

Universidade do Minho
Escola de Ciências

João Pedro Cardoso Ribeiro

New Generation Antidiabetics: what are the
implications on spermatogenesis nutritional
support?

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implications on spermatogenesis nutritional
support?**

Dissertação de Mestrado
Bioquímica Aplicada
Especialização em Biomedicina

Trabalho efetuado sob a orientação do
Professor Doutor Pedro Fontes Oliveira

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ANTIDIABÉTICOS DE NOVA GERAÇÃO: QUAIS SÃO AS IMPLICAÇÕES NO SUPORTE NUTRICIONAL DA ESPERMATOGÊNESE?

A incidência de doenças metabólicas como Diabetes *mellitus* tipo 2 (TD2M) e obesidade tem aumentado entre crianças e jovens adultos. Estas doenças estão intimamente ligadas, portanto, novos agentes farmacológicos têm como objetivo controlar a glicemia enquanto provocam perda de peso. A recentemente implementada terapia combinada de dapagliflozina (inibidor do cotransportador de sódio-glucose) e exenatida (análogo do *glucagon-like peptide 1*) tem sido prescrita para tratar T2DM. Este tratamento influencia o metabolismo da glucose em todo o corpo, portanto, também podem afetar o metabolismo dos tecidos responsáveis pela fertilidade masculina. A espermatogênese é altamente dependente da cooperação metabólica estabelecida entre as células germinativas em desenvolvimento e as células de Sertoli. Estas usam glucose extracelular para produzir lactato, que as células germinativas usam como combustível.

Neste trabalho, avaliamos os efeitos da dapagliflozina e exenatida no metabolismo das células de Sertoli. Para tal, células de Sertoli TM4 de murganho foram tratadas na ausência (controle) ou na presença de concentrações sub-farmacológicas, farmacológicas e supra-farmacológicas de dapagliflozina (50; 500; 5000 nM, respectivamente) ou exenatida (2,5; 25; 250 pM, respectivamente) ou ainda, com uma combinação das concentrações farmacológicas de ambas as drogas, durante 24 horas. A citotoxicidade destes tratamentos para as células de Sertoli foi avaliada através de ensaios de MTT, liberação da lactato desidrogenase (LDH) e SRB. O perfil glicolítico das células de Sertoli também foi determinado (espectroscopia de ^1H -RMN), bem como os níveis de expressão dos principais transportadores e enzimas como a LDH, transportadores de glucose 2 (GLUT2) e transportador de monocarboxilatos 4 (MCT4). Além disso, foi quantificado o conteúdo em glicogênio e as reservas lipídicas nas células de Sertoli TM4. A concentração farmacológica de dapagliflozina mostrou um leve efeito citotóxico que também foi observado no tratamento combinado. Além disso, a concentração farmacológica de dapagliflozina mostrou aumentar a eficiência glicolítica e aumentar a secreção de lactato pelas células de Sertoli, que é visto como um potencializador da espermatogênese. O tratamento combinado demonstrou que os diferentes fármacos parecem ter um efeito sinérgico para manter a homeostase metabólica das células de Sertoli. Assim, os nossos resultados sugerem que o tratamento com dapagliflozina, exenatida e o tratamento combinado de ambos os fármacos em concentrações farmacológicas podem ser adequados para homens na idade reprodutiva.

Palavras-chave: Células de Sertoli, Dapagliflozina, Doenças metabólicas, Exenatida, Infertilidade.

NEW GENERATION ANTIDIABETICS: WHAT ARE THE IMPLICATIONS ON SPERMATOGENESIS NUTRITIONAL SUPPORT?

The incidence of metabolic diseases such as type 2 Diabetes *mellitus* (T2DM) and obesity has been increasing among children and young adults. Both conditions are tightly linked thus new pharmacological agents aim to control glycemia while provoking weight loss. Recently implemented combined therapy of dapagliflozin (sodium-glucose cotransporter inhibitor) and exenatide (glucagon-like peptide 1 analogue) has been prescribed against T2DM. This treatment influences whole-body glucose metabolism, and thus can also impact the metabolism of tissues responsible for male fertility. Spermatogenesis is highly dependent on the metabolic cooperation established between Sertoli cells and germ cells. The former uses extracellular glucose to produce lactate, which germ cells use as fuel.

In this work, we evaluated the effects of dapagliflozin and exenatide on Sertoli cells metabolism. For this purpose, mice TM4 Sertoli cells were treated in the absence (control) or presence of sub-pharmacologic, pharmacologic and supra-pharmacologic concentrations of dapagliflozin (50; 500; 5000 nM, respectively), or exenatide (2,5; 25; 250 pM, respectively), or with a combination of the pharmacological concentrations of both drugs, during 24 hours. The cytotoxicity of these compounds on Sertoli cells was evaluated by MTT, lactate dehydrogenase (LDH) release, and SRB assays. The glycolytic profile of SCs was also determined (^1H -NMR spectroscopy), as well as the expression levels of key metabolite transporters and enzymes, such as LDH, glucose transporters 2 (GLUT2), and monocarboxylate transporter 4 (MCT4). To further pursue our aim, glycogen storage and lipid reserves were quantified in TM4 Sertoli cells. Pharmacological concentration of dapagliflozin showed a mild cytotoxic effect that was also seen in the combined treatment. In addition, the pharmacological concentration of dapagliflozin showed to enhance the glycolytic efficiency and increase lactate secretion by Sertoli cells, which is seen as a positive enhancer of spermatogenesis. The combined treatment demonstrated that the different drugs seem to have a synergic effect to maintain the metabolic homeostasis of Sertoli cells. Thus, our results suggest that treatment with dapagliflozin, exenatide and the combined treatment of both drugs at pharmacological concentrations may be suitable for males in reproductive age.

Keywords: Dapagliflozin, Exenatide, Infertility, Metabolic diseases, Sertoli cells.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BTB	Blood-testis barrier
cAMP	Cyclic adenosine monophosphate
DM	Diabetes <i>mellitus</i>
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
FSH	Follicle-stimulating hormone
GLP-1	Glucagon-like peptide 1
GLUT's	Glucose transporters
GnRH	Gonadotrophin-releasing hormone
LDH	Lactate dehydrogenase
LH	Luteinizing hormone
MCT's	Monocarboxylate transporters
NMR	Nuclear molecular resonance
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PKA	Protein kinase A
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SGLT's	Sodium glucose co-transporters
SRB	Sulforhodamine B
T1DM	Type 1 Diabetes <i>mellitus</i>
T2DM	Type 2 Diabetes <i>mellitus</i>
TCA	Tricarboxylic acid

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Chapter I

INTRODUCTION

1. INTRODUCTION

1.1. Diabetes mellitus

Diabetes *mellitus* (DM) is a chronic disease that has reached pandemic proportions and its incidence has been increasing dramatically over the last decades. Moreover, it is expected a growing trend in the number of individuals suffering from this metabolic disease. It has been estimated that a worrisome 693 million people will develop DM by 2045 (1). DM consists of multiple conditions related to deficient carbohydrate, lipid and protein metabolism. This dysregulation impacts multiple organs and systems such as the kidney, retina, circulatory system, and nervous system (2). Hence, acute complications in long-term DM patients can result in blindness, macrovascular and microvascular complications, and sexual dysfunction, among others (3). Moreover, DM increases the chance of strokes and coronary artery disease in undiagnosed or untreated individuals (4), which could result in death. Impaired glucose homeostasis results in hyperglycemia which is caused by hypoinsulinemia or insulin resistance (5).

Insulin is a hormone that is secreted into circulation after a meal rich in carbohydrates. Glucose levels are sensed with the participation of specific glucose transporters together with neural stimuli and incretin signaling. This leads to the activation of insulin secretion on β -cells in the pancreas. Insulin will act on other cells increasing their glucose uptake and decreasing glycemia levels (6). Besides, insulin also mediates meal-time and has a role in cell growth and differentiation (7). The plasmatic insulin is insufficient to maintain glucose homeostasis in DM patients, which can be caused by autoimmune β -cell elimination, known as type 1 diabetes *mellitus* (T1DM); or gradual loss of insulin sensitivity, classified as type 2 diabetes *mellitus* (T2DM) (8).

Patients with T1DM are increasing among children and has been reported that this growth in incidence does not only depend on genetic factors (9) but also in environmental factors (10) such as enterovirus infection or nutritional factors (e.g. vitamin D deficiency or premature contact with cow's milk proteins) (11). These factors can lead to the destruction of insulin-producing β -cells by T lymphocytes in an autoimmune manner. Additionally, nearly one-third of patients with T1DM suffer from diabetic ketoacidosis (8). This condition is a consequence of diminished insulin/glucagon ratio presence in circulation that activates ketogenic machinery in liver cells to produce glucose, but also ketones from long-chain fatty acids (12). Ketones such as acetoacetate and β -hydroxybutyrate increase in plasma and this result in metabolic acidosis (13) that in severe cases can lead to coma (14). Thus, to work around this condition the patients suffering from T1DM will depend on exogenous insulin for life (15), forcing them to continuous health care.

1.1.1. Type 2 Diabetes *mellitus*

T2DM is the most prevalent type of DM accounting approximately 90% of all occurrences (16) and is tightly linked with obesity, sedentary lifestyle (17), but also genetic factors (18). Usually, the risk of developing this condition increases with age (19). However, this epidemic is affecting an increasing number of individuals from younger group ages, which intensifies the health, sociologic, and economic problems associated with T2DM (20). This type of chronic hyperglycemia results from diminished insulin bioactivity, which can be due to anomalous insulin secretion, decreased insulin sensitivity, or a combination of both (21, 22). In a scenario with high glucose intake, a healthy individual would secrete proportional insulin to normalize glycemia levels. However, diabetic patients insulin secretion do not compensate the subnormal hormone effect on its receptors of skeletal muscle, pancreas, adipose tissue, and liver (23), promoting the increase of glucose levels on plasma thus, causing hyperglycemia.

Lifestyle modifications are essential to counter the effects of T2DM, including changing diet and exercise habits. Multiple drugs were also developed to counteract hyperglycemia and its associated comorbidities. However, a complex study is needed to find the most suitable antidiabetic therapy for each patient situation (20).

1.2. Obesity and its role in the origin of T2DM

According to the World Health Organization, a person is considered overweight when its Body Mass Index (BMI) score is above 25 and is considered obese when the score is superior or equal to 30. BMI is a crude measure for studying obesity in a population and can be calculated by dividing a person weight (kilograms) with the square of the person height (meters) (24). Through the years, BMI has increased in developed countries possibly by their easier access to high caloric meals. This excessive caloric intake is contributing to pandemic obesity never seen before. Most victims suffering from obesity also develop T2DM, since obesity already induces some degree of insulin resistance (25). In obese humans, the bioactivity of the circulating insulin is diminished by the excessive abdominal fat. Thus, hyperglycemia will aggravate and this causes more insulin secretion, increasing its concentration on plasma which worsens insulin resistance and promotes high blood pressure (26). Furthermore, fat cells can penetrate the pancreatic islets and influence insulin secretion in more advanced obese patients (26). Obesity is also known to dysregulate the secretion of hormones responsible for the all-body energy balance, mainly ghrelin, leptin, glucagon-like peptide (GLP-1), and insulin, being the former already discussed above. Ghrelin is a hormone

secreted by the endocrine cells of the gastrointestinal tract and it is known as the hunger hormone since it is related to the origin of appetite. Ghrelin levels in obese people plasma are reduced in relation to the ones of a healthy individual (27). Leptin, however, is released by white adipose tissue and its levels are increased in obese persons when compared with healthy individuals. It is believed that leptin plays a role in reducing appetite and together with ghrelin they help in the regulation of energy homeostasis, glucose metabolism, and reproductive function (28).

Weight-loss should be the main goal to control glycemia in obese T2DM individuals, even though this can be the most challenging aspect of the treatment (29). In 2015, Whitmore stated that managing weight in T2DM patients is comparable to a “juggling act” with balanced energy intake/output, glycemic control, hypoglycemia, medication and the negative sides that those factors can generate (30). DM treatment is constantly evolving, and new approaches are being developed to improve the health of the patients and their lifestyle (30). Some of the antidiabetic drugs used to regulate T2DM also have anti-obesogenic properties which provoke weight-loss in the patient, namely: low affinity/high capacity sodium-glucose cotransporter 2 (SGLT2) inhibitors (e.g. dapagliflozin) and GLP-1 receptor agonist (e.g. exenatide) (20). Both these pharmacological agents were already used separately or in combination with other drugs. Combined therapy of dapagliflozin (10 mg once daily) plus exenatide (2 mg injection once weekly) is being studied in the form of phase III clinical trial to compare the results of this *novel* therapy with more standardized therapies (31).

1.3. Sodium-glucose cotransporter 2 inhibitors

Kidneys role in glucose homeostasis starts when Malpighi glomerulus filters plasmatic glucose without restrains, followed by its reabsorption in the proximal tubule into circulation by specific glucose transport proteins. In individuals with chronic hyperglycemia, the renal threshold (180 mg/dL) is not enough to reabsorb all plasmatic glucose, forcing the remaining glucose to be excreted through the urine (32). This event can be explained by the SGLT2 activity and expression in the proximal tubule. This transporter is responsible for glucose resorption and its activity is insulin-dependent (33). In addition, kidneys are also involved in glucose metabolism homeostasis by performing gluconeogenesis, when humans are fasted for more than 14 hours, contributing with around 20% to 25% of total plasmatic glucose after that period (34).

SGLT2 is mainly found in the initial part of renal convoluted proximal tubules, more specifically in the apical membrane. This transporter is responsible for the reabsorption of approximately 90%

of glucose in the kidneys (35). The reabsorption is established when SGLT2 imports one sodium ion and one glucose molecule into the cytosol finally, glucose is then transported into the bloodstream through the action of glucose transporter 2 (GLUT2) (36) (Figure 1). Interestingly, patients with T2DM have increased expression levels of SGLT2 in proximal tubules cells. This fact suggests that T2DM patients have an above normal renal threshold (37). Thus, more glucose is reabsorbed into the bloodstream which will only aggravate hyperglycemia (32). Considering this, compounds capable of inhibiting SGLT2 could be a convenient way of controlling glycemic levels, blocking the reabsorption of this hexose into circulation, increasing its excretion through the urine, and diminishing the overall caloric intake of the individual.

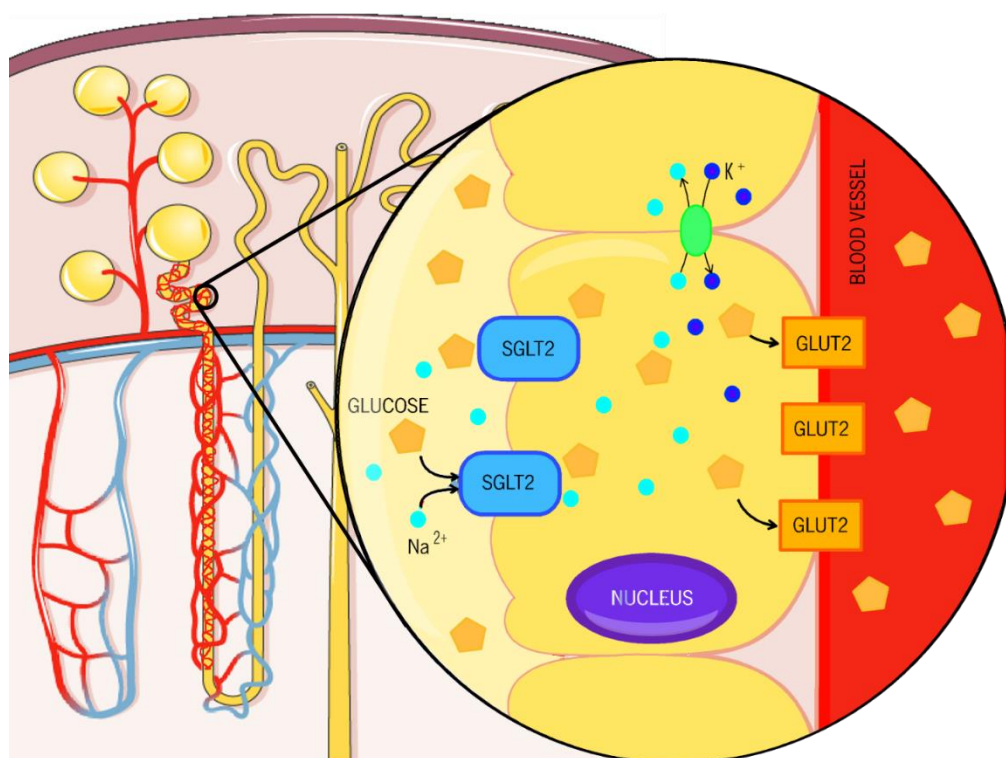


Figure 1: Schematic representation of glucose reabsorption by sodium-glucose cotransporter 2 (SGLT2) in the proximal tubule. Glucose and sodium ion are transported from the lumen of the tubule to the cytoplasm through the SGLT2. After this, glucose transporters 2 (GLUT2) transport the glucose into the circulation.

Dapagliflozin favors this management of glucose homeostasis. Thus, the European Union approved dapagliflozin for the treatment of T2DM in 2012 (38). Dapagliflozin is chemically described as (2S,3R,4R,5S,6R)-2-[4-chloro 3-(4-ethoxybenzyl) phenyl]-6-(hydroxymethyl) tetrahydro-2H-pyran-3,4,5-triol). Its molecular formula is $C_{21}H_{25}ClO_6$ which translates in a molecular

weight of 408.87 g/mol (38). Dapagliflozin structure is organized in one glucose molecule which is linked to an aglycone component through a carbon-carbon bond with the purpose of increasing its metabolic stability against glucosidase enzymes (39). This fact allows dapagliflozin to be prescribed for oral administration and can be found in tablets of 5 mg or 10 mg (39). It needs only approximately 1 hour (t_{max}) after the first dosage of 5 mg or 10 mg dapagliflozin to be absorbed. Dapagliflozin is also rapidly absorbed even when the treatment is made after a meal, due to its high aqueous solubility and great intestinal absorption (40). Its maximal plasma concentration can reach up to approximately 70 ng/ml (5 mg treatment) and 200 ng/ml (10 mg treatment) (38, 41) moreover, dapagliflozin half-life time ($t_{1/2}$) is approximately 17 hours (42, 43).

The efficiency and safety of dapagliflozin 10 mg once-daily therapy for obese with T2DM patients has already been studied in multiple clinical trials. DURATION-8 was a 28-week long phase III clinical trial in adults with inadequate glycemic control (HbA_{1c} 8-12%). During this clinical trial, 19% dapagliflozin treated group had a decreased on HbA_{1c} score by 7%, 20% of the group lost more than 5% of their weight, and systolic blood pressure decreased 1,8 mm Hg in relation to the baseline values at the beginning of the trial (44). In another phase III clinical trial with similar dosage, dapagliflozin showed to improve HbA_{1c} score and weight loss by a difference of -0,90% and -1.38 Kg, respectively, in comparison to individuals from the placebo group at the end of the 24-week trial duration (45). Moreover, 10 mg once daily therapy of dapagliflozin can be also used as a complementary agent to exogenous insulin treatment in patients with T1DM (46). In relation to the placebo group, patients treated with dapagliflozin improved their HbA_{1c} score by -0,45% in the first 4 weeks of the trial, maintaining that difference in the remaining time of the study. In addition, dapagliflozin (10 mg/day) treatment allowed the reduction of the exogenous insulin dosage by 9,8% per kilogram of bodyweight at week 24 of the study. Similarly, body weight also decreased by 3.72% in relation to the individuals from the placebo group, most of this reduction occurred in the first 8 weeks of the trial and was maintained until the end of the experiment (46).

As above demonstrated, dapagliflozin decreases hyperglycemia, body weight and blood pressure in DM patients, being that these are the key factors responsible for the most deleterious effects of DM, making this pharmacological agent a promising drug for the treatment of this condition and increasing the life quality of the user. However, like any drug in the market, dapagliflozin entails some negative side effects to the user. The main concern about this drug is its influence on renal and cardiovascular function of treated DM and obese patients. T2DM patients with normal or moderately impaired renal function were subjected to dapagliflozin therapy. Their

estimated glomerular filtration rate was calculated to assess the implications that dapagliflozin could generate to patients' renal function. Kohan and coworkers analyzed twelve clinical trials and stated that dapagliflozin (10 mg/day) decreased estimated glomerular filtration rate by $-4,13 \text{ mL/min/1,73m}^2$ in relation to the baseline value in the first week. Nevertheless, this value returned to baseline by week 24 and remained stable until the end of the experiment (47). Blood urea nitrogen values were significantly increased in relation to placebo at the end of the experiment which is justified by the osmotic diuretic effect that the drug entails (47) which also allows the reduction of blood pressure (48). Regarding cardiovascular function, a randomized clinical trial with 28 weeks of duration was performed. Dapagliflozin (10mg/day) was administered to patients with a high risk of cardiovascular problems and the results showed a greater occurrence of hypotension and dehydration in relation to the placebo group, with the usual improvement in glycemic levels and body weight (49). These results could be justified by a large number of plus 65 years old patients that participated in this study and by the fact that the patients already had high risk of cardiovascular problems. However, another study described that dapagliflozin treatment increased plasmatic levels of high-density lipoprotein 2-cholesterol, which are regarded as an advantageous cardiometabolic marker (50). On the other hand, low-density lipoproteins, which are considered a strong marker of possible cardiovascular complications, did not change between the dapagliflozin treated group and the placebo group (50). Moreover, SGLT2 inhibitors such as dapagliflozin have been also associated with risks of inducing diabetic ketoacidosis. This could be due to the increase of glucagon/insulin ratio levels in circulation which promote hepatic ketogenesis when in dapagliflozin therapy. Furthermore, treatment for T2DM with SGLT2 inhibitor combined with exogenous insulin increases the chances of developing diabetic ketoacidosis due to the need of lowering exogenous insulin dosage to prevent hypoglycemia, which only aggravates the glucagon/insulin ratio. This happens due to enhanced lipolysis caused by the lack of insulin, increasing ketones levels on plasma (51). SGLT2 inhibitors, such as dapagliflozin, are also linked with genitourinary tract infections caused by the enhanced glucose concentration in the urine (52).

