hypoglycemia and accelerates the time taken to reach hypoglycemia. Because of our less than ideal sample sizes, specifically in SGLT2 inhibitor-treated female animals, we could not confidently determine if these results applied to females as well. Additional animals are needed in order to establish if this decrease in glucagon secretion is sex-specific. It would also be beneficial to test for SGLT-2 inhibitor-associated changes in glucagon secretion in humans with T1D, since clinical trials are currently underway.

Unexpectedly, we uncovered sex differences in blood glucose and glucagon response to exercise. We showed that female rats with T1D respond to voluntary physical activity with a drop in both blood glucose levels and glucagon concentration, whereas males are unaffected

by voluntary physical activity in these regards. Although the difference in glucagon concentrations that we observed might explain the differing blood glucose responses, other counterregulatory hormones could have played a role as well. These sex differences should be further investigated and other counterregulatory hormones should be measured as well. Moreover, future studies should investigate whether these sex differences to exercise in T1D apply to humans as well.

Overall, our findings have important and potentially concerning implications for the use of SGLT2 inhibitors in T1D, and have also revealed sex differences in blood glucose response to exercise in rodent with T1D. Additional studies using larger sample sizes, euglycemic conditions, and clinical trials would give us a clearing and broader understanding of our finding and their possible applications.

		INACTIVE PLACEBO						ACTIVE PLACEBO						INACTIVE DRUG						ACTIVE DRUG				
	Animal ID	SW2	SW3	SW5	SW24	SW30	SW47	SW12	SW16	SW18	SW26	SW28	SW29	SW1	SW7	SW9	SW23	SW43	SW45	SW11	SW14	SW15	SW27	SW46
	Time (min)																							
Treatment →	-30	23.7	23.1	16.6	31.1	21.8	26.6	24.0	23.4	23.2	33.3	25.0	20.8	14.2	19.2	25.8	14.3	20.5	10.7	20.5	19.5	26.0	11.0	14.5
Insulin →	0	21.5	18.5	15.6	30.7	26.1	15.4	21.0	23.6	23.9	32.7	28.1	14.3	5.1	8.9	12.2	7.4	5.1	7.4	13.5	8.6	10.8	7.3	7.1
	5										30.1													
	10	14.3	10.7	12.9		23.0	9.5	15.8	19.6	23.3		18.3	9.9	3.7	5.5	9.7		3.8	3.4	8.0	6.3	12.3	3.6	5.9
	15																							
	20	9.4	4.7	6.6	23.5	13.1	6.0	9.1	14.2	17.2	22.4	12.9	6.9	2.8	5.4	4.2	3.0	3.3		5.1	3.9	4.8	2.5	3.7
	25												5.2											
	30	6.1	3.5	4.8	10.3	8.7	4.0	7.2	8.2	9.1	13.6		6.5		3.8	3.6				3.9	2.4	5.2		2.7
	35																							
	40	4.3		5.5	6.7	8.6	3.1	4.5		6.1	11.6	6.9	4.5		2.3	3.6				2.9		3.6		
	45								4.3							3.8								
	50	4.2		4.4	5.7	5.8		3.5		4.9	7.3	4.8	3.4			3.5						2.8		
	55								5.3															
	60	2.7		3.0	4.0	5.9		3.6		4.7	4.7	5.2												
	65								2.3															
	70				3.0	4.9		2.2		3.6	3.9	4.1												
	75																							
	80					3.9					3.2	3.1												
	85																							
	90					3.0																		
	Time to hypoglycemia (≤3.5 mmol/L)(min):	60	30	60	70	90	40	70	65	70	80	80	50	20	40	50	20	20	10	40	30	50	20	30

Supplementary Figure 1: Blood glucose concentrations of male animals during the insulin-induced hypoglycemic challenge. Blood glucose measurements are shown for individual animals throughout the insulin-induced hypoglycemic challenge (mmol/L). Drug (10mg/kg empagliflozin) or placebo treatment (H_2O) was given at -30 minutes via oral gavage and insulin aspart (10U/kg) was injected subcutaneously at 0 minutes, as indicated. Time to hypoglycemia was calculated as the minutes taken to reach a blood glucose measurement of ≤3.5 mmol/L.

		INACTIVE PLACEBO						ACTIVE PLACEBO							INACTIVE DRUG				ACTIVE DRUG			
	Animal ID	SW36	SW39	SW52	SW53	SW56	SW59	SW20	SW42	SW50	SW58	SW62	SW63	SW64	SW32	SW37	SW40	SW54	SW35	SW49	SW55	SW61
	Time (min)																					
Treatment →	-30	26.1	26.6	23.6	32.9	33.3	22.0	20.7	12.7	19.9	25.2	26.2	26.9	23.2	19.3	10.6	23.3	27.3	23.5	10.1	17.7	24.9
Insulin →	0	20.8	17.8	10.2	17.1	31.4	18.3	21.4	7.7	16.8	18.8	28.8	24.5	23.8	13.1	9.9	18.1	13.6	13.8	6.5	12.8	14.0
	5			9.3	13.4													8.0			10.7	
	10	24.3	13.5	7.8	9.5	25.0	12.2	16.9	2.9	10.8	17.1	26.0	20.9	16.9	10.6	6.3	12.9	5.5	8.0	5.1	10.1	10.1
	15			5.8	6.8		9.3							11.4							7.2	6.7
	20	8.3	8.8	5.1	4.2	14.6	5.7	5.9		5.8	10.2	12.7	11.1	7.4	5.7	3.2	6.7	3.4	6.5	2.1	4.7	4.5
	25			3.7	4.3						4.8	8.0	10.4	5.1					5.1		4.7	3.1
	30	6.0	5.4	3.2	4.0	8.2	3.9	6.9		3.9	3.3	7.4	6.5	4.5	4.4		5.5		4.7		3.4	
	35				2.9	4.6	3.4			3.9		6.6	4.0	4.1								
	40							4.5		3.4		5.4	2.9	3.7	3.3				3.4			
	45	3.7	2.0			4.4								3.3			3.2					
	50	3.3				3.7		3.2				7.7										
	55					3.0						4.6										
	60											4.6										
	65											4.4										
	70											3.5										
	75																					
	80																					
	Time to hypoglycemia (≤3.5 mmol/L)(min):	50	45	30	35	55	35	50	10	40	30	70	40	45	40	20	45	20	40	20	30	25

Supplementary Figure 2: Blood glucose concentrations of female animals during the insulin-induced hypoglycemic challenge. Blood glucose measurements are shown for individual animals throughout the insulin-induced hypoglycemic challenge (mmol/L). Drug (10mg/kg empagliflozin) or placebo treatment (H_2O) was given at -30 minutes via oral gavage and insulin aspart (10U/kg) was injected subcutaneously at 0 minutes, as indicated. Time to hypoglycemia was calculated as the minutes taken to reach a blood glucose measurement of ≤3.5 mmol/L.

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