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High-Energy Diets and Diabetes Mellitus: a Threat for Male Fertility

Luís Pedro Ferreira Rato

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Supervisor: Carlos Pedro Fontes Oliveira, PhD
Co-supervisor: José Eduardo Brites Cavaco, PhD
Co-supervisor: Ana Isabel Marques Duarte, PhD

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Dedication

A todos aqueles que se dedicam arduamente no avanço da saúde reprodutiva masculina.

"Every sperm is sacred", Monthy Python in *The Meaning of Life*.

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Resumo

A sobrevivência do ser humano reside numa fertilidade saudável, no entanto, nos últimos anos tem-se observado um declínio na fertilidade masculina. Este problema tem particular incidência nas sociedades modernas, e a curto prazo estará presente nos países em desenvolvimento. Factores externos associados ao estilo de vida, tais como maus hábitos alimentares, particularmente a ingestão excessiva de dietas de alta energia, têm contribuído para o aumento de doenças metabólicas, nomeadamente a obesidade e a diabetes mellitus (DM). Na verdade, a combinação de factores como: mudanças na composição de alimentos; aumento do consumo de dietas de alta energia; consumo de alimentos com altos níveis de açúcar e gorduras saturadas de elevada palatibilidade; o sedentarismo e a falta de atividade física são a principal causa para o incremento destas patologias. A obesidade e a DM são problemas de saúde pública nos países desenvolvidos e a sua incidência tem vindo a aumentar rapidamente entre os homens em idade reprodutiva, contribuindo para o surgimento da subfertilidade e infertilidade nesses indivíduos. A desregulação metabólica e endócrina associada a estes estadios patológicos compromete a função reprodutora masculina, uma vez que o eixo hipotálamo-hipófise-testículo, também conhecido como eixo reprodutivo, é sensível a alterações metabólicas.

O tecido testicular é composto por uma população heterogénea de células somáticas e germinativas. As células germinativas estão dependentes do apoio nutricional fornecido pelas células de Sertoli, e distúrbios metabólicos podem perturbar essa cooperação metabólica. O metabolismo das células testiculares, em particular o das células de Sertoli, apresenta características únicas, uma vez que estas células são capazes de metabolizar vários substratos (*e.g.*: glucose, ácidos gordos, corpos cetónicos). As células de Sertoli metabolizam preferencialmente a glucose, sendo que a maioria desta é convertida a lactato, e não oxidada através do ciclo de Krebs. Os mecanismos que regulam o metabolismo das células de Sertoli são essenciais para a espermatogénese, e este processo metabólico é controlado por vários fatores, entre eles a insulina e as hormonas esteróides sexuais.

Doenças metabólicas, como a DM, apresentam na sua origem resistência à insulina e/ou ausência de insulina, bem como uma incapacidade de as células responderem de forma eficiente à estimulação por esta hormona. Dada a importância da insulina no metabolismo da glucose e o facto de as células de Sertoli expressam receptores específicos para esta hormona, avaliámos o comportamento metabólico das células de Sertoli em situações de privação de insulina. Nestas condições, as células de Sertoli alteram o seu metabolismo glicolítico, diminuíndo a taxa de produção de lactato através da modulação da expressão de proteínas associadas à produção e exportação de lactato, sugerindo que são afectadas na sua actividade metabólica em condições patológicas associadas à desregulação da insulina, como é o caso da DM.