Besides that, studies suggest that dapagliflozin is a relatively safe pharmacological agent and even entails some advantages to the patient, such as the ability to protect pancreatic β -cells of hyperglycemia adverse effect, conserving their function (53). Moreover, this drug is associated with weight loss which results from the enhanced glucose excretion, maximizing caloric loss yet, this fact can originate an increase in appetite (54). In addition, a study stated that dapagliflozin (5mg/day) treatment for six months decreased total body weight, without significantly changing

skeletal muscle mass and soft lean mass in relation to the placebo group (55). Dapagliflozin also minimizes the chances of hypoglycemia since SGLT2 inhibitors do not interfere directly with insulin secretion (56), unlike other antidiabetic drugs. Thus, dapagliflozin can be complemented and used simultaneously with other antihyperglycemic drugs capable of modulating insulin secretion (44), such as exenatide which belongs in the GLP-1 receptor agonist class of antidiabetic drugs.

1.4. Glucagon-like peptide-1 receptor agonist

GLP-1 is an incretin derived from a different path of post-transcriptional modification of proglucagon in enteroendocrine L-cells, which can be found along the gastrointestinal tract with greater abundance in the distal gut, ileum, and colon (57). Oral ingestion of nutrients is the major physiologic stimuli for the secretion of GLP-1 by L-cells, although its concentration in the bloodstream depends on the composition of the meal. Meals richer in carbohydrates and fats stimulate greater GLP-1 secretion than meals rich in proteins (58). It is believed that GLP-1 secretion is mediated by direct and indirect sensing since GLP-1 levels rise in circulation within minutes. Normally, this time is insufficient for the food to reach the L-cells in the distal gut. Thus, it is believed that some of these cells are situated in the upper gut to sense the nutrients ingested and share that information with the richest L-cells regions for a rapid and efficient GLP-1 release into circulation (57). After this, and when it binds to its G-protein coupled receptors on pancreatic β -cells, GLP-1 stimulates insulin secretion by activating the second messenger cyclic adenosine monophosphate (cAMP) which, in its turn, activates the insulin effectors protein kinase A (PKA) (59) (Figure 2). Interestingly, as the incretin effects are proportional to the glucose intake, also insulin secretion will be mediated by the ingested glucose (57). The ability of such peptide to modulate insulin secretion and suppress excessive glucagon secretion (reducing hepatic gluconeogenesis) (60), shows that GLP-1 receptors agonists could be an interesting approach to control glycemia in individuals with T2DM (61).

Exenatide is an example of one GLP-1 receptor agonist composed of 39 amino acids with a molar mass of 4186.6 g/mol (62), originally extracted from the salivary excretion of the *Heloderma suspectum* (Gila monster) and commonly named exadin-4 (63). When compared with GLP-1, exenatide shares 53% of sequence homology (64), has a 20 to 30-fold optimized half-life and 5500 times greater potency in decreasing glycemic levels in T2DM patients (65). This significant difference in half-life time is due to the enzyme DPP-4 present in circulation, which cleaves GLP-1 in the second amino acid residue (alanine). In exenatide, this alanine was altered to a glycine

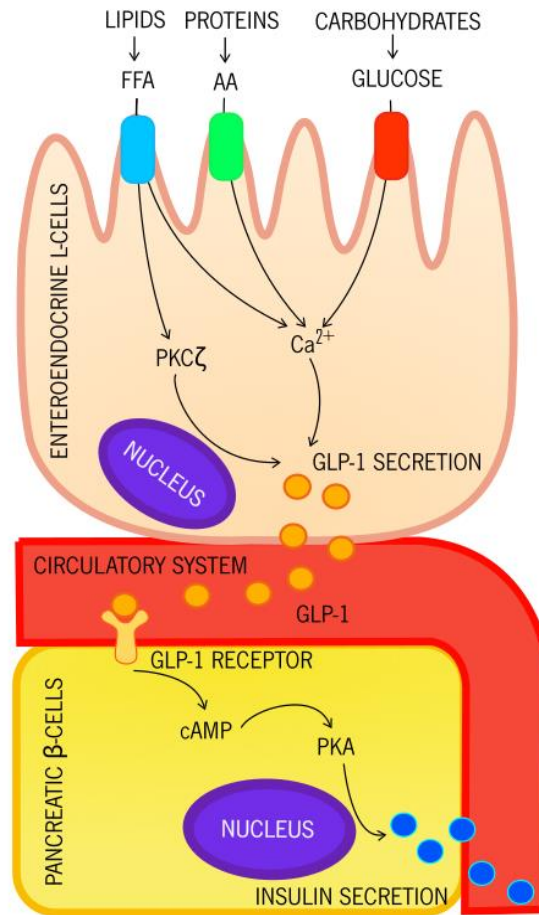


Figure 2: Scheme of the mechanism of glucagon-like peptide 1 (GLP-1) release from L-cells and insulin secretion pathway activation. In the presence of different nutrients, L-cells secrete GLP-1 through different metabolic pathways. After, entering in circulation, GLP-1 binds to its receptor on pancreatic β -cells and activate the pathway of insulin secretion. FFA, free fatty acids; AA, amino acids; PKC ζ , protein kinase C ζ ; cAMP, cyclic adenine monophosphate; PKA, protein kinase A.

residue (Figure 3) to enhance the biodisponibility of the drug (58). This GLP-1 receptor agonist is administered by subcutaneous injection in dosages of 5 μ g or 10 μ g of exenatide twice daily (66) or 2 mg once weekly and has a maximal concentration found in plasma around 90 pg/ml (for dosage of 5 μ g) or 210 pg/ml (for dosage of 10 μ g and 2mg) (67-69). Moreover, when is prescribed the long action exenatide microspheres, the drug diffusion lasts for approximately 2 weeks and drug clearance around 7 weeks since the injection (67).

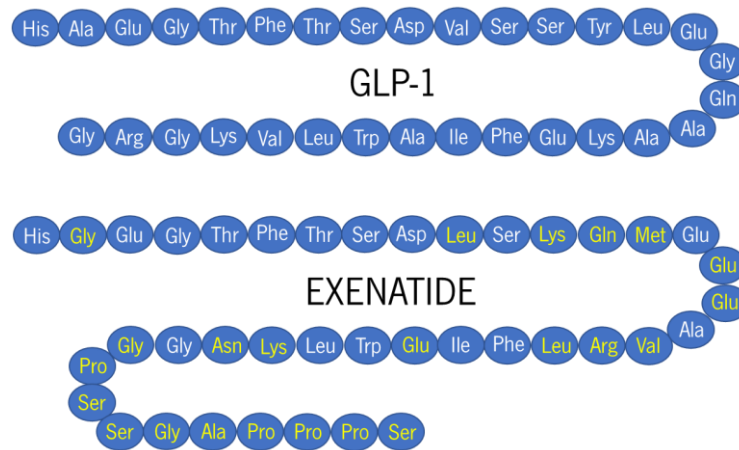


Figure 3: GLP-1 amino acid sequence and the differences in the sequence (amino acids in yellow) that allow exenatide increased half-life time.

When comparing the two major dosage schedules of exenatide treatment (exenatide 10 μ L twice daily or exenatide 2 mg once weekly), a study referred that exenatide 2 mg once weekly demonstrated the best results in lowering glycemic levels, and a significantly lower incidence in adverse effects in relation to the twice-daily dose in a 28-week long study (70). With this in mind, and together with a more convenient dosage schedule, we prioritized studies that evaluated the impact of the treatment of exenatide 2 mg once weekly. DURATION-8 clinical trial studied the effect of exenatide 2 mg once weekly on glycemic control, weight, and blood pressure in a similar manner to dapagliflozin treatment. The results showed that exenatide decreased the baseline score of HbA1c by 7% in 27% of the individuals. Some patients (14%) loss more than 5% of their total body mass and also decreased the systolic blood pressure by -1,2 mm Hg in the 28 weeks of the trial in the individuals treated with exenatide (44). In another study, exenatide 2 mg once weekly therapy was compared to a therapy of another GLP-1 receptor agonist (liraglutide) in a single daily administration of 1.8 mg. Exenatide was less efficient in regulating HbA1c score, weight loss and systolic blood pressure (-0,36 %; -0,90 kg; and -0,87 mm Hg, respectively), when compared with liraglutide therapy. Still, the occurrence of adverse effects on exenatide treated group was less common in almost all categories (71).

Metabolic diseases, such as DM and obesity, already entail cardiovascular complications and have been described that some antidiabetic drugs could enhance the probability of those complications (20). However, studies have suggested that this is not the case for exenatide. In fact, exenatide showed to decrease arterial stiffness in T2DM patients averaging 64 years of age and improved diastolic function in relation to the placebo group. Unfortunately, this did not result in a significant increase in exercise capacity of the patients. Still, exenatide showed to have a protective

effect on cardiac function, which is welcome on T2DM obese patients (72). On the other hand, GLP-1 receptor agonists have been known for causing side effects of gastrointestinal nature, such as moderate nausea, diarrhea, and vomiting. These adverse effects are dependent on the dosage and normally attenuate over time (73). This class of antihyperglycemic drugs has also been associated with higher risks of pancreatitis incidence and pancreatic and thyroid cancer, but no significant relation was found in relation to the treatment with other antidiabetic drugs (74). Another potential problem associated with this drug is the appearance of antibodies against exenatide in patients plasma, yet, no difference was shown in the glycemic control in patients with or without exenatide antibodies (58).

The anti-obesogenic properties present GLP-1 receptor agonists such as exenatide are another advantage for overweight T2DM patients. The weight loss is provoked by the ability of exenatide to slow gastric emptying, thus reducing intestinal nutrient absorption, as well as reducing glucose and lipids levels in postprandial plasma (75). Gastric emptying is slowed by the inhibition of small intestine motor function and gastric acid secretion interfering with nutrient absorption and decreasing appetite, possibly by an interaction between exenatide and the enteric nervous system (76) not yet fully understood. On the other hand, studies have shown that exenatide (0,05 µg/100 nL) injection on ventral tegmental area decreased palatable high-fat diet intake of Sprague-Dawley rats by decreasing the amount of food ingested per meal. The authors of this study stated that the activation of the GLP-1 receptor modulates the activity of the dopaminergic neurons in the ventral tegmental area. Thus, the injection of exenatide increased dopamine secretion, which is also associated with an anorectic effect (77).

1.5. Combined therapy of exenatide plus dapagliflozin

As mentioned above, dapagliflozin (10 mg once daily) plus exenatide (2 mg injection once weekly) combination treatment for obese patients with insulin resistance is being evaluated and compared with more standardized therapies (31). Is worth noticing that the combined therapy of both drugs seems complementary to each other on their effects in the body. Dapagliflozin can increase appetite yet, exenatide had shown to have anorectic effect in the patients. Exenatide stimulates insulin secretion in a direct way, while dapagliflozin does not interfere with hormone secretion in a direct manner. This, together with the fact that both have antidiabetic and anti-obesogenic properties, evidences that the combined therapy of these two pharmacological agents seem logical, promising and directed for T2DM obese patients. With this in mind, other clinical

trials are currently recruiting to further elucidate the effects of this combined therapy on human metabolism. For example, the influence of this combined therapy on food-related stimuli will be assessed in a phase IV randomized trial in obese T2DM patients (78). Despite that, both classes of drugs are considered safe and even seem to have some beneficial impacts on metabolism such as provoking weight loss with low risks of hypoglycemia and protective effects in heart and kidney tissue (79). In addition, other clinical trials with this therapy have already been performed and showed promising results.

In a recent study, Lundkvist and co-workers examined the anti-obesogenic potential of the combined treatment of dapagliflozin plus exenatide, on obese people without DM (80). Of 50 participants, 73.5% had prediabetes which is a major risk factor for DM and is characterized by impaired glucose metabolism but with HbA1c scores below the ones seen in DM (81). The results showed an average reduction on body weight of 4,13 Kg in relation to the placebo group, mainly in visceral adipose tissue, in a 24-week long study, as well as a reduction on prediabetes cases within the participants (34,8% versus 85% for the placebo group). The authors also proposed an additive effect in the combined therapy since the weight loss was greater than with each monotherapy (80). In another study, the same authors continued the project for 52 weeks and noticed that the decrease in body weight, prediabetes percentage, and systolic blood pressure were attenuated after the 24 weeks until 52-weeks of treatment. In the first 24 weeks, the average weight decreased 4,5 Kg, while in the next 28 weeks the difference was -1,2 Kg, of which 1,5 L were total adipose tissue volume. The proportion of individual in the study with prediabetes by the 52-week mark also increased by a thin margin of 0,5%. However, the systolic blood pressure decreased 12 mm Hg in the 52 weeks in relation to the baseline value. This shows that dapagliflozin plus exenatide improve multiple cardiometabolic risk factors, ideal in the treatment of metabolic diseases such as DM and obesity (82). Is also worth noticing that in this trial, as well as in the DURATION-8 trial, the subgroups with greater baseline values were the ones that also had a more accentuated decrease of body weight and systolic blood pressure (83). In DURATION-8 trial the safety of the administration of exenatide plus dapagliflozin was compared with that of exenatide plus placebo, and dapagliflozin plus placebo efficiency. In all groups the more common adverse effects were of gastrointestinal nature, varying from 19,9% (exenatide plus placebo) to 14,2% (dapagliflozin plus placebo) in incidence, with these adverse effects occurring in 17,7% of the individual in the combined treatment. In addition, not major hypoglycemic events occur in all trial

duration. A maximum of 0,4% of individual suffered cardiovascular problems (84) which confirms the notion that this therapy is relatively safe and without major side effects.

Tatarkiewicz and colleagues determined that exenatide (0,03 mg/kg/day) plus dapagliflozin (1 mg/kg/day) combined therapy optimized kidney function by reducing creatinine on plasma and improving glomerular filtration rate in obese diabetic mice (85). For the noted increase in cardioprotection, Ferrannini *et al*/ hypothesized that moderate but constant levels of ketones (β -hydroxybutyrate) in circulation are oxidized by the myocardium instead of fatty acids and this can yield better oxygen consumption, improving myocardium function. The hyperketonemia highlighted above can be observed in treatments with SGLT2 inhibitors, showing that this class of antihyperglycemic drugs can provide heart protection however, hyperketonemia can have serious implications (86). In a different study, Charokopou stated that treatment with dapagliflozin and exenatide has shown to be cost-effective (within established United Kingdom parameters) for individuals that made an unsuccessful treatment with metformin (87).

Although there is still a small number of authors evaluating the impact of this drug combination, all of them stated that dapagliflozin and exenatide in complementary treatment seem viable and have advantages for T2DM patients, especially in obese subjects. However, in all the studies, there were also some undesired side effects, especially of gastrointestinal nature. Scarce studies were published describing the effect of dapagliflozin or exenatide in male reproductive tissues (88) and none assessing the effect of combined therapy on spermatogenesis. The study of the impact of antidiabetic and anti-obesogenic drugs on male reproductive function is imperative once both classes of drugs modulate glucose metabolism, which is essential for normal spermatogenesis, hence for male fertility. The effect of other antidiabetic drugs, such as metformin, has already been studied and the results obtained showed that metformin seems to protect spermatogenesis on diabetic individuals. However, in healthy individuals, metformin seems to influence male fertility in a negative way (89, 90). Thus, it seems convenient to evaluate the effect of the *nove*/therapy on spermatogenesis and enlighten if this therapy can worsen or increase the fertility rates in males.

1.6. Male reproductive function and spermatogenesis

Male reproductive tract is constituted by the testes, efferent ducts, epididymis and vas deferens. These heterogeneous tissues are protected by the scrotum, which helps maintain the ideal temperature for the formation of gametes. Inside the scrotum resides the testes. Testes are the organ responsible for the creation of the microenvironment that allows the spermatogenesis

(91). These organs are encapsulated by the tunica albuginea, which forms septations separating the human testis into lobules. Besides coating the testes, tunica albuginea also regulates the interstitial pressure and the blood flow through the testes (92). The multiple conical lobules include interstitial tissue (tissue between the tubules) and coiled seminiferous tubules, being the later responsible for 60-80% of the total volume of the testis (93). The end of all seminiferous tubules converges to the rete testis, where the secretions of the tubules are collected and then sent through the epididymis tubule (94). Inside the seminiferous tubules, we can find the blood-testis barrier (BTB), which is formed by adjacent Sertoli cells. The interstitial tissue consists of Leydig cells, leukocyte, fibroblasts and blood vessels (95). Leydig cells are capable of performing steroidogenesis, being the ones responsible for testosterone synthesis, which is essential for the occurrence of spermatogenesis (96). It is worth noting that all processes of spermatogenesis occur inside the seminiferous tubules. Spermatozoa are then guided from rete testis into the efferent ductules, which lead into the epididymis. Finally, spermatozoa enter the vas deferens and are forced into the ejaculatory duct by peristaltic movements (97).

Spermatogenesis can be defined as a set of complex and coordinated processes that allow the transmission of genetic information to the next generation (98). About 40 millions of spermatozoa are formed daily in a fertile man and a complete spermatogenic cycle is concluded in 74-76 days (99). Four major phases are involved in the preparation of genetic information and each phase is controlled by endocrine, paracrine, and autocrine factors. The first phase (mitotic phase) involves a series of spermatogonia type A and type B stem cells divisions (100). Spermatogonium is a diploid cell that suffers mitotic divisions in the periphery of seminiferous tubules to originate spermatocytes and can be divided into three categories: type A Dark and type A Pale that does not exhibit heterochromatin; and its successor (type B) that presents packed chromatin (101). Spermatogonia type A Dark are the least differentiated from the stem cells and are capable of dividing into other spermatogonia type A Dark or differentiate into a type A Pale spermatogonium, which will start a new process of spermatogenesis. Then, the type A Pale is differentiated into type B spermatogonia giving place to the second phase (meiotic phase). This phase consists of the maturation of type B spermatogonia into pre-leptotene and then into leptotene spermatocytes. Spermatocyte goes through two consecutive meiotic cycles, where it is transformed into zygotene and pachytene spermatocytes (102). In these sub-phases, homologous recombination is established and after the meiotic processes, the result is a haploid round spermatid. The third phase (spermiogenesis), round spermatids suffer major morphologic changes, being transformed

into elongated spermatids (98), which will develop into highly specialized and functional sperm cells (102). Even though the genetic information is ready to be passed on in rounded spermatids, in natural conditions, it is not sufficient for oocyte fertilization (103). To accomplish natural fertilization, round spermatids have to suffer morphological changes in their transformation into spermatozoa, including condensation and elongation of the nucleus, development of the flagellum and acrosome, and loss of cytoplasm to facilitate the mobility and fertilization (104). The development of the acrosome has the purpose to support the final stage of fertilization. In the moment of contact between sperm cell and oocyte, the acrosome binds to the oocyte and secretes enzymes to allow the fecundation (105). Furthermore, the reorganization of mitochondria in the spermatozoa midpiece allows adenosine triphosphate (ATP) synthesis through the oxidative phosphorylation, which is essential for the flagellum movement (106). After this morphologic specialization, Sertoli cells release the fully developed spermatozoa (Figure 4) into the epididymis to continue the maturation, in a process called spermiogenesis, which is the fourth and final phase of spermatogenesis (104). In the epididymis, immature sperm cells suffer a change in their membrane proteomics providing them with motility and more fertilization competency however, this process is not yet fully understood (107). In addition, the spermatozoa are stored in the epididymis tail before entering the vas deferens to be ejaculated. Mobility and mitochondrial function in spermatozoa are essential to male fertility (108). All these processes take place inside

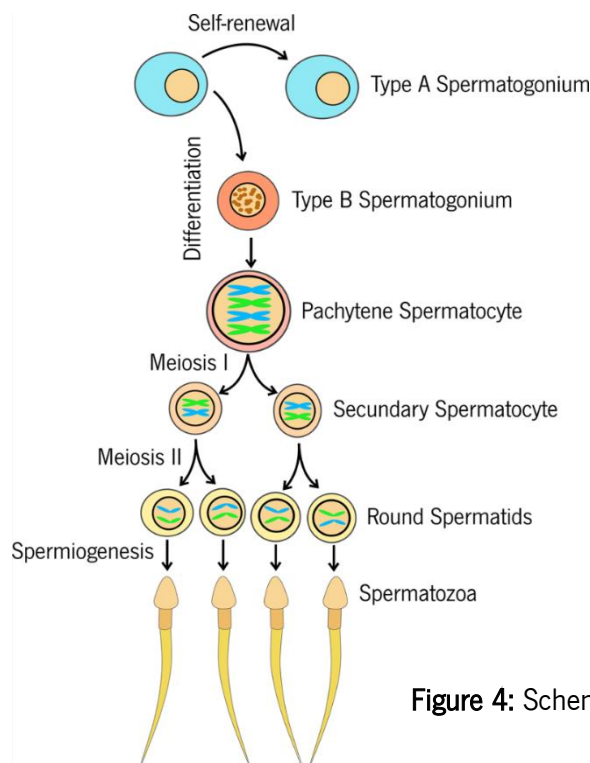


Figure 4: Scheme of the different stages of spermatogenesis.

the seminiferous tubules that provide the conditions to enhance all phases of spermatogenesis. These conditions are ensured by Sertoli cells, which form the BTB.

The BTB supports the development of the germ cells. BTB can be divided into basal and apical section (109). Cells in different phases of spermatogenesis can be found in different sections of the barrier. While the basal section supports the mitosis and differentiation of spermatogonia, which is more exposed to the circulatory and lymphatic systems, the apical section supports the meiosis and the morphologic specialization of spermatocytes and spermatids, respectively (110). This process takes place away from circulatory and lymphatic systems creating an immune free micro-environment (Figure 5). This physical barrier together with immuno-suppressors creates a privileged environment able to protect the haploid cells from the immune system that could identify them as strange cells and destroy them (111).

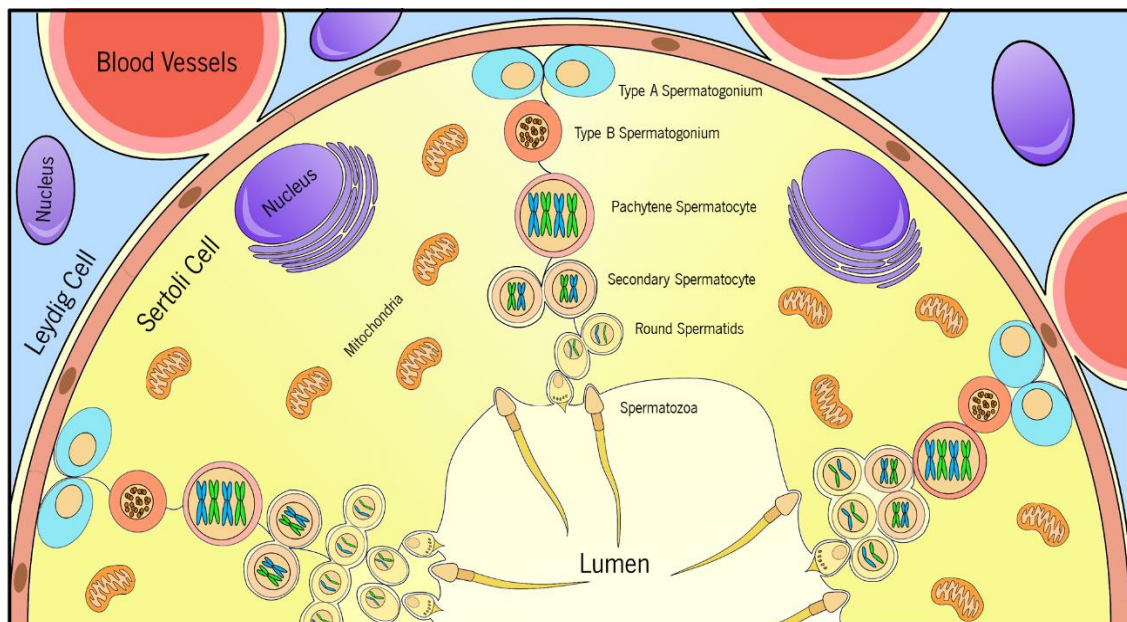


Figure 5: Schematic representation of the blood-testis barrier (BTB) and the different stages of spermatogenesis.

BTB (or Sertoli cell barrier) is primarily formed by Sertoli cells, with small contributions by other cells like peritubular myoid and endothelial cells (112). Specialized junctions, such as tight junctions, basal ectoplasmic specializations, gap junctions, basal tubular complex, and desmosome-like junctions can be found between adjacent Sertoli cells forming the barrier (112, 113). Tight junctions are known to allow the transport of biomolecules depending on the size of its pores (113). Ectoplasmic specializations are highly packed filaments of actin and can be found between spermatids and Sertoli cells (apical section), which function to anchor the spermatids

during maturation. Ectoplasmic specializations are also found together with gap junctions, tight junctions, and desmosomes-like junctions to create the already mentioned immune free environment unique to BTB (114). For the occurrence of the different phases of spermatogenesis, the BTB structure must suffer some changes to allow the developing germ cells to transit from basal to apical sections, to be released as viable spermatozoa (110). It will also need the decoupling of BTB in the spermiation (115). All these processes take place without compromising the structure and function of the BTB (110).

BTB is also impermeable to several drugs, preventing their action in the developing germ cells. Thus, possibly interfering in the treatment of some infertility conditions, decreasing drug efficiency and detain the development of contraceptive pills for men.

1.6.1. Sertoli cells and spermatogenesis

Spermatogenesis depends on Sertoli cells which occupy up to 40% of the volume of seminiferous epithelium in humans (116). Hence, a subnormal number of Sertoli cells is related to subnormal testis size and a diminished function and efficiency of Sertoli cells will result in subnormal number of viable germ cells (117). Sertoli cells are responsible for the support and nourish of all the cells during the processes of spermatogenesis. One man at reproductive age has about 800 to 1200 million of Sertoli cells (118). These cells are also involved in the transportation of spermatids into the lumen of seminiferous tubules and are the mediators and producers of endocrine and paracrine factors, which modulate the development of germ cells.

Sertoli cells are able to supply the germ cells with their nutritional requirements for proper development. In the different stages of spermatogenesis, the metabolism of the developing germ cells will differ (119). In more detail, the main pathway utilized by spermatogonia to obtain energy is glycolysis and pentose phosphate pathway. Contrastingly, spermatocytes and spermatids obtain their energy mostly from the substrates like lactate and pyruvate. Finally, spermatozoa utilizes glucose/fructose to produce ATP in a similar manner as spermatogonia (120).

Hence, for spermatids to develop properly, Sertoli cells metabolism need to perform a “Warburg-like” metabolism to supply the developing germ cells with their required substrates (121), establishing what has been named as the testicular metabolic cooperation. These somatic cells use a less cost-effective metabolism by using the glycolytic pathway which transforms a glucose molecule into 4 ATPs molecules and lactate, instead of directing the resultant pyruvate through the TCA cycle which would result into a max of 36 ATPs molecules (121). Thus, β -oxidation

of lipids followed by TCA cycle is the main metabolic pathway used by Sertoli cells to produce ATP to itself once almost all glucose is used to produce lactate, which is the preferred substrate for energy synthesis in developing germ cells (119).

To internalize the glucose, cells rely on two different major families of membrane glucose transporters, GLUTs, and SGLTs. SGLTs expression has already been identified on testes tissue (122) and multiple isoforms of GLUTs were already described on Sertoli cells, namely: GLUT1 (123), GLUT2 (124), and GLUT3 (125). Once the glucose molecule enters Sertoli cell cytoplasm, the majority of it is metabolized into two molecules of pyruvate through glycolysis (119), followed by the metabolization (approximately 95%) of pyruvate into lactate for the developing germ cells. The lactate is secreted through specific monocarboxylate transporters (MCTs) to germ cells that use it as their main energy source. However, Sertoli cells can utilize other substrates in a glucose-free environment to continue the production of lactate, energy, and ATP (119). In fact, to maintain the high levels of ATP and lactate needed to ensure spermatogenesis in the absence of glucose, Sertoli cells can metabolize lipids and glycogen (119). Fatty acids and amino acids are metabolized into energy by the TCA cycle, and the majority of the resulting ketone bodies are also transformed into energy by this metabolic pathway (112). Moreover, glycogen can also be mobilized and undergo glycolysis originating pyruvate, which is used to produce ATP through TCA cycle (119) or is metabolized into lactate by lactate dehydrogenase (LDH) (126). The developing germ cells consume the lactate with specialized transporters in the plasmatic membrane (MCT 2), which internalize the lactate into the cytoplasm. Therein, a specific LDH isoform (LDH-C4) metabolizes lactate into pyruvate to be transferred to the mitochondrion to start a new TCA cycle (via acetyl coenzyme A) (Figure 6) to produce ATP (127). Besides being the preferred energy source for the developing germ cells, lactate can also modulate gene expression (126), modify the expression of important proteins for spermiogenesis in spermatids (128), and has been reported to have anti-apoptotic properties for the developing germ cells (129). To support, nourish, and facilitate the cooperation with all the developing germ cells, Sertoli cells cytoplasm is stretched enough to reach fully developed germ cells. This allows direct communication and better development management of germ cell development (130).

Sertoli cells also produce and secrete glycoproteins with different functionalities. The ones with the highest secretion rate are transporting enzymes, such as transferrin and androgen binding protein. Proteases and protease inhibitors, important for the morphologic changes of germ cells, as well as insulin-like growth factor (131). This cellular type also secretes into circulation inhibin B

(132). This protein is a marker of Sertoli cell activity and is important in the regulation of gonadotrophic hormones.

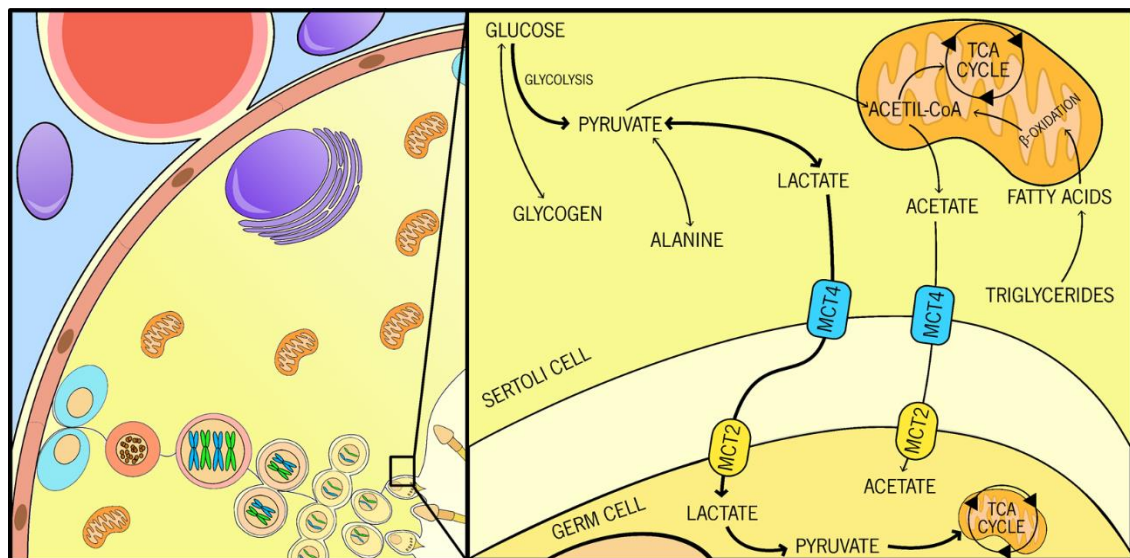


Figure 6: Mechanisms of Sertoli cells glucose metabolism and its metabolic cooperation with the germ cells. Sertoli cells can use multiple substrates to produce energy, but the main substrate used is glucose. Glycolysis uses glucose to produce pyruvate that can be transformed in lactate, alanine or in acetyl-CoA to start the tricarboxylic acid (TCA) cycle. Lactate and acetate are transported out of Sertoli cells and into the germ cells cytoplasm by monocarboxylate transporters (MCT4 and MCT2, respectively). Thicker arrow indicates prioritized pathway by Sertoli cells and germ cells.

Spermatogenesis and Sertoli cell metabolism are tightly regulated by the hypothalamus-pituitary-testis axis. Specialized neurons in the hypothalamus area create secretion impulses of gonadotropin-releasing hormone (GnRH), which influences the anterior pituitary to produce follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (133). Both hormones are secreted by the pituitary and have an impact on spermatogenesis (134). Sertoli cells are regulated by FSH and Leydig cells by LH (135) nevertheless, both cell types regulate each other activity (136). LH influences the secretion of androgens by Leydig cells to control spermatogenesis (135). Without testosterone or androgen receptors in Sertoli cells, spermatogenesis is not possible due to the inability of sperm cells develop past the meiosis phase, unsuccessful spermiation, and BTB structural problems (137). Moreover, testosterone can be also converted into estrogens by aromatase present in Leydig cells. It has been shown that this hormone has an important role in the regulation of testosterone and LH secretion (138). On the other hand, FSH plays a role in Sertoli cells proliferation during fetus development and also regulates Sertoli cells differentiation

after puberty (139). Androgens and estrogens, such as 5 α -dihydrotestosterone and 17 β -estradiol, respectively, also affect Sertoli cells metabolism. It has been reported that these hormones affect the glycolytic flux and the production of acetate (via acetyl coenzyme A) in a different manner. While 5 α -dihydrotestosterone decreases acetate production, 17 β -estradiol increases acetate production (140). Acetate is an important substrate for Sertoli cells once it is described that is involved in the formation of lipids by the Sertoli cells for the formation of new sperm cells (141).

In sum, Sertoli cell metabolism is tightly regulated by endocrine, paracrine and autocrine factors, and these will contribute ultimately to the formation of new sperm cells and hence to the establishment of male reproductive potential. Metabolic diseases such as obesity and DM and what they entail are able to dysregulate the balance of this system and impact male fertility. In the next chapter, the main factors that negatively influence male fertility will be further discussed.

1.7. Negative influence of metabolic diseases on male fertility

Infertility can be defined as a disease of the reproductive system which prevents a couple of achieving clinical pregnancy and can be diagnosed after one year of regular sexual intercourse without contraceptive measures (142). Around the globe, approximately one in six couples encounter some form of infertility (143). In western countries, about half of those cases, the hurdle comes from the male part, either by himself or in combination with the female partner (144).

Alterations in three major features of the semen are frequently associated with male infertility: the concentration (145), the motility, and the morphology of spermatozoa (146). Obese males are more susceptible to these alterations due to anatomical features, dysfunctional metabolism and impaired hormone secretion (147, 148). An obese male has more fat deposits in suprapubic and upper thigh regions which can difficult testicular cooling and so, obese male testis are more likely to have a superior scrotal temperature when compared with a male with normal BMI (148). This factor can cause heat stress on the testis and influence gene expression of germ cells as well as DNA repair (149), production of free reactive oxygen species (ROS), and apoptosis (150). The changes in gene expression profile and DNA repair can ultimately lead to a different proteomic profile in the spermatocyte. Some of the up-regulated genes are translated into proteins with specific roles in cellular apoptosis. However, a study described that this up-regulation stops after the testes reach normal temperature for a few hours to prevent the elimination of all sperm cells, in mice models (151). In a different manner, the increased ROS levels also increase apoptosis risk

by oxidation of DNA and lipids (150). Moreover, DNA polymerase beta and DNA ligase III have reduced expression in hyperthermia, which compromises DNA repair (150).

In another perspective, obese males excessive adipose tissue transforms androgens into estrogens via aromatization at a higher rate than lean males. This process, similar to the one that occurs in the testis, often results in free testosterone reduction and the dysregulation of the hypothalamic-pituitary negative feedback, which compromises spermatogenesis (152). Thus, testosterone deficiency can result in diminished testes volume and functionality (147). In addition, the continuous formation of new adipose tissue characteristic of obese males enhances the release of cytokines (ILs and TNF- α), resulting in a constant inflammation status, which recruits leucocytes that enhance the production of ROS (148). This chronic inflammatory status and the secretion of TNF- α observed on long term obese males also has a negative impact on rodent Leydig cells steroidogenesis (153).

White adipose tissue in excess will secrete an abnormal quantity of leptin, which is the principal hormone secreted by white adipose tissue (154). It was described that this hormone plays a role in the regulation of appetite, glucose metabolism, and has an impact on reproductive and immune systems (155). Reports described that leptin is associated with increased LDH activity and GLUT2 expression levels in human Sertoli cells (156). This metabolic effect was corroborated by another study that stated that leptin can modulate Sertoli cells metabolism by conditioning cellular glucose transport (141) and the production of acetate in human Sertoli cells (141). In addition, the excess of adipocytes and consequent increase of leptin secretion was described to diminish free androgen levels (157) and consequently increase the apoptosis in germ cells (158). The dysfunctional androgen response is due to a subnormal transition of 17-OH-progesterone to testosterone (157) and the increase in apoptosis is due to the production of ROS and TNF- α . In fact, increased ROS levels can still be found in seminal fluid (152).

Ghrelin is another hormone which secretions are impacted by the BMI of the person. A higher BMI usually indicates diminished ghrelin levels. This 28-amino acid peptide is secreted by the stomach and is known to promote food consumption in humans. Its secretion is more accentuated in the fasting state when the person is in food deprivation. In fact, ghrelin has an important role in regulating energy expenditure, which directly and indirectly modulates Sertoli cell metabolism and spermatogenesis (159). Tena-Sempere and co-workers studied the effect of ghrelin on testosterone secretion by rat Leydig cells. This group of work discovered that ghrelin at concentrations of 10^{-7} M had an inhibitory role on testosterone secretion, by decreasing the gene expression of proteins

responsible for steroidogenesis (160). A distinct study described that ghrelin could upregulate apoptosis in germ cells. This increase in apoptosis rates is associated with the increased translation of apoptosis regulator Bcl-2-associated X protein and decrease in the expression of the proliferating cell nuclear antigen (161).

On the other hand, a defective insulin secretion also impacts Sertoli cell metabolism (162). Oliveira and co-workers reported that Sertoli cells deprived of insulin showed decreased glucose and pyruvate consumption. They also described that when these cells were subjected to longer periods of insulin deprivation, lactate production was also affected (163). These results were explained by the ability of insulin to enhance some GLUTs and MCTs activity, modulating glucose and lactate transport, respectively. In a different study performed by the same group, insulin deprivation diminished acetate production by Sertoli cells. Thus, insulin deprivation affects Sertoli cells metabolism and energy synthesis, which could ultimately influence spermatogenesis (140). Furthermore, Sertoli cells apoptotic pathways dependent on caspases. This class of proteins is enhanced under insulin absence, promoting apoptosis of Sertoli cells (164) and concurrently diminishing germ cell numbers (132).

1.8. Exenatide and Dapagliflozin impact on male fertility

Exenatide and dapagliflozin, like the other pharmacological agents prescribed for the treatment of DM and obesity, modulate energy expenditure and the glucose metabolism in multiple tissues and cellular systems. Still, almost no data is available regarding the effects of each of these two drugs on male fertility. In addition, no study has been made to evaluate the effect of the combined therapy of exenatide plus dapagliflozin on male reproductive function.

Unfortunately, not only the impact of exenatide and GLP-1 receptors agonists on male fertility have been overlooked, but also that of the incretin GLP-1. One of the few studies available indicated that GLP-1 interfered with the hypothalamic-pituitary-gonadal axis, which resulted in decreased testosterone secretion (165). This reduced testosterone production can result in arrested germ cell development. However, in a more recent study, exenatide treatment (1 nmol/kg) showed to protect spermatogenesis from ROS by increasing antioxidant enzyme activity (166). Furthermore, exenatide showed the ability to increase testosterone levels in aging mice, expanding their fertile period. In another study, exenatide influence on testis inflammation of induced obese mice was accessed (167). In this study, exenatide seemed to decrease the expression of pro-inflammatory cytokines. However, these results were attributed to the ability of exenatide to reduce weight, hence

reducing inflammation (167). Another example of this indirect effect was described by Topyildiz and coworkers. In their 2016 study, Topyildiz workgroup described that exenatide decreased ghrelin levels in circulation in individuals with T2DM to values near those seen in healthy individuals after a meal (27, 168).

In regarding Sertoli cells metabolism, a study published by our laboratory described that, when these cells were exposed to 1 nM of GLP-1, glucose consumption was decreased. An increase in lactate production was also noticed when the Sertoli cells were exposed to either 0.01 or 1 nM of GLP-1 (61). Being lactate a critical substrate for the proper progress of spermatogenesis and having anti-apoptotic properties for the developing germ cells (129), it is possible that this metabolic change provoked by GLP-1 enhanced spermatogenesis. In addition, 1 nM of GLP-1 also increased acetate secretion that has been described as an important substrate for the formation of lipids for the developing germ cells (141).

In the case of SGLT2 inhibitors and dapagliflozin, the available published work accessing the effect of this class of antidiabetic drug on any type of tissue is scarce, even more addressing its effect on male fertility. This could be explained by the fact that dapagliflozin and the inhibition of the SGLT2 is a novel therapy and the fact that is also considered safe. Nevertheless, the more common side effect in dapagliflozin treatment is the urinary tract infection caused by the excess of glucose in urine (52). This factor can compromise male fertility by pathogenic infection present on semen (169), namely *Candida* (170). Interestingly, individuals subjected to treatment with dapagliflozin (10mg per day) have increased values of ketones in circulation (171). In fact, increased seminal plasma lipid oxidation is considered to be an indicator for sperm malfunction and subfertility (172). However, to the best of our knowledge, no data exists that links the circulating plasmatic and the seminal plasmatic fluid ketone levels.

Hopefully, this study will bring more insight into the effect of this class of drugs on Sertoli cells and on their impact on male fertility. It will be interesting to comprehend if the blockage of SGLT2 will alter glucose metabolism by Sertoli cells. In fact, understanding the influence that both exenatide and dapagliflozin will have on these cells, if any, will make us understand more about Sertoli cell glucose metabolism and the way of action of the pharmacological agents.

Chapter II

OBJECTIVES

2. OBJECTIVES

The treatment of metabolic diseases, such as T2DM and obesity, involves the administration of drugs, like dapagliflozin and exenatide, that are prescribed to assist in the regulation of glucose homeodynamics and to lessen the comorbidities associated with these pathological conditions. Glucose metabolism is essential for the normal development of spermatogenesis. However, no data is available on the impact of the antidiabetic drugs dapagliflozin and exenatide on male fertility and how they influence Sertoli cells metabolism and their function in nursing and supporting the spermatogenesis. Furthermore, the influence of the combined therapy of dapagliflozin and exenatide on the male reproductive tract and particularly on Sertoli cells is even more scarce.

The aim of this project is to study the influence of the exposure of Sertoli cells to dapagliflozin and/or exenatide, with a particular focus on their metabolism and viability. The target of the study will be the glucose metabolism in Sertoli cells when the antidiabetic and anti-obesogenic drugs are present in the medium. Therefore, we will focus on the following objectives:

1. Determine the expression of SGLT2 and GLP-1 receptor in murine Sertoli cells (TM4 cell line);
2. Evaluation of the cytotoxic profile of dapagliflozin and/or exenatide in TM4 cell line;
3. Determine Sertoli cells (TM4 cell line) glycolytic profile after the exposure to dapagliflozin and/or exenatide;
4. Evaluate of the expression of key metabolic enzymes and transporters in Sertoli cells (TM4 cell line) after the exposure to dapagliflozin and/or exenatide;
5. Evaluate the oxidative status of Sertoli cells (TM4 cell line) after the exposure to dapagliflozin and/or exenatide;
6. Determine Sertoli cells (TM4 cell line) glycogenic and lipidic reserves after the exposure to dapagliflozin and/or exenatide.

Chapter III

METHODS

3. METHODS

3.1. Chemicals

TM4 Sertoli cell line was obtained from ATCC (Manassas, VA, USA). Fetal Bovine Serum (FBS) was purchased from Biochorm AG (Berlin, Germany). Insulin-transferrin-sodium selenite supplement and gentamicin were obtained from Life Technologies (Carlsbad, CA, USA). Dapagliflozin and exenatide were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Testosterone was purchased from Honeywell Fluka (Charlotte, NC, USA). LDH-Cytox™ Assay Kit was purchased from BioLegend (San Diego, CL, USA). MTT was obtained from Amresco (Parkway Solon, OH, USA). Sulforhodamine B (SRB) was purchased from Biotum (Hayward, CA, USA). JC-1 dye was purchased from Life Technologies (Gaithersburg, MD, USA). BCA Protein Assay kit was purchased from Thermo Scientific (Waltham, MA, USA). NZY M-MuLV Reverse transcriptase, random hexamer primers, NZTaq 2x Green Master Mix, dNTPs, Reaction Buffer, and Greensafe were all obtained from NZYTech (Lisbon, Portugal). Dulbecco's Modified Eagle Medium Ham's Nutrient Mixture F12 (DMEM: Ham's F12), DNase, trypsin-ethylenediaminetetraacetic acid (EDTA), Oil Red O Staining and other chemicals were all purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

3.2. Mouse Sertoli cell line TM4 culture

TM4 Sertoli cell line is derived from immature BALB/c mouse testes. The cell line maintains some original key features of normal Sertoli cells, such as the need for testosterone and FSH in the culture medium for normal function (173). The cells were grown in 75 cm² T-flasks with Sertoli cell culture medium (DMEM: Ham's F-12 1:1, containing 15mM HEPES, 50 U/ml penicillin, 50 mg/ml streptomycin sulphate, 0,5 mg/ml fungizone, 50 mg/ml gentamicin, and 10% FBS inactivated by heat). They were handled in a laminar flow chamber and incubated at 37 °C with a 5% CO₂ humidified atmosphere.

3.3. Experimental groups

To evaluate the potential effect of each pharmacological agent on TM4 Sertoli cells metabolism, 10 experimental groups were designed. Cells were seeded in 10 cm plates and then treated in the absence (control group) or in the presence of sub-pharmacologic, pharmacologic, and supra-pharmacologic concentrations of dapagliflozin (50 nM, 500 nM, and 5000 nM, respectively) or exenatide (2.4 pM, 24 pM, and 240 pM, respectively) with a duration of 24 hours. These

concentrations were chosen considering dapagliflozin and exenatide pharmacological concentration in plasma (41, 67). For the study of the effects of combined therapy, the pharmacological concentrations of dapagliflozin (500 nM) and exenatide (25 pM) were added simultaneously in the medium. Pharmacological agents were added to ITS treatment medium (DMEM: Hams's F-12 1:1, containing 15 mM HEPES, 50 U/ml penicillin, 50 mg/ml streptomycin sulphate, 0,5 mg/ml fungizone, 50 mg/ml gentamicin, 100 U/L FSH (Puregon™), 5 µmol/L testosterone, and insulin-transferrin-sodium selenite supplement) (insulin 10 µg/mL; transferrin 0,55 µg/mL; sodium selenite 0,0067 µg/mL) and stored at -20 °C. After the incubation time, extracellular media was collected for posterior analysis (¹H-NMR) and the cells were removed from the plates with a trypsin-EDTA solution. After homogenization, the cells were counted with a Neubauer chamber and collected for protein extraction and glycogen quantification.

3.4. Evaluation of cytotoxic profile of the compounds

TM4 Sertoli cells were seeded in 24-well and 48-well plates after 24 h of exposure to the treatment mediums of exenatide, dapagliflozin, and exenatide plus dapagliflozin. For the duration of the treatment, the cells stayed incubated at 37° C, with 5% CO₂ humified atmosphere. To evaluate the cytotoxicity of the different treatments were used three different methods: SRB assay, MTT assay, and LDH release assay.

3.4.1. SRB cytotoxic assay

SRB is an aminoxanthene dye with pink color that binds to basic amino-acids residues in acidic conditions. This technique allows the quantification of cell mass due to its direct proportion with the amount of dye extracted from stained cells (174). This allows to compared cell proliferation when treated with drugs and assess their cytotoxicity. Cell line TM4 proliferation was evaluated by the colorimetric SRB assay when treated with exenatide and dapagliflozin as well as their combined treatment, with the concentrations in study. In brief, cells were cultured in a 24-wells plate and treated with the concentrations of exenatide, dapagliflozin, and exenatide plus dapagliflozin. After the 24h of treatment, the treatment medium was removed, and the cells were washed with phosphate-buffered saline (PBS). Then, cells were fixed with a solution of 1% acetic acid in 99% of methanol for at least 1h at -20° C followed by the incubation with a solution of SRB at 0.05% prepared with 1% of acetic acid in water, for 1h at 37° C. After being washed with 1% of acetic acid in water, the dye was removed from the cells with a 10 mM Tris solution with pH 10 and its optical

density was determined at 490 nm using the BioTek Synergy HT (BioTek, Winooski, VT, U.S.A). Resulting values were divided by the mean of the control group and expressed in fold variation versus the control group.

3.4.2. MTT cytotoxic assay

MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) is used to measure metabolic cell viability by the evaluation of mitochondrial viability. This is possible by the conversion of tetrazolium compound into formazan. The first compound is soluble in water, but it is converted by dehydrogenases present in the mitochondria, into insoluble crystals (formazan). Thus, the greater conversion will mean greater viability, and that can be measured by the change of color from yellow (MTT) to purple (formazan crystals) through spectrometry (175).

After the cells were seeded and treated in a 48-wells plate, the treatment medium was removed and replaced with 500 μ L Sertoli cell medium plus 50 μ L of MTT solution at 5 mg/mL and were incubated for 3h at 37° C protected from light. After incubation, the medium was removed and 250 μ L of dimethyl sulfoxide was added to dissolve the MTT crystals. Then, 50 μ L of the crystal solution was transferred to a 96-wells plate and diluted in 50 μ L of dimethyl sulfoxide. The absorbance was measured at 570 nm and 655 nm through a BioTek Synergy HT (BioTek, Winooski, VT, U.S.A). Resulting values were divided by the mean of the control group and expressed in fold variation versus the control group.

3.4.3. LDH release assay

LDH assay allows evaluating plasma membrane integrity. LDH is an intracytoplasmic enzyme and if this enzyme is encountered in the extracellular media can be assumed that the plasmatic membrane is damaged (176). LDH-Cytox™ Assay Kit was used for the quantification of LDH in the extracellular medium according to the manufacturer's instructions. In brief, Sertoli cells were incubated with the different treatments for 24h in 48-well plates. After the treatment, 50 μ L of the extracellular medium were transferred to 96-well plates and added 50 μ L of Reaction Mix. Then, the plates were incubated in the dark for 30 minutes at room temperature. At the end of the incubation, the reaction was stopped with the STOP Solution and the absorbance was measured at 490 nm using BioTek Synergy HT (BioTek, Winooski, VT, U.S.A). Resulting values were divided by the mean of the control group and expressed in fold variation versus the control group.

3.5. Protein extraction and quantification

TM4 Sertoli cells total protein was extracted using radioimmunoprecipitation assay (RIPA) buffer formed with 0,5% deoxycholate, 0,1% sodium dodecyl sulfate dissolved in PBS. This solution was then complemented with 1% of proteases inhibitors cocktail and sodium orthovanadate at approximately 10 mM. At that point, 50 μ L of RIPA buffer was added to the cell homogenate and suffered 20 minutes of agitation at 4 °C. Then, samples were centrifuged for 20 minutes at 14000 g at 4 °C. Protein quantification was performed using the Pierce™ Microplate BCA Protein Assay Kit according to the manufacturer's instructions. Calibration line was calculated by using different bovine serum albumin concentrations and samples absorbance was measured at 560 nm using BioTek Synergy HT (BioTek, Winooski, VT, U.S.A).

3.6. Intracellular LDH activity assay

Intracellular LDH activity was determined using the same commercial kit used on LDH release assay. The protocol was performed following the instructions of the manufactures of LDH-Cytotox™ Assay Kit. Briefly, on the same day that the proteins were extracted from the cellular pellets, 5 μ g of protein were diluted in PBS to make up to 50 μ L. To this was added 50 μ L of LDH assay substrate, then all the samples were incubated at 37 °C in a dark environment for approximately 10 minutes. The plate had to be constantly checked to avoid absorbances above 1. After this, the absorbance of the samples was measured at 490 nm using the BioTek Synergy HT (BioTek, Winooski, VT, U.S.A). The time from the addition of LDH assay substrate until absorbance reading was measured to calculate LDH enzymatic activities in units per milligram of protein, using the molar extinction factor (ϵ).

3.7. Nuclear Magnetic Resonance spectroscopy

Nuclear magnetic resonance (NMR) allows the study of the metabolites in certain solution by the interpretation of the ^1H -NMR spectra (177). The procedure used to obtain the spectra was similar to the one described by Alves and collaborators in 2013 (178). Briefly, samples of 220 μ L were used to obtain the spectra with a Bruker Avance 600 MHz spectrometer (Bruker Biospin, Karlsruhe, Germany) equipped with a 3-mm Indirect Detection probe (Varian, Inc., Palo Alto, CA, USA) in a magnetic field of 14,1 T at 25 °C. Sodium fumarate with a final concentration of approximately 2 mM was used as an internal reference (singlet, 6,50 p.p.m.) to quantify the diverse metabolites in the extracellular medium: H1- α glucose (duplet, 5,22 p.p.m.), glutamine (multiplet,

2,44 p.p.m.), pyruvate (singlet, 2,35 p.p.m.), acetate (singlet, 1,9 p.p.m.), alanine (duplet, 1,45 p.p.m.), and lactate (singlet, 1,33 p.p.m.). Spectra were manually phased, baseline corrected, followed by the quantification of the relative areas of ^1H -NMR resonances using the curve fitting routine in the NUTSpro spectral analysis program (Acorn NMR, Inc., Fremont, CA, USA). Results were normalized in relation to the quantities of the metabolites on the medium before incubation and in relation to the number of cells.

3.8. Reverse Transcriptase Polymerase Chain Reaction

The tRNA obtained from each sample was reversely transcribed in a mixture containing 2,5 μL of Random Hexamer primers (50 ng/ μL), 1 μL of dNTPs (10 mM), 1 μg of tRNA, and sterile H_2O to make up 17 μL . The mixture was initially incubated for 5 minutes at 65 $^\circ\text{C}$. After this, 1 μL of NZY M-MuLV Reverse transcriptase and 2 μL of Reaction Buffer were added and incubated sequentially at 25 $^\circ\text{C}$ for 10 minutes, 37 $^\circ\text{C}$ for 50 minutes and 70 $^\circ\text{C}$ for 15 minutes. The resulting cDNA was used with exon-exon spanning primer sets to amplify SGLT2 and GLP-1 receptor cDNA fragments. Polymerase chain reaction (PCR) was performed using 1 μL of cDNA in 12,5 μL of the final volume of a mixture which contained 6,25 μL of NZYTaq Green Master Mix 2x, 1 μM of each primer and sterile H_2O . Optimal annealing temperature and optimal number of cycles required for exponential amplification were discovered for each primer using standard procedures (Table 1). Then, samples were run in 1 % agarose gel electrophoresis with 2 μL of Greensafe in 200 mL, during approximately 30 minutes at 120 V. Together with the samples, a DNA ladder VI was run in the gel for size comparison of the products. Agarose gel was visualized using a Bio-Rad QuemiDoc XRS (Bio-Rad, Hemel Hempstead, UK).

Table 1: Genes, oligonucleotide sequence and respective conditions for polymerase chain reaction amplification of GLP1-receptor and SGLT2 genes.

Gene	Sequence (5'-3')	AT ($^\circ\text{C}$)	Amplicon (bp)	Cycles
GLP-1 receptor NM_021332.2	Sence: GATGCTGCCCTCAAGTGGAT Antisence: TAACGAACAGCAGCGGAACT	61	253	37
SGLT2 NM_133254.3	Sence: ATGGAGCAACACGTAGAGGC Antisence: ATGACCAGCAGGAAATAGGCA	63	104	35

3.9. Western Blot

Western Blot is a technique that allows the detection of a target protein in a complex mixture (179). Western Blot procedure was performed as previously described by Alves and collaborators in 2011 (180). In brief, 20 µg of protein samples were incubated for 10 minutes at 37 °C. After this, proteins were fractionated by weight in a 15% polyacrylamide gels with 0,75 mm width and the electrophoresis was performed during approximately 120 minutes at 100 V. Then, proteins were electrotransferred from the gel into a polyvinylidene difluoride membrane with a Trans-Blot® Turbo™ Transfer System (Bio-Rad, Hemel Hempstead, UK) for 7 minutes at 1.3 mA and 25 V. To ensure protein transfer, the membranes were incubated with Ponceau S staining solution (NZYtech, Lisbon, Portugal) and total protein was detected with Bio-Rad QuemiDoc XRS (Bio-Rad, Hemel Hempstead, UK) and calculated with Image Lab™ software (BioRad, Hemel Hempstead, UK). The membrane was then blocked for 90 minutes in a 5% non-fat milk solution of tris-buffered saline solution at room temperature. After this, the membranes were incubated overnight at 4 °C with goat anti-LDH (1:10000, Abcam, Cambridge, UK), or goat anti-GLUT2 (1:5000, Abcam, Cambridge, UK), or goat anti-MCT4 (1:5000, Abcam, Cambridge, UK). The immune-reactive proteins were detected with goat anti-rabbit IgG-HRP (1:5000, Merck Millipore, Burlington, MA, USA). Membranes were then incubated with WesternBright™ ECL Chemiluminescent HRP Substrate (Advansta, Menlo Park, CA, USA) following manufactures instructions. Briefly, we mixed WesternBright™ ECL components 1:1 to make up 4 mL per membrane and incubate the membranes for 2 minutes. Finally, the membranes were read with Bio-Rad QuemiDoc XRS (Bio-Rad, Hemel Hempstead, UK). Protein band was quantified using Image Lab™ standard procedures. Band density was divided by total protein previously acquired and normalized with control group value.

3.10. Colorimetric Method for Glycogen Quantification

Quantification of glycogen was performed using the colorimetric methodology described by Dubois and coworkers (181) with some optimizations for TM4 Sertoli cells and the scale of its glycogen storage. Briefly, 50 µL solution of cellular pellet from each condition were treated with 250 µL of KOH at 0,5 M under agitation. The solution was kept at 4 °C for 30 minutes. At the 15-minute mark of this incubation, the cells were sonicated for 1 minute. After this, was added 150 µL of H₂O to the samples and then suffered centrifugation of 16000 g for 15 minutes at 4 °C. The supernatant was transferred to another tube and this solution had its pH neutralized by the addition

of 80 μL of phosphoric acid 10%. Following this, the samples were centrifuged at 16000 g for 15 minutes at 4 °C. Then, 250 μL of the supernatant was collected to a new tube, where was added 250 μL of ethanol 100% and incubated overnight at 4 °C. In the next day, the samples were centrifuged at 16000 g for 15 minutes at 4 °C and the supernatant was discarded. The resultant glycogen pellet was dissolved in 100 μL of worm water. After dissolution, the samples were treated with 100 μL of phenol at 6,5% followed by 500 μL of concentrated sulfuric acid. The samples were left at room temperature until they cooled and reached room temperature. Finally, the calibration line was calculated by using different glycogen concentrations and the absorbance of the samples was read at 490 nm using BioTek Synergy HT (BioTek, Winooski, VT, U.S.A). Results were normalized in relation to protein concentration of the sample and the results were presented in nmol of glycogen per mg of protein.

3.11. Oil Red O Staining

Oil Red O staining technique allows the detection and quantification of lipid droplets in cultured cells (182). The red dye will accumulate in cells lipid droplets that can be removed with isopropanol 100% for quantification. With this in mind, we developed this technique based on the protocol used by Xie Ge *et al*/ (183) in TM4 Sertoli cells however, with some modifications. In brief, cells were seeded and treated with the treatments for 24 hours in 12-wells plates with coverslips previous placed in the bottom. After this, cells were washed with PBS with Ca^{2+} and Mg^{2+} and then fixated with a buffered solution of formalin 10%, for 60 minutes. Cells were washed with PBS with Ca^{2+} and Mg^{2+} and incubated with isopropanol 60% for 5 minutes. After this, the stock solution of Oil Red Staining O™ at 0,05% was diluted in water for a final concentration of 0,02% and then filtrated. This solution was incubated with the fixated Sertoli cells for 20 minutes. After this incubation, the cells were washed with a solution of isopropanol at 50% and then with PBS until the oil red residues disappear. Then, the coverslips were removed from the wells and placed in a clean 12-wells plate and dried at 37 °C. Finally, 150 μL of isopropanol were added for the removing of the oil red accumulated in the lipid droplets of the cell and then transferred to a 96-wells plate for quantification through absorbance at 510 nm using BioTek Synergy HT (BioTek, Winooski, VT, U.S.A). Resulting values were divided by the mean of the control group and expressed in fold variation versus the control group.

3.12. Mitochondrial membrane potential

Mitochondrial membrane potential reveals the functional status of the mitochondria and cell health (184). Depolarization of the mitochondrial membrane means that the organelle is damaged and that is not able to satisfy cellular energy demands. Hyperpolarization of the mitochondrial membrane is a marker of cellular damage, being the increased presence of ROS the main culprit of the damage (185). 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide or JC-1 is a dye which allows to measure mitochondrial membrane potential. Unhealthy cells emanated green fluorescence caused by JC-1 monomers. On the other hand, healthy cells emitted red fluorescence from the aggregates of JC-1. TM4 Sertoli cells were seeded in black 96-well plates and incubated with the different treatments' medium. After the 24h treatment with dapagliflozin, exenatide, and the combined therapy, the treatment medium was removed and replaced with Sertoli cell medium with JC-1 at 2 μ M diluted in FBS (1% of medium volume). Then, the cells were incubated at 37 °C for 30 minutes in a dark environment. After incubation, the medium was removed and media without JC-1 was added to allow the measurement of the fluorescence of each sample at the excitation wavelength of 485 nm and 535 nm and an emission wavelength of 530 and 590 nm with BioTek Synergy HT (BioTek, Winooski, VT, U.S.A). Results were treated as the ration aggregates/monomers, divided by the mean of the control group and expressed in a fold variation versus the control group.

3.13. Statistical analysis

Statistical significance among the experimental groups' samples was assessed by ordinary one-way ANOVA plus uncorrected Fisher's LSD, with a single pooled variance test. This test was performed in all the results except when were compared the results from the combined therapy of dapagliflozin plus exenatide with its control. In this situation was performed an unpaired T-test student. All experimental data are shown as mean $\pm$ SEM. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA). Outliers were identified using the Grubbs method with Alpha equal to 0,2 and the results were considered significant when $p < 0,05$.

Chapter IV

RESULTS

4. RESULTS

4.1. GLP-1 receptor and SGLT2 are expressed in Sertoli cells

GLP-1 receptor has already been identified in mouse testes (167) and in human Sertoli cells (186). Herein, we examined if GLP-1 receptor was expressed in TM4 Sertoli cell line derived from mouse testes. We evaluated the mRNA expression for this receptor, using specific primer set, and obtained a 253 bp amplicon (Figure 7A). Similarly, SGLT2 expression in human testes was also already described (122). In the TM4 Sertoli cell line we were able to detect the presence of SGLT2 mRNA, using a specific primer set, which resulted in a 104 bp amplicon (Figure 7B).

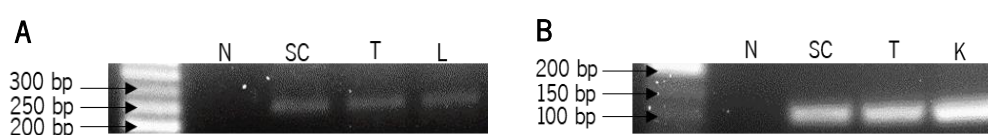


Figure 7: Confirmation of the expression of GLP-1 receptor (A) and SGLT2 (B) in TM4 Sertoli cells by reverse transcriptase polymerase chain reaction. Mouse lung, testis, and kidney total RNA were used as positive controls. Legend: N - negative control; SC - TM4 Sertoli cells; T - Testis; L - Lung; K - Kidney.

4.2. Exposure of TM4 Sertoli cells to exenatide

4.2.1. Exenatide decreased Sertoli cell membrane integrity and metabolic viability, without affecting cell proliferation

Results from techniques such as SRB, LDH release and MTT assays are essential in studies that aim to evaluate the cytotoxic effect of a chemical or drug. In our case, cytotoxicity assays allowed us to determine if the different treatments would affect cell viability or metabolic viability in a way that would invalidate further studies.

SRB assay data showed that exenatide at concentrations of 2,5 pM ($1,05 \pm 0,03$ – fold variation to control), 25 pM ($1,06 \pm 0,05$ – fold variation to control), or 250 pM ($1,03 \pm 0,04$) did not have any significant effect on TM4 Sertoli cells proliferation, as compared with Sertoli cells on control conditions (Figure 8A). In the case of the LDH release assay, exenatide decreased membrane integrity in relation to control at every concentration tested. Exenatide showed to increase LDH leakage by TM4 Sertoli cells at the concentrations of 2,5 pM ($1,44 \pm 0,10$ – fold variation to control), 25 pM ($1,68 \pm 0,12$ – fold variation to control), and 250 pM ($1,82 \pm 0,12$ – fold variation to control) (Figure 8B). Finally, data obtained from of the MTT assay showed that the cells incubated with the pharmacological concentration (25 pM) of exenatide did not have a

significantly different metabolic viability ($1,00 \pm 0,03$ – fold variation to control) in relation to control. However, the Sertoli cells exposed to the other exenatide concentrations showed a decrease in the metabolic viability. The cells exposed to exenatide 250 pM ($0,87 \pm 0,03$ – fold variation to control) were the ones in which metabolic viability decreased the most. The cells exposed to the concentration of 2,5 pM ($0,88 \pm 0,02$ – fold variation to control) also showed a significant decreased of their metabolic viability in relation to the ones of the control group (Figure 8C).

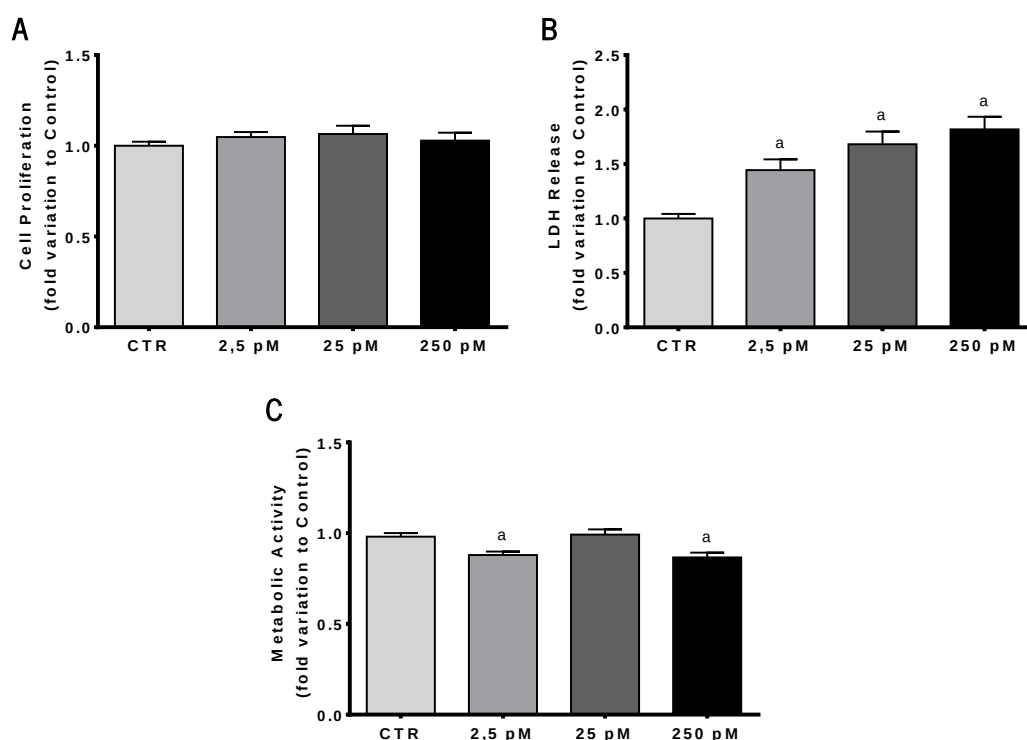


Figure 8: Evaluation of the cytotoxic profile of the different concentration of exenatide on TM4 Sertoli cells. The data shows results from the experimental groups of sub-pharmacological concentration (2,5 pM), pharmacological concentration (25 pM), and supra-pharmacological concentration (250 pM) of exenatide. To evaluate the treatments cytotoxicity, the effect of the different concentrations on cellular proliferation (A), membrane integrity (B), and metabolic viability (C) was studied through SRB assay, LDH release assay, and MTT assay, respectively. The data is organized in pooled data of independent experiments and the results are expressed as mean $\pm$ SEM (N=6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to control (CTR).

4.2.2. Exenatide maintained glucose consumption by Sertoli cells while increasing the expression of GLUT2, when at pharmacological concentrations

Sertoli cells nourish developing sperm cells through all the steps of spermatogenesis. With this in mind, glucose consumption is essential for the formation of new spermatozoa. Ideally, Sertoli cells should be able to maintain glucose consumption to sustain the delivery of metabolites such as lactate and acetate to developing germ cells.

In our study, the data showed no statistical differences in the Sertoli cell glucose metabolism of cells treated or not with exenatide. Taking the glucose consumed by the control group as reference (12127 ± 4212 nmol/ 10^6 cells), the treatment with exenatide at 2,5 pM (6589 ± 1497 nmol/ 10^6 cells), at 25 pM (6416 ± 1154 nmol/ 10^6 cells), and at 250 pM (8313 ± 1734 nmol/ 10^6 cells) seemed to decrease glucose consumption although this tendency did not reach statistical significance (Figure 9A). In contrast, GLUT2 expression levels were increased in cells treated with exenatide concentration of 25 pM ($3,18 \pm 1,02$ – fold variation to control). On Sertoli cells exposed to the concentrations of 2,5 pM and 250 pM, the enhanced expression was not significantly different ($2,59 \pm 0,49$ and $2,63 \pm 0,75$ – fold variation to control, respectively) from that observed in the cells from the control group (Figure 9B).

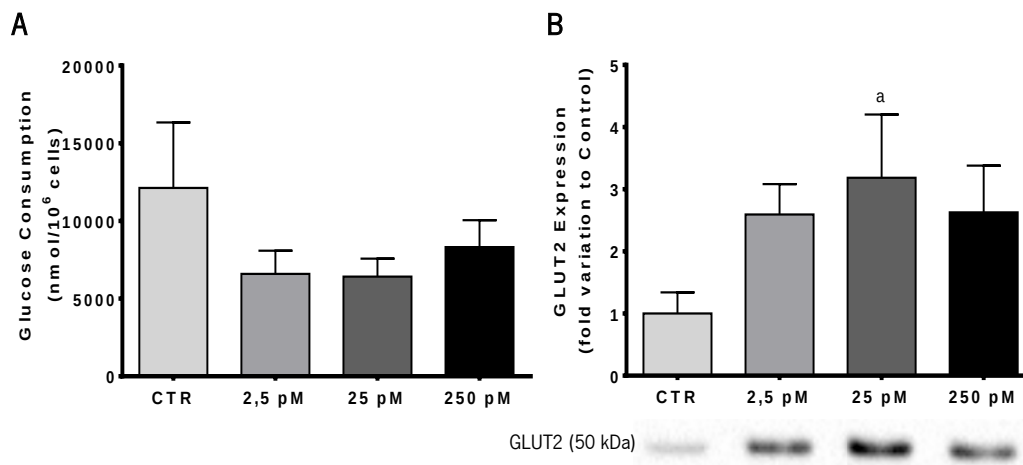


Figure 9: Effect of exenatide at 2,5 pM, 25 pM, and 250 pM on glucose consumption by TM4 Sertoli cells. The figure shows pooled data of independent experiments, indicating glucose consumption (A) and protein levels of glucose transporters 2 (GLUT2) and a representative immunoblot (B) performed with Sertoli cells treated with the different concentrations of exenatide and compared with the control group. The results are expressed as mean $\pm$ SEM (N=6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to control (CTR).

4.2.3. Exenatide treatment maintained the secretion of monocarboxylates and alanine in TM4

Sertoli cells

Lactate and acetate are two monocarboxylates essential for spermatogenesis. The secretion of these metabolites by TM4 Sertoli cells exposed to exenatide did not show any difference in relation to those of the control group. Lactate production by Sertoli cells exposed to 2,5 pM of exenatide (21682 ± 3934 nmol/ 10^6 cells), or 25 pM (24447 ± 4990 nmol/ 10^6 cells), or 250 pM (18532 ± 4493 nmol/ 10^6 cells) did not reach statistical difference, when compared that of cells from the control group (15370 ± 2397 nmol/ 10^6 cells) (Figure 10A). Concurrently, the results obtained for the expression levels of LDH in the TM4 Sertoli cells of the different experimental groups treated with exenatide did not vary significantly. Sertoli cells treated with 2,5 pM presented an LDH expression level of $1,50 \pm 0,26$ – fold variation to cells of the control group. Similarly, cells treated with 25 pM and 250 pM had LDH expression levels of $1,30 \pm 0,34$ and $1,54 \pm 0,41$ – fold variation to control group, respectively (Figure 10B). LDH activity also did not show any difference in the diverse treatments. When compared to the control group ($90,1 \pm 17,5$ nmol/min/mg of protein), Sertoli cells treated with exenatide at 2,5 pM (112 ± 23 nmol/min/mg of protein), at 25 pM ($99,4 \pm 21,4$ nmol/min/mg of protein), and at 250 pM (101 ± 18 nmol/min/mg of protein) maintained LDH activity (Figure 10C).

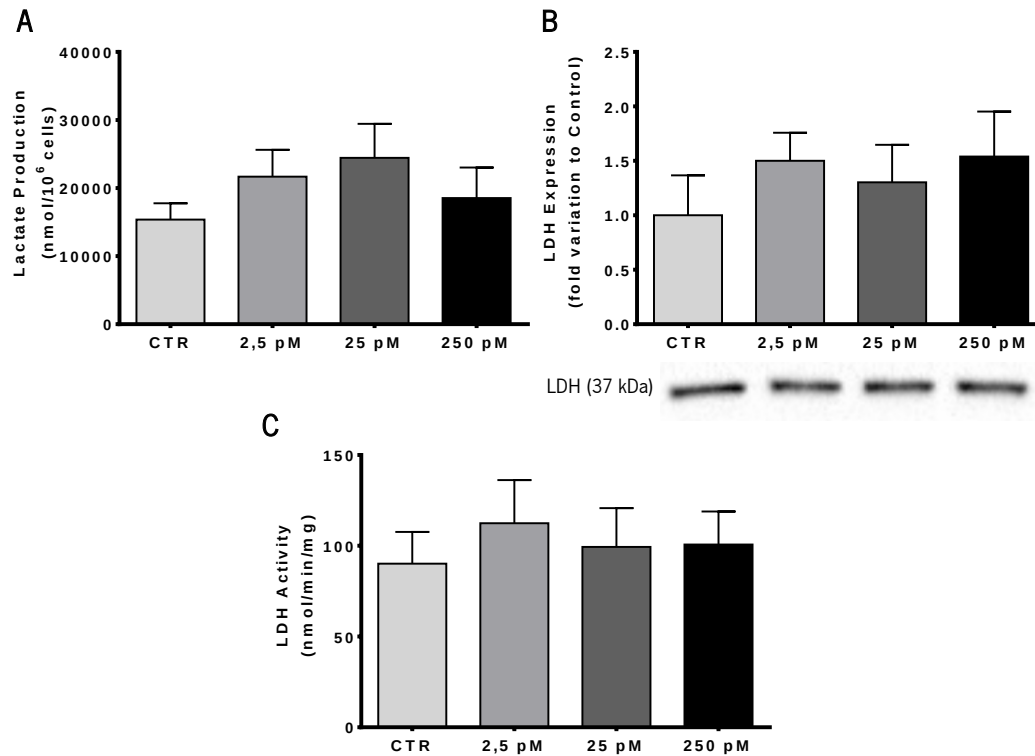


Figure 10: Effect of exenatide concentrations (2,5 pM, 25 pM, and 250 pM) on lactate production in TM4 Sertoli cells. The figure shows pooled data of independent experiments, indicating lactate production (A), the quantification of lactate dehydrogenase (LDH) expression levels and representative immunoblot (B), and intracellular LDH activity (C) by Sertoli cells under the different exenatide treatments and compared with the control group (CTR). The results are expressed as mean $\pm$ SEM (N=6 for each condition).

Acetate is also an important substrate for the development of the germ cells. Our results showed that exenatide did not alter acetate secretion by Sertoli cells in a statistically significant way (Figure 11A). However, treatment of TM4 cells with 2,5 pM (-26 ± 67 nmol/10⁶ cells) and 25 pM (-32 ± 64 nmol/10⁶ cells) reverted the production trend of acetate, observed in the cells of the control group ($115 \pm 28,6$ nmol/10⁶ cells) in which acetate is being produced. It is worth noticing that this fact could be due to the low concentration of acetate in the medium. The remaining supra-pharmacological concentration led to an acetate production of $89,9 \pm 60,5$ nmol/ 10⁶ cells, which is not statistically different from that observed in the cells from the control group. We also determined the MCT4 expression levels in TM4 cells of the different experimental groups. The cells exposed to the concentrations of 2,5 pM ($2,78 \pm 1,48$ – fold variation to control), 25 pM ($1,44 \pm 0,41$ – fold variation to control), and 250 pM ($1,72 \pm 0,61$ – fold variation to control), although

showing an increasing tendency, did not exhibit an alteration on the expression of MCT4 in relation to the control group (Figure 11B). Similarly, the expression of the MCT4 B1ATM2 isoform (Uniprot: B1ATM2) with lower molecular weight remained unchanged in the TM4 Sertoli cells after exposure to exenatide. In cells exposed to 2,5 pM of exenatide, MCT4 B1ATM2 isoform levels were $1,66 \pm 0,42$ – fold variation to control and in those exposed to 25 pM of exenatide the expression levels were $2,0 \pm 0,5$ – fold variation to control, whereas cells exposed to supra-pharmacological concentrations (250 pM) of exenatide had an expression level of $1,54 \pm 0,27$ – fold variation to control (Figure 11C) for this MCT4 isoform.

Like lactate and acetate, alanine is also an important substrate for developing germ cells. In addition, the equilibrium between lactate and alanine is important for the redox state of the cell (187). In Sertoli cells cultured in control conditions, alanine production was 376 ± 85 nmol/ 10^6 cells. When TM4 Sertoli cells were treated with 2,5 pM of exenatide, the production of alanine was 383 ± 58 nmol/ 10^6 cells, and in those treated with 25 pM, the production was 471 ± 122 nmol/ 10^6 cells. Finally, in cells treated with 250 pM of exenatide, we observed an alanine production of 342 ± 60 nmol/ 10^6 cells, statistically similar to that observed in the other experimental groups (Figure 11D).

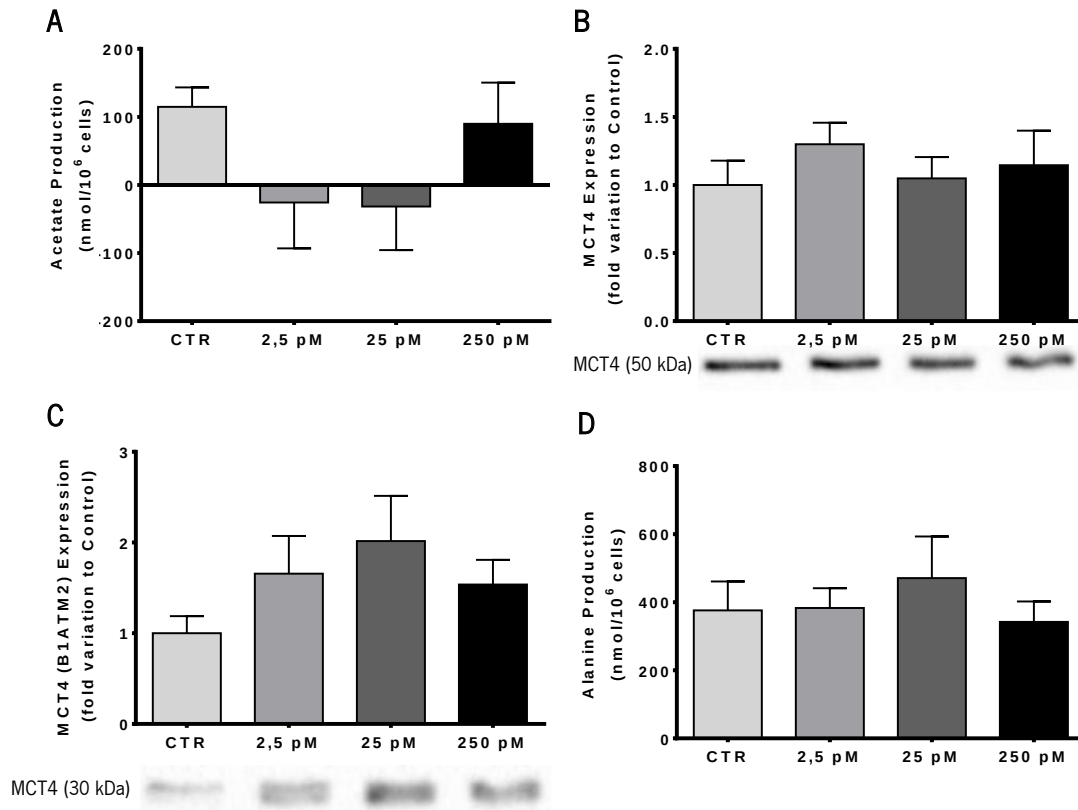


Figure 11: Effect of exenatide concentrations (2,5 pM, 25 pM, and 250 pM) on metabolite production and secretion in TM4 Sertoli cells. The figure shows pooled data of independent experiments, indicating acetate production (A), monocarboxylate transporter 4 (MCT4) expression levels and representative immunoblot (B), MCT4 B1ATM2 isoform expression levels and representative immunoblot (C), and alanine production (D) by Sertoli cells under the different exenatide treatments and compared with the control group (CTR). The results are expressed as mean $\pm$ SEM (N=6 for each condition).

4.2.4. Sub-pharmacological concentration of exenatide decreases mitochondrial membrane potential in TM4 Sertoli cells

Sertoli cells need to ensure not only the energetic requirements of germ cells during spermatogenesis but also its own metabolic and energetic needs. To do so, Sertoli cells utilize the TCA cycle and the pentose phosphate pathway (121). A proper mitochondrial function is imperative to produce energy through the TCA cycle. Impaired mitochondrial membrane potential is a marker of unhealthy mitochondria and dysregulated energy synthesis. JC-1 assay allows us to measure the depolarization of mitochondrial membrane.

Interestingly, sub-pharmacological concentration of exenatide (2,5 pM) significantly decreased the JC-1 monomers/aggregates ratio to $0,82 \pm 0,05$ – fold variation to control in TM4 Sertoli cells. This indicates that the cells subjected to this treatment had a depolarization in the mitochondrial membrane. However, pharmacological (25 pM) and supra-pharmacological (250 pM) concentrations of exenatide did not significantly altered the JC-1 ratio ($0,96 \pm 0,03$ and $1,15 \pm 0,07$ – fold variation to control, respectively) in TM4 Sertoli cells (Figure 12A).

Lactate/alanine ratio is often related to the redox state of the cell. This is due to the linkage between the ratio lactate/alanine and the ratio of NADH/NAD⁺ in the cytosol (187). When compared to the control group ($31,8 \pm 2,0$ – lactate/alanine ratio), cells treated with exenatide at the concentration of 2,5 pM showed to have a ratio of lactate/alanine of $43,4 \pm 4,2$, which is significantly higher when compared with the one of the control group. Similarly, to the results of the JC-1 ratio, the cells exposed to the other two concentrations of exenatide, 25 pM ($39,6 \pm 1,2$ – ratio lactate/alanine) and 250 pM ($36,5 \pm 1,3$ – ratio lactate/alanine), exhibited lactate/alanine ratios that were not different from those observed in the cells of the control group (Figure 12B).

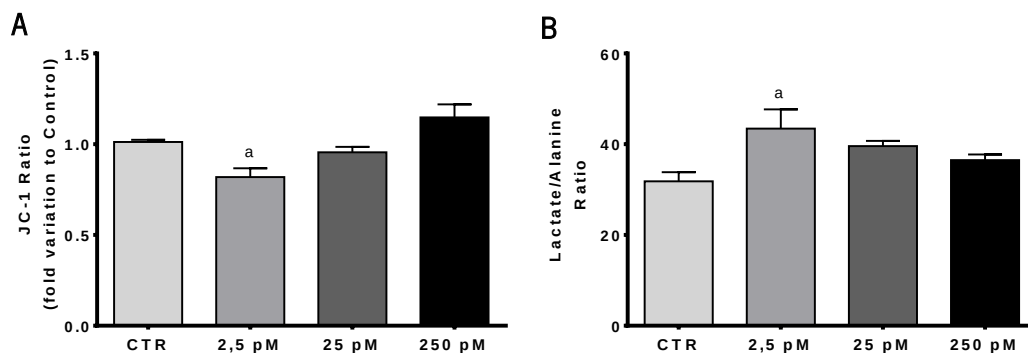


Figure 12: Effect of exenatide treatments (2,5 pM, 25 pM, and 250 pM) on the mitochondrial function of TM4 Sertoli cells. The data shows the ratio of the JC-1 assay (A) and the ratio of lactate/alanine (B) of Sertoli cells incubated with the different concentrations of exenatide. The data is organized in pooled data of independent experiments and the results are expressed as mean $\pm$ SEM (N=6 for each condition). Significantly different results (P < 0.05) are indicated as: a – relative to control (CTR).

4.2.5. Incubation with exenatide maintained glycogen and lipid droplet reserves on TM4 Sertoli cells

Even without observing any differences in the metabolic performance of TM4 Sertoli cells after exposure to the different concentrations of exenatide, we further evaluated the intracellular reserves of glycogen and lipid droplets in these cells. The data resulting from the glycogen quantification showed no statistical difference between the cells treated with exenatide and those of the control group. Glycogen reserves of the cells from the control group were $15,2 \pm 1,4$ nmol/mg protein, while the cells treated with 2,5 pM of exenatide had a glycogen reserve of $12,7 \pm 1,8$ nmol/mg protein. TM4 cells treated with 25 pM of exenatide had glycogen reserve of $12,4 \pm 1,2$ nmol/mg protein, and those treated with 250 pM of exenatide had a glycogen reserve of $14,1 \pm 1,6$ nmol/mg protein (Figure 13A). Similarly, lipid droplet quantification also showed no significant alterations among the TM4 Sertoli cells treated with exenatide and those from the control group. Sertoli cells treated with exenatide at a concentration of 2,5 pM, 25 pM, and 250 pM ($0,98 \pm 0,04$; $0,95 \pm 0,05$; and $0,97 \pm 0,04$ – fold variation to control, respectively) showed no difference in intracellular lipid accumulation (Figure 13B). Lipid droplets that allowed the quantification can be seen as red dots in TM4 Sertoli cells under control conditions in Figure 13C. Sertoli cells incubated under control conditions without oil red o staining incubation are represented in the Figure 13D.

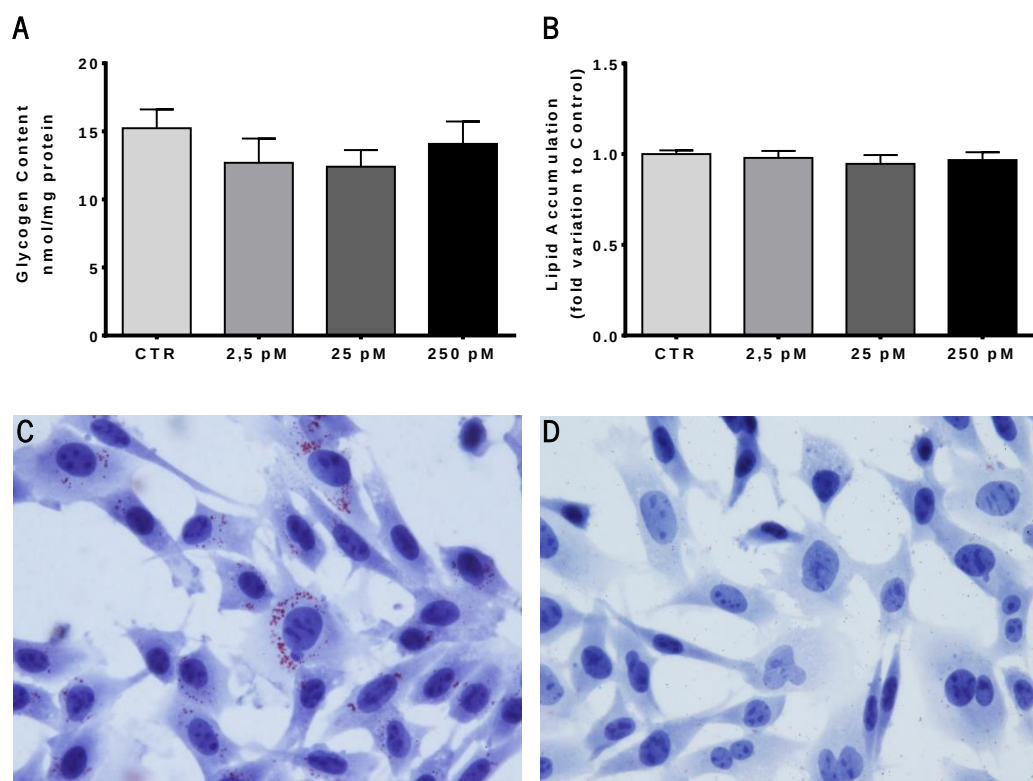


Figure 13: Effect of exenatide treatments (2,5 pM, 25 pM, and 250 pM) on carbon storage by TM4 Sertoli cells. The data shows the concentration of glycogen reserves (A) and the quantification of lipid droplets (B) of Sertoli cells when incubated with the different concentrations of exenatide and compared with the control group (CTR). Lipid droplets can be seen as red dots in Sertoli cells under control conditions incubated with (C) and without (D) oil red staining solution with amplification of 400 times. The data is organized in pooled data of independent experiments and the results are expressed as mean $\pm$ SEM (N=6 for each condition).

4.3. Exposure of TM4 Sertoli cells to dapagliflozin

4.3.1. Dapagliflozin decreases Sertoli cell proliferation, membrane integrity, and metabolic viability when at pharmacologic concentrations

The cytotoxic assays described previously were used to determine the cytotoxicity of the different concentrations of dapagliflozin on TM4 Sertoli cells. SRB assay showed that the exposure to the pharmacological (500 nM) and supra-pharmacological (5000 nM) concentration of dapagliflozin resulted in a significant decrease of TM4 Sertoli cell proliferation ($0,85 \pm 0,04$ and $0,85 \pm 0,06$ – fold variation to control, respectively) when compared with the cells from the control group. On the other hand, exposure to 50 nM of dapagliflozin did not alter cell proliferation ($1,07$

$\pm 0,05$ – fold variation to control) when compared with the cells from the control group (Figure 14A).

LDH release assay allowed us to determine that exposure of Sertoli cells to dapagliflozin at the concentration of 50 nM, 500 nM, and 5000 nM decreases membrane integrity ($2,25 \pm 0,17$; $2,34 \pm 0,15$; and $2,93 \pm 0,23$ – fold variation to control, respectively) (Figure 14B). MTT assay also showed that the exposure to any of the concentrations of dapagliflozin used led to a decrease in the metabolic viability of Sertoli cells when compared with the cells from the control group. TM4 Sertoli cells exposed to 50 nM of dapagliflozin showed a decreased metabolic viability to $0,85 \pm 0,03$ – fold variation to control, while those exposed to 500 nM dapagliflozin had their metabolic viability diminished to $0,86 \pm 0,03$ – fold variation to control. Finally, the exposure of Sertoli cells to 5000 nM of dapagliflozin lead to a decrease of the cellular viability to $0,79 \pm 0,04$ – fold variation to control (Figure 14C).

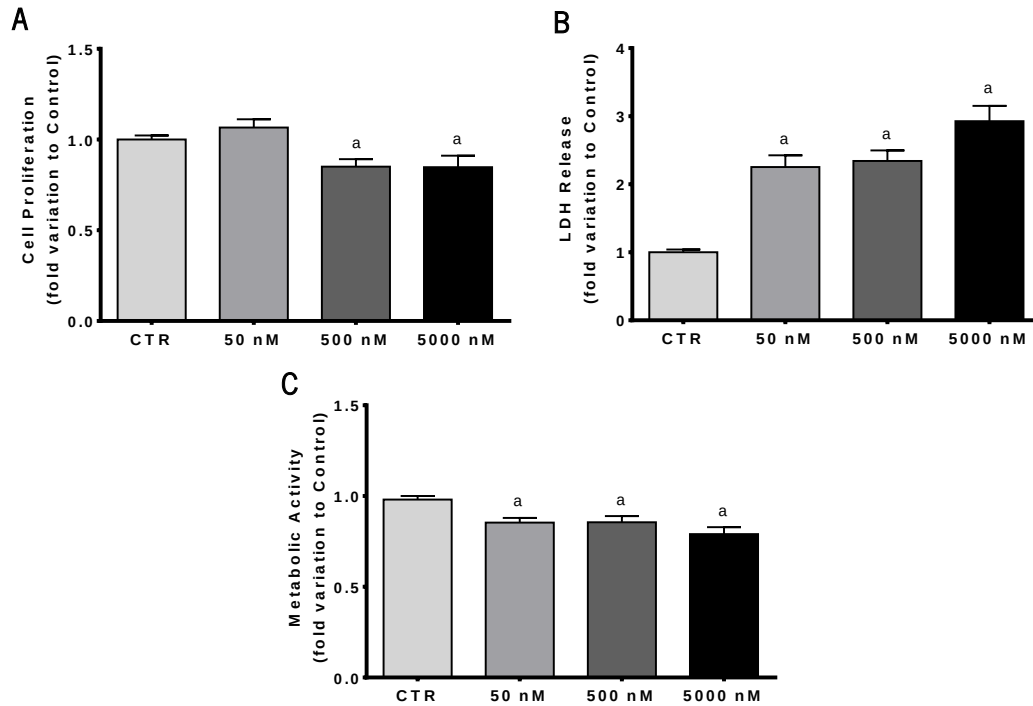


Figure 14: Evaluation of the cytotoxic profile of the different concentration of dapagliflozin on TM4 Sertoli cells. The data shows results from the experimental groups of the sub-pharmacological (50 nM), pharmacological (500 nM), and supra-pharmacological concentration (5000 nM) of dapagliflozin. The cytotoxic effect of the different concentrations of dapagliflozin on cellular proliferation (A), membrane integrity (B), and metabolic viability (C) was studied through SRB assay, LDH cytotoxic release assay, and MTT assay, respectively. The data is organized in pooled data of independent experiments and the results are expressed as mean $\pm$ SEM (N=6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to control (CTR).

4.3.2. Dapagliflozin does not alter glucose consumption but increases GLUT2 expression in TM4 Sertoli cells

Dapagliflozin is an SGLT2 inhibitor. The main function of the SGLT2 is the import of glucose into the cytoplasm. The exposure of cells to such drug may result in decreased glucose consumption.

In our work, glucose consumption by Sertoli cells treated with the different dapagliflozin concentrations remained constant. Sertoli cells exposed to the control medium had a glucose consumption of 12127 ± 4212 nmol/ 10^6 cells, whereas cells treated with 50 nM dapagliflozin had a glucose consumption of 14825 ± 4408 nmol/ 10^6 cells, and those treated with 500 nM, and

5000 nM of dapagliflozin had a glucose consumption of 14725 ± 2265 nmol/ 10^6 cells, and 10643 ± 1434 nmol/ 10^6 cells, respectively (Figure 15 A).

Additionally, as observed in the cells treated with exenatide, the exposure to dapagliflozin also led to an increase of GLUT2 expression levels. In this case, the supra-pharmacological concentration of dapagliflozin (5000 nM) was responsible for an increase of GLUT2 expression levels ($4,53 \pm 1,93$ – fold variation to control) in TM4 Sertoli cells. The other dapagliflozin concentrations (50 nM and 500 nM) did not altered significantly the GLUT2 expression levels ($3,32 \pm 1,18$ and $3,0 \pm 1,0$ – fold variation to control, respectively) in TM4 Sertoli cells (Figure 15B).

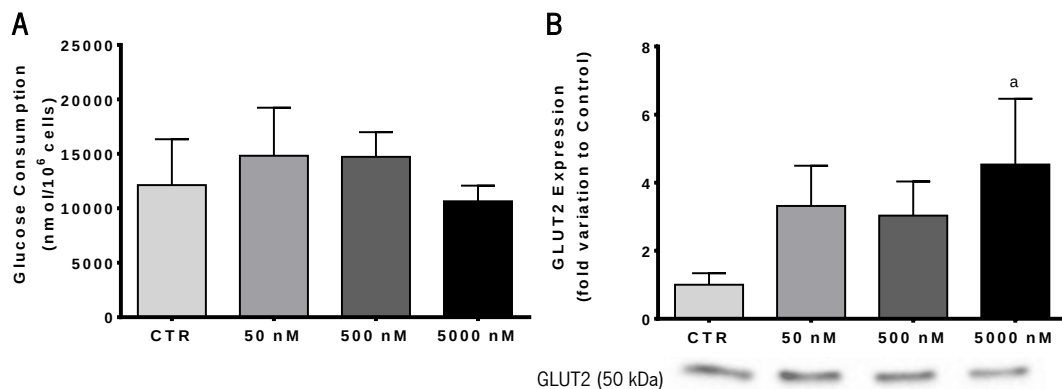


Figure 15: Effect of dapagliflozin (50 nM, 500 nM, and 5000 nM) on glucose consumption by TM4 Sertoli cells. The figure shows pooled data of independent experiments, indicating glucose consumption (A) and protein levels of glucose transporters 2 (GLUT2) and the representative immunoblot (B) performed with Sertoli cells treated with the different concentrations of dapagliflozin and compared with the control group. The results are expressed as mean $\pm$ SEM (N=6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to control (CTR).

4.3.3. Pharmacological concentration of dapagliflozin increases the secretion of lactate and alanine by TM4 Sertoli cells

As mentioned before, the preferred metabolic substrate of the developing germ cells is lactate. This means that Sertoli cells have to ensure the secretion of lactate to the germ cells, in order to sustain spermatogenesis. In the Sertoli cells treated with dapagliflozin at pharmacological concentration (500 nM), was noticed a significant increase in the secretion of lactate (26342 ± 4450 nmol/ 10^6 cells) in relation to the cells of the control group (15370 ± 2397 nmol/ 10^6 cells). Concentrations of 50 nM (18746 ± 3836 nmol/ 10^6 cells) and 5000 nM (18840 ± 3177 nmol/ 10^6 cells) of dapagliflozin did not significantly alter the secretion of lactate by TM4 Sertoli cells when

compared with that of cells from the control group (Figure 16A). Interestingly, LDH expression shows a tendency to increase when cells were treated with increasing concentrations of dapagliflozin, without ever reaching statistical significance. TM4 Sertoli cells treated with 50 nM of dapagliflozin had an LDH expression of $1,32 \pm 0,40$ – fold variation to control, whereas those treated with 500 nM of dapagliflozin had an LDH expression of $1,48 \pm 0,37$ – fold variation to control. Finally, cells treated with 5000 nM of dapagliflozin had an LDH expression of $2,10 \pm 0,57$ – fold variation to control (Figure 16B). Similarly, intracellular LDH activity did not show any significant change when cells were treated with increasing concentrations of dapagliflozin in relation to the control group. Intracellular LDH activity in Sertoli cells treated with control conditions ($90,1 \pm 17,5$ nmol/min/mg of protein) did not had a significant variation in relation to the Sertoli cells treated with 50 nM (101 ± 15 nmol/min/mg of protein), with 500 nM (105 ± 17 nmol/min/mg of protein), or with 5000 nM (103 ± 16 nmol/min/mg of protein) of dapagliflozin (Figure 16C).

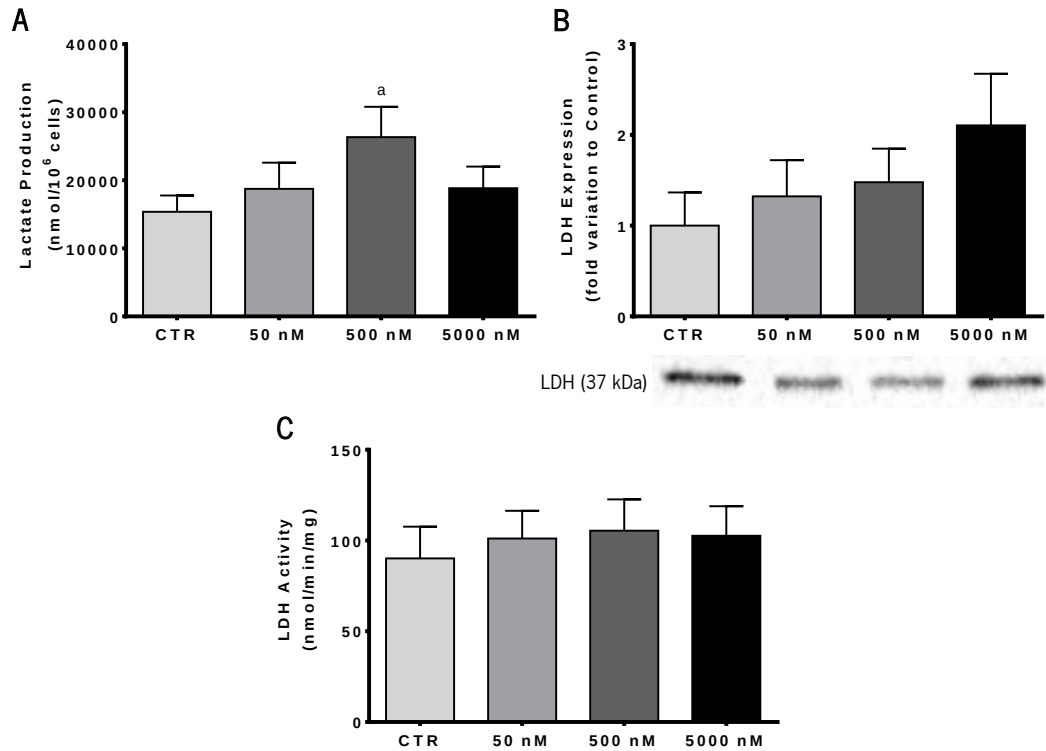


Figure 16: Effect of dapagliflozin concentrations (50 nM, 500 nM, and 5000 nM) on lactate production in TM4 Sertoli cells. The figure shows pooled data of independent experiments, indicating lactate production (A); the expression levels of lactate dehydrogenase (LDH) and representative immunoblot (B); and intracellular LDH activity (C) by Sertoli cells under the different treatments of dapagliflozin and compared with the control group. The results are expressed as mean $\pm$ SEM (N=6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to control (CTR).

On the other hand, acetate production was not significantly different between the cells of the various experimental groups. Sertoli cells from the control group exhibited an acetate production of 115 ± 28 nmol/10⁶ cells, whereas those of the groups treated with 50 nM, 500 nM, and 5000 nM exhibited an acetate production of 196 ± 34 ; 206 ± 41 ; and 202 ± 33 nmol/10⁶ cells, respectively (Figure 17A). Moreover, MCT4 expression levels in cells treated or not treated with dapagliflozin was not altered. When incubated with 50 nM dapagliflozin, the TM4 Sertoli cells' expression levels of MCT4 were $1,57 \pm 0,28$ – fold variation to control. Those treated with dapagliflozin at a concentration of 500 nM had MCT4 expression levels of $1,68 \pm 0,37$ – fold variation to control, while those treated with 5000 nM of dapagliflozin had MCT4 expression levels of $1,68 \pm 0,40$ – fold variation to control (Figure 17B). Contrastingly, the expression of the MCT4

B1ATM2 isoform was altered in the presence of dapagliflozin. Interestingly, there was a significant increase in the isoform expression when cells were treated with the pharmacological concentration (500 nM) of dapagliflozin, reaching a $2,18 \pm 0,42$ – fold variation to control. The treatment of TM4 Sertoli cells with 50 nM ($1,94 \pm 0,44$ – fold variation to control) and 5000 nM ($1,27 \pm 0,36$ – fold variation to control) of dapagliflozin did not alter significantly the expression levels of the MCT4 B1ATM2 isoform when compared with that of the cells from the control group (Figure 17C).

Similarly to what happened with lactate, alanine production also peaked when Sertoli cells were treated with the pharmacological concentration (500 nM) of dapagliflozin. When incubated with the control medium, Sertoli cells alanine production was 376 ± 85 nmol/ 10^6 cells. However, when the cells were treated with the pharmacological concentration of dapagliflozin (500 nM), alanine production was significantly enhanced to 863 ± 174 nmol/ 10^6 cells. Contrastingly, treatment with the 50 nM of dapagliflozin (539 ± 102 nmol/ 10^6 cells) and 5000 nM of dapagliflozin (728 ± 178 nmol/ 10^6 cells) did not change significantly the production of alanine in relation to that of cells from the control group (Figure 17D).

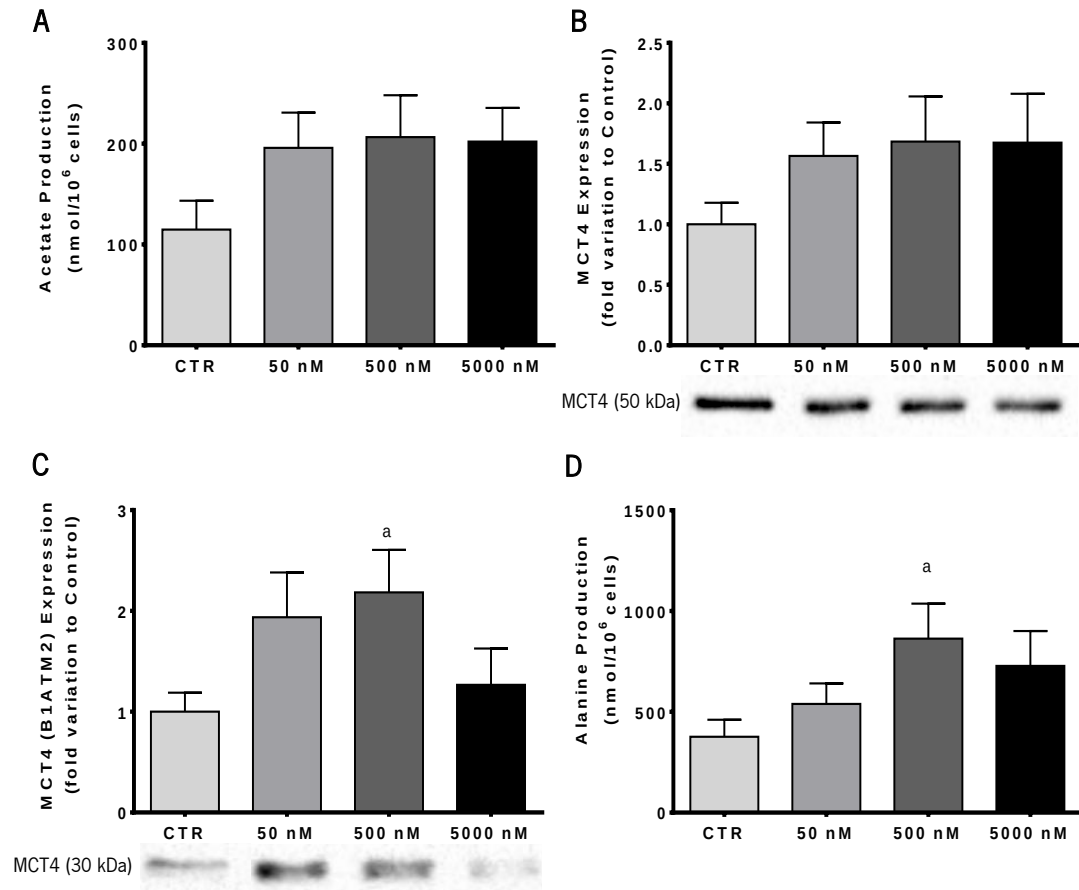


Figure 17: Effect of dapagliflozin concentrations (50 nM, 500 nM, and 5000 nM) on acetate and alanine production in TM4 Sertoli cells. The figure shows pooled data of independent experiments, indicating: acetate production (A); the monocarboxylate transporter 4 (MCT4) expression levels and representative immunoblot (B); the expression levels of MCT4 B1ATM2 isoform and representative immunoblot (C); and alanine production (D) by Sertoli cells under the different treatments of dapagliflozin and compared with the control group. The results are expressed as mean $\pm$ SEM (N=6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to control (CTR).

4.3.4. Supra-pharmacological concentration of dapagliflozin decreases the amount of lipid droplets on TM4 Sertoli cells

Our data showed that dapagliflozin increased the secretion of lactate and alanine without increasing glucose consumption. This fact made us investigate which could be the source of the extra carbons used for the synthesis of the extra substrates noticed at pharmacological concentrations. First, we evaluated the glycogen reserves of the Sertoli cells because glycogen can be quickly mobilized and converted into glucose to undergo glycolysis.

The quantification of glycogen on the cells from the different experimental groups showed no differences in glycogen content. The Sertoli cells from the control group had similar glycogen content ($15,2 \pm 1,4$ nmol/mg protein) to those treated with concentration of 50 nM of dapagliflozin ($13,9 \pm 1,1$ nmol/mg protein), to those treated with 500 nM of dapagliflozin ($12,5 \pm 0,6$ nmol/mg protein), and to those treated with 5000 nM of dapagliflozin that had a glycogen content of $11,5 \pm 1,6$ nmol/mg protein (Figure 18A).

We also focused our attention on the mobilization of cytoplasmic lipid droplets. Oil red o staining assay results showed that the cells treated with dapagliflozin at supra-pharmacological concentrations of 5000 nM ($0,74 \pm 0,03$ – fold variation to control) had decreased content of lipid droplets when compared to those of the control group. Cells from the other experimental groups did not show any alteration in the lipid droplets content in relation to cells from the control group, with the cells exposed to 50 nM of dapagliflozin showed a lipid content of $0,92 \pm 0,07$ – fold variation to control, and those exposed to 500 nM of dapagliflozin showed a lipid content of $0,89 \pm 0,08$ – fold variation to control (Figure 18B).

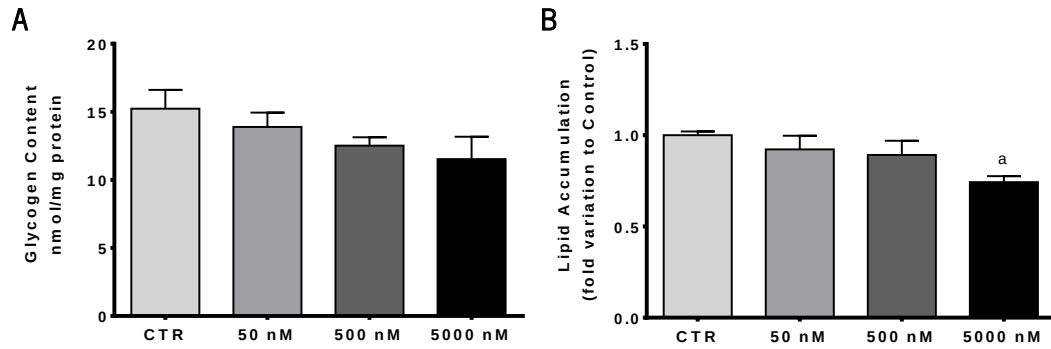


Figure 18: Effect of dapagliflozin treatments (50 nM, 500 nM, and 5000 nM) on carbon storage by TM4 Sertoli cells. The data shows the concentration of glycogen reserves (A) and the quantification of lipid droplets (B) of Sertoli cells when incubated with the different concentrations of dapagliflozin. The data is organized in pooled data of independent experiments and the results are expressed as mean $\pm$ SEM (N=6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to control (CTR).

4.3.5. Dapagliflozin maintains the mitochondrial membrane potential of Sertoli cells

Dapagliflozin at a supra-pharmacological concentration decreases lipid droplets in TM4 Sertoli cells, which could indicate that β -oxidation could be favored. To evaluate this hypothesis, cells treated with the different concentrations of dapagliflozin had their mitochondrial membrane potential analyzed by JC-1 assay. The data obtained from the cells treated with dapagliflozin at 50 nM (1.13 ± 0.28 – fold variation to control), at 500 nM (1.15 ± 0.33 – fold variation to control), and at 5000 nM (1.12 ± 0.24 – fold variation to control) did not significantly differ from that obtained for the cells of the control group (Figure 19A).

Similarly, lactate/alanine ratio was also maintained in treated TM4 Sertoli cells. Control group had a ratio of 31.8 ± 2.0 , whereas Sertoli cells treated with 50 nM, 500 nM, and 5000 nM had a ratio of 34.3 ± 2.5 ; 31.6 ± 1.9 ; and 33.7 ± 1.3 ; respectively, never reaching statistical relevance (Figure 19B).

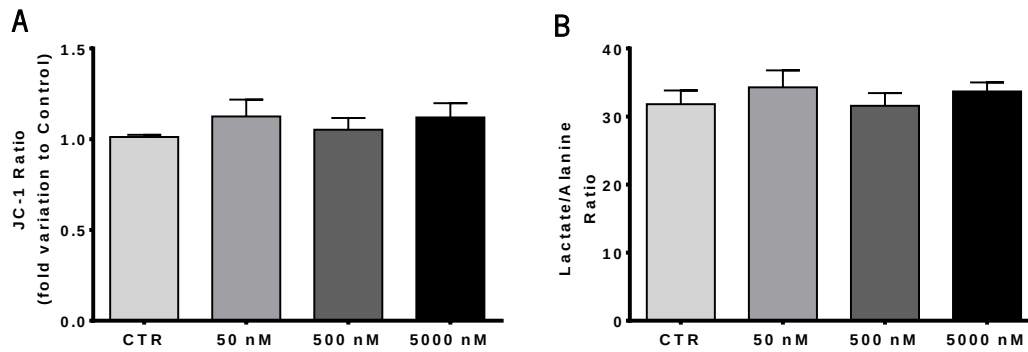


Figure 19: Effect of dapagliflozin (50 nM, 500 nM, and 5000 nM) on the mitochondrial function of TM4 Sertoli cell line. The data shows the ratio of the JC-1 assay (A) and the ratio of lactate/alanine (B) of Sertoli cells incubated with the different concentrations of dapagliflozin and compared with the control group (CTR). The data is organized in pooled data of independent experiments and the results are expressed as mean $\pm$ SEM (N=6 for each condition).

4.4. Exposure of TM4 Sertoli cells to exenatide plus dapagliflozin

4.4.1. Combined treatment with exenatide plus dapagliflozin decreases Sertoli cells proliferation and metabolic viability without decreasing membrane integrity

With this study, we tried to evaluate the impact that the treatment with the combination of pharmacological concentrations of both exenatide (25 pM) and dapagliflozin (500 nM) could have on Sertoli cell development and metabolism.

First, we assessed the cytotoxicity of this combined treatment on Sertoli cell proliferation. SRB assay results showed that the combined treatment with exenatide plus dapagliflozin decreased cell proliferation ($0,81 \pm 0,03$ – fold variation to control) in relation to the control group (Figure 20A). Moreover, a cytotoxic effect was also noted when evaluating the metabolic viability through MTT assay. Sertoli cells treated with the combination of exenatide plus dapagliflozin ($0,92 \pm 0,02$ – fold variation to control group) presented decreased metabolic viability (Figure 20B). Contrastingly, LDH release did not significantly change in relation to the control group when the cells were treated with the combination of exenatide plus dapagliflozin ($0,93 \pm 0,04$ – fold variation to control) (Figure 20C).

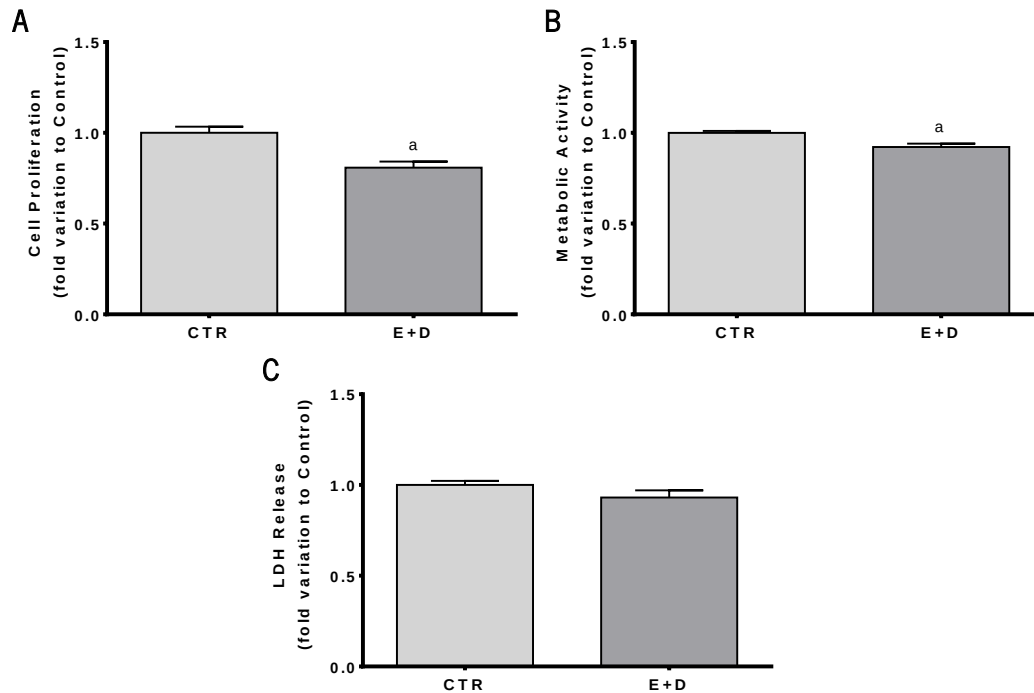


Figure 20: Evaluation of the cytotoxicity profile of the combined treatment (E+D) (25 pM exenatide plus 500 nM dapagliflozin) on TM4 Sertoli cells. To evaluate the cytotoxicity of the combined treatment on cellular proliferation (A), metabolic viability (B), and membrane integrity (C) were evaluated using the SRB assay, MTT assay, and LDH release assay, respectively. The data is organized in pooled data of independent experiments and the results are expressed as mean $\pm$ SEM (N=6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a – relative to control (CTR).

4.4.2. Combined treatment with exenatide plus dapagliflozin maintains the glucose consumption and GLUT2 expression on TM4 Sertoli cells

The exposure of TM4 Sertoli cells to the combination of the pharmacological concentration of exenatide and dapagliflozin did not alter glucose consumption. Sertoli cells from the control group exhibited a glucose consumption of 22390 ± 7834 nmol/ 10^6 cells, whereas, when cells were treated with the drug combination, the glucose consumption was 41303 ± 11800 nmol/ 10^6 cells (Figure 21A). GLUT2 expression levels of cells treated with exenatide plus dapagliflozin (1.04 ± 0.26 – fold variation to control) did not differ from that observed in cells from the control group (Figure 21B).

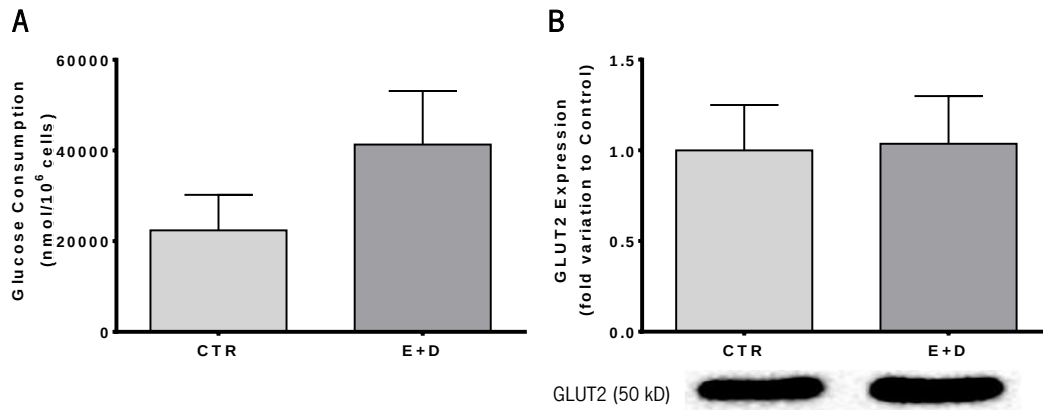


Figure 21: Effect of the combined treatment (E+D) (25 pM exenatide plus 500 nM dapagliflozin) on glucose consumption by TM4 Sertoli cells. The figure shows pooled data of independent experiments, indicating glucose consumption (A) and protein levels of glucose transporters 2 (GLUT2) and the respective representative immunoblot (B) of Sertoli cells under the treatment of the combined treatment and compared with the control group (CTR). The results are expressed as mean $\pm$ SEM (N=6 for each condition).

4.4.3. Combined treatment with exenatide plus dapagliflozin maintained the production of monocarboxylates and alanine on TM4 Sertoli cells

Cells exposed to control medium exhibited a lactate production of 31518 ± 10556 nmol/10⁶ cells, while those treated with the combination of exenatide plus dapagliflozin had a lactate production of 48730 ± 12274 nmol/10⁶ cells, which were not statistically different (Figure 22A). Similarly, LDH expression and activity levels were not altered by exposure to the combined treatment with exenatide plus dapagliflozin. Sertoli cells treated with the combination exenatide plus dapagliflozin showed an LDH expression level of $0,90 \pm 0,19$ – fold variation to control (Figure 22B). In addition, cells exposed to exenatide plus dapagliflozin had an intracellular LDH activity (106 ± 20 nmol/min/mg of protein) similar to those of the control group (96 ± 11 nmol/min/mg of protein) (Figure 22C). Moreover, neither MCT4 or MCT4 B1ATM2 isoform levels expression were altered in relation to the control group ($0,75 \pm 0,05$ and $0,43 \pm 0,05$ – fold variation to control, respectively) by the combined treatment with exenatide plus dapagliflozin (Figure 22D). These transporters are also capable of export other monocarboxylates that are produced by the cells, namely acetate. The amount of acetate exported by Sertoli cells exposed to the combined treatment (108 ± 45 nmol/10⁶ cells) was not different from that exported by cells from the control group (296 ± 116 nmol/10⁶ cells) (Figure 22E). The production of alanine by Sertoli cells was also

statistically similar between the cells from the group exposed to the combined treatment with exenatide plus dapagliflozin (1428 ± 304 nmol/ 10^6 cells) to that from cells from the control group (637 ± 258 nmol/ 10^6 cells) (Figure 22F).

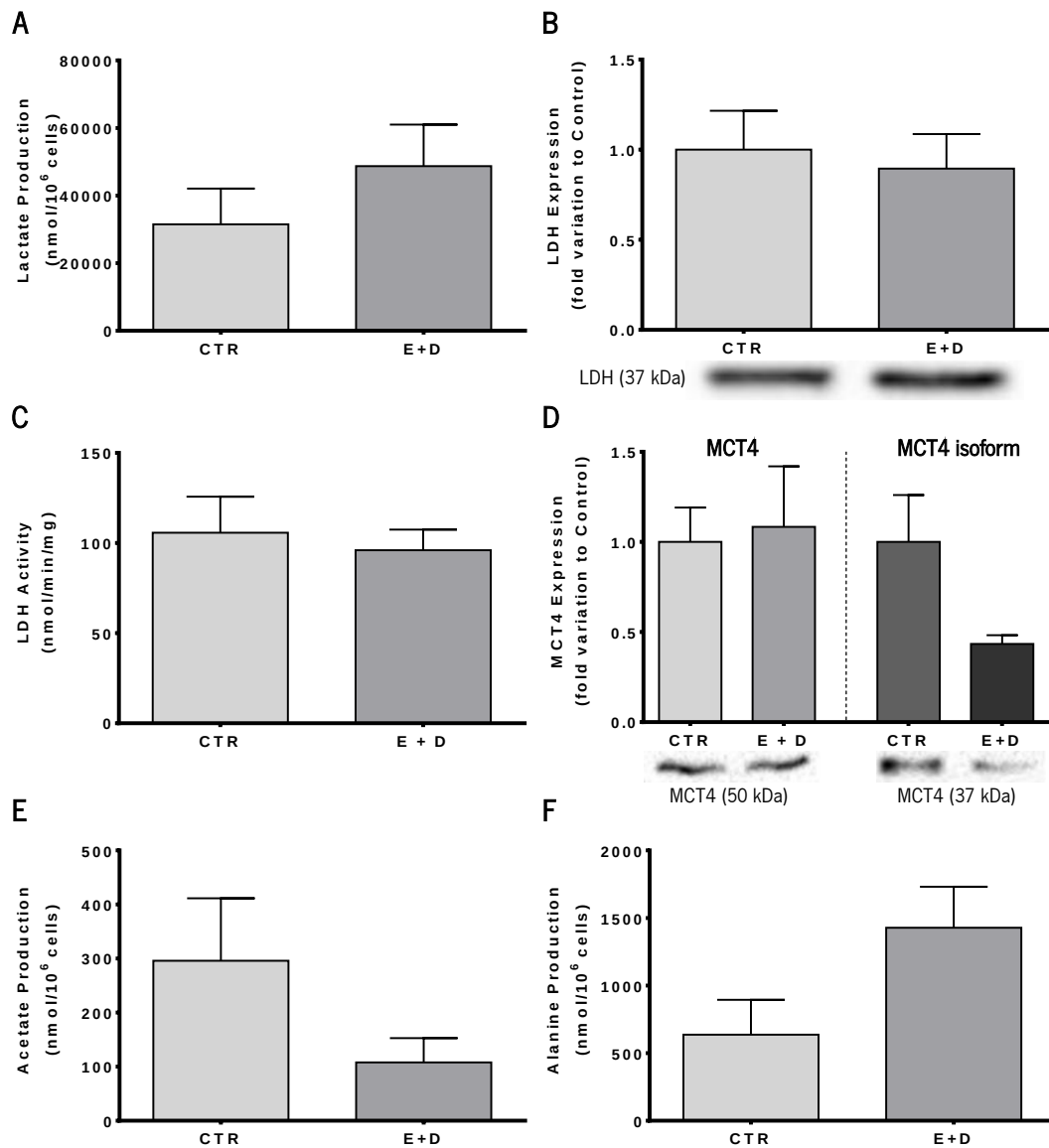


Figure 22: Effect of the combined treatment (E+D) (25 pM exenatide plus 500 nM dapagliflozin) on monocarboxylates (lactate and acetate) and alanine production in TM4 Sertoli cells. The figure shows pooled data of independent experiments, indicating lactate production (A), quantification of lactate dehydrogenase (LDH) expression levels and respective representative immunoblot (B), intracellular LDH activity (C), the expression levels of monocarboxylate transporter 4 (MCT4) and MCT4 B1ATM2 isoform and respective representative immunoblots (D), acetate production (E) and alanine production (F) by Sertoli cells under the treatment of the combined treatment and compared with the control group (CTR). The results are expressed as mean $\pm$ SEM (N=6 for each condition).

4.4.4. Glycogen and intracellular lipid contents were maintained in Sertoli cells after the combined treatment with exenatide plus dapagliflozin

With no differences found between the metabolome of cells treated with the combination of exenatide plus dapagliflozin and those in control conditions, we further investigated any differences in the content of glycogen between the cells of the two groups. Sertoli cells from the control group had a glycogen content of $13,0 \pm 1,2$ nmol/mg protein and those of the treated group had glycogen content of $12,3 \pm 1,9$ nmol/mg protein (Figure 23A), which did not differ significantly. Concurrently, the data obtained from the quantification of cytoplasmic lipid droplets using the oil red o staining showed no differences between the cells from the control group and the treated group ($0,91 \pm 0,06$ – fold variation to control) (Figure 23B).

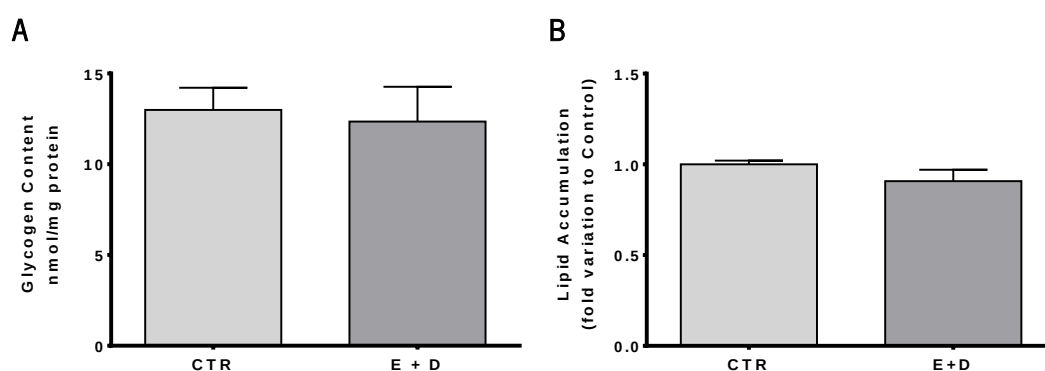


Figure 23: Effect of the combined treatment (E+D) (25 pM exenatide plus 500 nM dapagliflozin) on carbon storage by TM4 Sertoli cells. The data shows the content of glycogen reserves (A) and the quantification of lipid droplets (B) of Sertoli cells when incubated with the combination of exenatide and dapagliflozin and compared with the control group (CTR). The data is organized in pooled data of independent experiments and the results are expressed as mean $\pm$ SEM (N=6 for each condition).

4.4.5. Combined treatment with exenatide plus dapagliflozin did not alter mitochondrial membrane potential of TM4 Sertoli cells

The treatment of Sertoli cells with exenatide plus dapagliflozin did not promote any alteration of the mitochondrial membrane potential (as determined by the JC-1 assay) when compared with that of the cells from the control group. JC-1 monomers/aggregates ratio of the cells treated with the combination of exenatide plus dapagliflozin was $1,01 \pm 0,04$ – fold variation to control and was not significantly from that observed in the cells from the control group (Figure 24A). Moreover, lactate/alanine ratio also remained statistically unchanged. Cells from the control group had a ratio

of $46,7 \pm 7,3$ and the cells treated with the combination of exenatide plus dapagliflozin had a similar ratio of $34,9 \pm 4,4$ (Figure 24B).

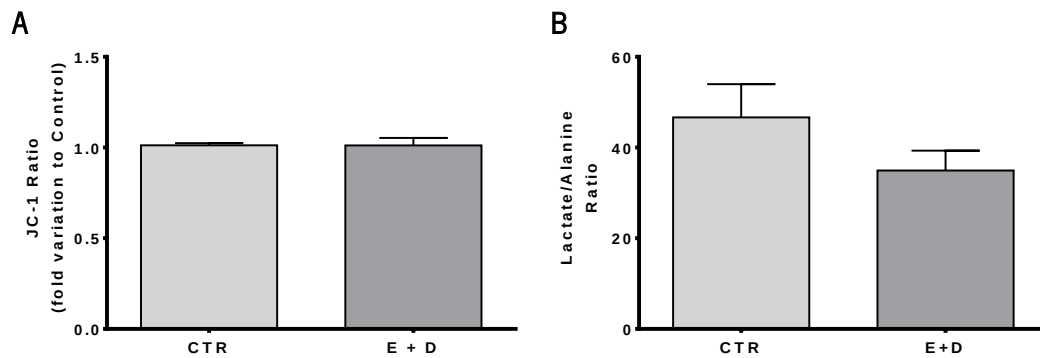


Figure 24: Effect of the combined treatment (E+D) (25 pM exenatide plus 500 nM dapagliflozin) on the mitochondrial function of TM4 Sertoli cells. The data shows the JC-1 monomers/aggregates ratio (A) and the ratio of lactate/alanine (B) of Sertoli cells incubated with the combination of exenatide plus dapagliflozin and those from the control group (CTR). The data is organized in pooled data of independent experiments and the results are expressed as mean $\pm$ SEM (N=6 for each condition).

Chapter V

DISCUSSION

5. DISCUSSION

Metabolic diseases like DM and obesity are increasing worldwide and recent estimations show that the tendency is to aggravate (188). With the increasing of this health and sociological problem, pharmaceutical companies are constantly trying to develop new ways of treating metabolic diseases in the more effective manner. However, the impact that these therapies have on the metabolic phenotype of different cellular systems is sometimes overlooked.

Male fertility is negatively affected by metabolic diseases (189), which is linked with the decreasing fertility rates evident in western society (190). Taking this in consideration, it is important to determine if the ability that antidiabetic and anti-obesogenic drugs have on modulating whole-body glucose metabolism is also reflected on glucose homeodynamics of the testicular environment, and particularly on the metabolic phenotype of the cells responsible for spermatogenesis: the Sertoli cells.

Exenatide is a drug with antidiabetic and anti-obesogenic properties that are showing promising results for the treatment of obese and diabetic patients (61). After thorough bibliographical research, it became evident a lack of studies focusing on the effect of the treatment with exenatide on male fertility. Like exenatide, dapagliflozin also has the ability to regulate glycemia levels and provoke weight loss in overweight diabetic patients. Thus, these pharmacological agents modulate whole-body glucose metabolism and energy homeostasis (38, 61). One of the objectives of this study was to evaluate the impact that the treatment with each one of these drugs has on Sertoli cells viability and glucose metabolism. Moreover, the combination of these pharmacological agents has gain importance in the treatment of metabolic diseases due to their efficiency in regulating glycemia and provoking weight loss. Perhaps due to their distinct mechanism of action, exenatide and dapagliflozin combined therapy seem to have an additive effect in relation to the treatment of the drugs separately (80). Thus, it seemed relevant to study if the combined therapy of these two drugs also had an additive effect on Sertoli cells metabolism.

Cytotoxic assays showed that exenatide treatment has no impact on TM4 Sertoli cell proliferation. Similar results had already been described in adult rodent insulin producing cells (191) although, in the same study exenatide increased proliferation of immature mice β -cells. In another study, GLP-1 receptor agonist liraglutide decreased mice vascular smooth muscle cells proliferation (192). These studies point to the ability of exenatide modulating cell proliferation may differ in relation to cell type or metabolic age. In addition, our data showed that exenatide decreased metabolic viability of Sertoli cells when present at sub-pharmacological concentrations (2,5 pM)

and supra-pharmacological concentrations (250 pM), but not when at pharmacological concentrations (25 pM). In the case of dapagliflozin, our results showed that pharmacological (500 nM) and supra-pharmacological (5000 nM) concentrations of dapagliflozin negatively impact TM4 Sertoli cells proliferation rate. These results are supported by a recent study which described that an SGLT2 inhibitor (canagliflozin) inhibited the proliferation of mice endothelial cells at concentrations of 50 μ M (193). In addition, all the concentrations of dapagliflozin tested in this study affected TM4 Sertoli cells metabolic viability. As referred above, the exposure of TM4 Sertoli cells to the pharmacological concentrations of exenatide did not change cell proliferation or metabolic viability. Contrastingly, when at a pharmacological concentration, dapagliflozin was able to decrease both cell proliferation and metabolic viability on TM4 Sertoli cells. Moreover, the combined treatment of these cells with exenatide plus dapagliflozin also led to a decrease in cell proliferation and metabolic viability, probably due to the presence of dapagliflozin at 500 nM. These results indicate that exenatide was incapable of protecting the Sertoli cells from the mildly cytotoxic effect of dapagliflozin when these two drugs were used in combination. Studies have been published describing that GLP-1 receptor agonists are capable of protecting mitochondrial malfunction from disrupting agents (e.g. TNF- α and iron ions) (194, 195). In our experiments, the GLP-1 receptors agonist used (exenatide) did not seem to have a positive influence on TM4 Sertoli cell proliferation, once the restrictive effect observed in the treatment with dapagliflozin prevailed in the combined treatment.

In addition, exenatide showed to decrease membrane integrity of TM4 Sertoli cells in every concentration used. Previous studies with this GLP-1 receptor agonist made in rodent pancreatic tissue described that exenatide (5 μ g/kg) provoked changes in pancreatic cell morphology and increased cellular necrosis (196). Dapagliflozin demonstrated a similar result, decreasing plasmatic membrane integrity of TM4 Sertoli cells in all concentrations studied. However, concerning this drug and the class of drugs it belongs to, little information is available about their effect on cellular membrane integrity. Interestingly, the cells subjected to the combination treatment showed that the membrane integrity of the cells from this group was preserved, when compared to that of the cells from the control group. The drug combination avoided the damages in the cellular membrane observed when the cells were exposed to the drugs separately.

The metabolism of glucose in Sertoli cells is essential for spermatogenesis in order to ensure the secretion of metabolic substrates for the developing germ cells. In healthy Sertoli cells, the vast majority of the glucose consumed suffers glycolysis and is metabolized into pyruvate and only a

very small fraction of the glucose is used for glycogen synthesis (197). Moreover, only a small part of the pyruvate is oxidized via TCA cycle, being that the vast majority (95% of the total consumed glucose) is used for the synthesis of lactate, which is secreted for the developing germ cells. Lactate is the preferential substrate for the cells that are undergoing spermatogenesis, being also an antiapoptotic factor for these cells (119). Therefore, any compound that disrupts this metabolic dynamic can be detrimental to male fertility.

GLP-1 is an incretin that has been shown to modulate human Sertoli cells glucose metabolism in a previous study developed by our research group (61). Exenatide is an analogue of GLP-1, being a synthetic version of Exendin-4, a peptide hormone found in the saliva of the Gila monster. In our experimental work, the exposure of TM4 Sertoli cells to this synthetic peptide in the culture medium seemed to maintain the glycolytic flux in these cells. Our results indicate that all the concentrations of exenatide used were able to maintain glucose consumption, the production of the monocarboxylates (lactate and acetate) and alanine by TM4 Sertoli cells, when compared to that of the cells from the control group. Also, LDH expression and activity was not altered in the presence of exenatide. These results suggest that exenatide does not alter the ability of Sertoli cells to supply high quantities of lactate to the developing germ cells in any of the concentrations tested in this study. Sertoli cells also secrete acetate for the developing germ cells, which use it for lipid synthesis (156). Our results showed consumption of acetate by Sertoli cells exposed to the concentrations of 2,5 pM and 25 pM of exenatide, instead of a production noted in the control group. This change did not reach statistical relevance possibly due to the reduced amounts of acetate that is either produced or consumed in our study model (TM4 Sertoli cell line). Previous studies using *in vitro* cultured human Sertoli cells exposed to GLP-1 at physiological post-prandial concentrations (10 pM) showed a reduction on glucose consumption and an increase of lactate production, after 6 hours of exposure (186). The discrepancy between these results and the ones we obtained might be due either to the difference in the exposure time and/or to different sensitivity to these two peptides (exenatide and GLP-1). It is worth noticing that exenatide has a half-life time 20 to 30 times superior when compared to GLP-1 (65). This fact can also explain the increased transcription of GLUT2 observed when TM4 Sertoli cells were treated with the pharmacological concentration (25 pM) of exenatide. GLUT2 is described to be one of the most predominant transporters of glucose in Sertoli cells (124) and its expression can modulate glucose consumption (20, 187). Thus, increasing GLUT2 expression could be the mechanism by which Sertoli cells resorted to balance a hypothetical decreased glucose consumption, like the one reported in human

Sertoli cells in the study mentioned above. This fact is one more example of the adaptative abilities and metabolic plasticity of the Sertoli cells to different external conditions (186, 187). Without an alteration of the glucose consumption, as observed in our study, TM4 Sertoli cells did not have the necessity of mobilizing their glycogen and lipid reserves as we report. However, TM4 Sertoli cells were not insensitive to exenatide. Sub-pharmacological concentrations (2,5 pM) increased the lactate/alanine ratio. This ratio is often associated with the redox state of the cell (187). Interestingly, lactate/alanine ratio increased in the Sertoli cells exposed to this same concentration, which further suggests a decreased metabolic viability in these cells. In fact, when TM4 Sertoli cells were exposed to exenatide at concentrations of 2,5 pM, we also observed depolarization of the mitochondrial membrane. With this data, we hypothesized that exenatide at 2,5 pM enhanced NADH/NAD⁺ ratio, which decreased the metabolic viability demonstrated by the mild depolarization of the mitochondrial membrane on TM4 Sertoli cells. In the cells exposed to the other exenatide concentrations, lactate and alanine production and the ratio NADH/NAD⁺ was not altered, which did not correlate with any mitochondrial membrane depolarization.

The study of the metabolic secretome of TM4 Sertoli cells exposed to dapagliflozin showed that glucose consumption did not suffer any significant change. One of the main pharmacological property of this drug is to block the SGLT2 and it has been shown that SGLT2 inhibitors decrease cellular glucose consumption in other cellular systems (198). However, our study revealed that cells incubated with 5000 nM of dapagliflozin had enhanced GLUT2 expression levels, one important glucose mobilizer on Sertoli cells (124). Enhanced expression of GLUT2 could compensate for the blockage of the SGLT2 and sustain the glucose consumption in these Sertoli cells. Moreover, even without increasing glucose consumption, exposure to pharmacological concentrations of dapagliflozin (500 nM) enhanced the lactate secretion on mice Sertoli cells. This fact could be positive for male fertility since lactate is the preferential energy substrate for developing germ cells (20, 187). MCTs are the membrane transporters responsible for the secretion of the lactate to the developing germ cells. The major MCT in Sertoli cells is MCT4 and its expression levels were not changed when TM4 Sertoli cells were treated with dapagliflozin. Still, we are able to describe for the first time the expression of on specific cataloged MCT4 isoform (Uniprot: B1ATM2), which has a very similar primary and secondary structure in relation to the original isoform. However, the molecular weight is approximately 30 kDa in contrasts with the one of the original MCT4 isoform which has a molecular weight of approximately 50 kDa. Moreover, the transcription of B1ATM2 isoform gene was only found in mice (199) up until now. The B1ATM2

MCT4 isoform had its expression levels increased after the treatment with pharmacological concentrations (500 nM) of dapagliflozin. This fact can explain the superior lactate levels observed in the extracellular medium. However, due to the lack of studies assessing the 3D structure and functional properties of this MCT4 protein isoform further studies are needed to support this suggestion.

To further understand the mechanism of action of dapagliflozin, we investigated if other carbon sources were being used for the increased production of lactate observed in the cells exposed to the pharmacological concentrations (500 nM). It has been described that Sertoli cells are capable of utilizing glycogen for the production of lactate in conditions with lower glucose availability and maintain their energetic needs (119, 189). Our results showed no changes in TM4 Sertoli cells glycogen content exposed to dapagliflozin. We must highlight that our study was performed with a culture medium supplemented with high glucose levels, mimicking the ones found in hyperglycemic individuals (3). In these conditions, it has been described that rodent Sertoli cells increased their glycogen reserves (200). Hence, we can hypothesize that both drug treatments and the hyperglycemic medium had opposite effects on glycogen reserves which could explain the non-alteration of the glycogen levels on TM4 Sertoli cells. After this, we evaluated the quantity of lipid droplets in the TM4 Sertoli cells after the treatment with dapagliflozin. It is widely described that Sertoli cells can metabolize lipids and mobilize its reserves when needed (119, 201). However, this was not the case for TM4 Sertoli cells treated with pharmacological concentrations of dapagliflozin (500 nM). Thus, our hypothesis for the enhanced lactate and alanine production on TM4 Sertoli cells exposed to 500 nM of dapagliflozin is that this treatment enhances the glycolytic efficiency of the cells. This could be proved through alanine production which is often considered a marker of glycolytic activity (202). Alanine synthesis is fundamental for the maintenance of the lactate/alanine ratio, which is not altered in the Sertoli cells exposed to the pharmacological concentrations of dapagliflozin (500 nM). Although our results showed that LDH expression and activity was maintained in these conditions. It is known that this enzyme is able to catalyze not only the forward reaction but also the reversible reaction. Thus, LDH can be prioritizing lactate synthesis when cells are exposed to this concentration of dapagliflozin. Is also worth noticing that dapagliflozin has the ability to diminish lipid droplets on TM4 Sertoli cells at supra-pharmacological concentrations. This evidences the anti-obesogenic ability of the dapagliflozin. In fact, it has already been described that people undergoing dapagliflozin treatment have enhanced levels of ketone bodies in circulation (171). In addition, diabetic patients that added dapagliflozin (5 mg/day) to its

natural treatment had their liver fat accumulation decreased during the 6-month treatment (203). Our study points out that dapagliflozin at supra-pharmacological concentrations (5000 nM) is also able to decrease intracellular fat deposits in Sertoli cells.

Combined treatment of exenatide plus dapagliflozin did not alter the glycolytic flux of TM4 Sertoli cells. Moreover, enhanced lactate and alanine production and increased MCT4 B1ATM2 isoform expression levels observed in cells treated with pharmacological concentration of dapagliflozin were not evident when treated with the same dapagliflozin concentration but also in the presence of exenatide. Taking these results in consideration, combined therapy of exenatide and dapagliflozin does not seem to have an additive effect at a cellular level, at least in the glucose metabolism of TM4 mice Sertoli cells. The presence of exenatide in the medium seemed to promote the metabolic homeostasis of TM4 Sertoli cells. In fact, the drugs seem to have more of a synergic effect to each other at a cellular level rather than the additive effect seen in human clinical trials. Still, we must be aware that our study was developed using TM4 Sertoli cell line, which is an immortalized cell line. TM4 Sertoli cells maintain the hormone responsiveness yet, it is not the same magnitude of healthy human Sertoli cells. Moreover, TM4 Sertoli cells are under constant division which contrasts with post-pubertal humans' Sertoli cells that stop their division, which can also alter the cell metabolism. However, we believe that besides these limitations our work is indicative of what happens in Sertoli cells when treated with the different therapies here tested.

Chapter VI

CONCLUSION

6. CONCLUSION

Metabolic diseases, such as DM and obesity, are rising among males in reproductive age. Along with this increase, subfertility and infertility incidence is also rising. To counteract this tendency, prevention with a healthy lifestyle should be the priority. However, in specific cases, pharmacological treatments allow to control glycemia levels and provoke weight loss in a safe manner. Often, two target pharmacological agents used in those treatments are the GLP-1 receptor agonist exenatide and the SGLT2 inhibitor dapagliflozin. Both can be used in combined treatment and have shown to efficiently control metabolic diseases comorbidities with relative safety. Although, as they modulate glucose metabolism, they can impact Sertoli cells glucose metabolism and influence spermatogenesis. To evaluate that, we incubated TM4 Sertoli cells with sub-pharmacological, pharmacological, and supra-pharmacological concentration of both exenatide and dapagliflozin. Moreover, the combination of both pharmacological concentrations was also tested and its effect on Sertoli cell viability and glucose metabolism were evaluated.

In sum, with this work, we were able to notice that pharmacological concentrations of exenatide do not modulate Sertoli cell division or metabolic viability. Likewise, Sertoli cell glucose metabolism was maintained after exposure to 25 pM of exenatide. Dapagliflozin pharmacological concentration caused a mildly detrimental effect on Sertoli cell proliferation and metabolic viability. However, the same concentration was capable of increasing lactate production on Sertoli cells due to enhanced glycolytic efficiency, which can be a good indicator for spermatogenesis. Moreover, dapagliflozin at 5000 nM showed to have anti-obesogenic properties at a cellular level. Interestingly, the combined therapy, while it still had an impact on cell division and metabolic viability, it restored the glycolytic flux, indicating that both drugs have a synergic effect on maintaining metabolic homeostasis on treated Sertoli cells. Further studies will be needed to confirm the hypothesis presented in this work and to further unveil the mechanism of action of each drug on Sertoli cells metabolism and male fertility.

As future perspectives would be interesting to evaluate the effect of the therapies at shorter time points with human Sertoli cells or animal models and evaluate if the alterations observed in the metabolism of these cells can jeopardize spermatogenesis or steroidogenesis. These studies would increase our knowledge about the side effects of the pharmacological agents used in this study.

Chapter VII

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7. REFERENCES

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