No entanto, a DM induz uma desregulação endócrina generalizada. Uma consequência directa da DM na função testicular é a inibição da síntese de testosterona (T). Neste trabalho, demonstramos que os esteróides sexuais, particularmente a testosterona (e o seu metabolito 5α-di-hidrotestosterona) e o 17β-estradiol (E_2), modulam o metabolismo glicolítico das células de Sertoli, favorecendo o consumo de glucose, sem contudo promoverem a síntese de lactato. De facto, a produção de lactato, que é o principal substrato para o desenvolvimento das células germinativas, encontrava-se diminuída pela acção androgénica, o que poderá resultar num comprometimento da cooperação metabólica testicular.
Quanto mais severo é o estado de progressão da DM, maior é a redução dos níveis da T. Desta forma, estudámos os efeitos da desregulação dos níveis da T induzidos por diferentes estadios da DM, pré-diabetes e diabetes mellitus tipo 2 no metabolismo glicolítico das células de Sertoli. Os resultados mostraram que quanto mais avançado é o estado da doença, mais a via glicolítica está comprometida, verificando-se igualmente uma alteração mais acentuada na maquinaria celular associada à produção de lactato em células em cultura com níveis de T associados à diabetes mellitus tipo 2. Curiosamente, as células de Sertoli em cultura em condições de T similares ao observado no estado de diabetes mellitus tipo 2 mostram que são capazes de adoptar mecanismos que promovem o uso de substratos alternativos, como é o caso do glicogénio.
Ao nível do testículo, evidenciou-se que o estado de pré-diabetes induzido pelo consumo de dietas de alta energia também altera o metabolismo glicolítico. Nestas condições, a via glicolítica está favorecida devido ao aumento da expressão e actividade de proteínas essenciais que intervêm nessa via metabólica. Também a expressão de proteínas associadas à produção de lactato está aumentada, o que parece contribuir para o aumento observado no lactato testicular. No entanto, e apesar da adaptação metabólica evidenciada, os parâmetros reprodutivos são seriamente afectados, o que pode resultar do favorecimento de um ambiente testicular oxidativo. De facto, nessas condições observou-se uma diminuição significativa da expressão de proteínas envolvidas tanto na manutenção da biogénese mitocondrial, como na activação do sistema de defesa contra as espécies reactivas de oxigénio. O potencial antioxidante testicular diminuído, assim como, a alteração na respiração mitocondrial testicular contribuíram igualmente para uma deficiente capacidade bioenergética e um aumento do ambiente oxidativo. Já em estadios mais avançados da DM, como é o caso da diabetes mellitus tipo 2, observou-se que o metabolismo glicolítico testicular é seriamente comprometido. A actividade da lactato desidrogenase está severamente diminuída, contribuindo para uma diminuição do conteúdo em lactato neste tecido. Porém, nestas condições destaca-se a adaptação verificada no metabolismo testicular, que promoveu um aumento do conteúdo de glicogénio nos testículos. Estes resultados indicam que a diabetes mellitus tipo 2 induz uma reprogramação metabólica testicular, promovendo vias metabólicas alternativas. No entanto, os parâmetros espermáticos dos indivíduos que sofriam desta condição estavam comprometidos, visto que a

motilidade e viabilidade dos espermatozóides estavam acentuadamente diminuídas e o número de espermatozóides com anomalias na morfologia era elevado.
Em conclusão, este trabalho demonstra que as doenças metabólicas, particularmente a DM, podem contribuir para uma diminuição do potencial reprodutivo masculino induzindo alterações profundas no metabolismo celular do testículo, e em particular no metabolismo das células de Sertoli. O processo da espermatogénese é complexo e, do ponto de vista fisiológico, o metabolismo glicolítico é essencial para o sucesso deste evento celular. A regulação do metabolismo da glucose nas células de Sertoli é alvo de vários factores, e tanto nos estadios iniciais da DM, como nos estadios mais avançados, sofre alterações, sendo que são mais pronunciadas em estadios avançados da doença. De facto, verificou-se que em estadios iniciais da DM o metabolismo testicular tende a adaptar-se de modo a assegurar a produção de lactato para as células germinativas em desenvolvimento. Todavia com a progressão da doença, a produção desse metabolito energético é seriamente comprometida. De facto, estas alterações metabólicas progressivas estão ainda associadas a uma diminuição dos parâmetros espermáticos, que seguramente serão responsáveis pelo declínio da saúde reprodutiva masculina.

Palavras-chave

Fertilidade masculina; Pre-diabetes; Diabetes mellitus; Diabetes mellitus Tipo 2; Metabolismo testicular, Células de Sertoli, Dietas de alta energia.

Resumo Alargado

A sobrevivência do ser humano reside numa fertilidade saudável, no entanto, nos últimos anos tem-se observado um declínio na fertilidade masculina. Este problema tem particular incidência nas sociedades modernas, e a curto prazo estará presente nos países em desenvolvimento. Factores externos associados ao estilo de vida, tais como maus hábitos alimentares, particularmente a ingestão excessiva de dietas de alta energia, têm contribuído para o aumento de doenças metabólicas, nomeadamente a obesidade e a diabetes mellitus (DM). Na verdade, a combinação de factores como: mudanças na composição de alimentos; aumento do consumo de dietas de alta energia; consumo de alimentos com altos níveis de açúcar e gorduras saturadas de elevada palatibilidade; o sedentarismo e a falta de atividade física são a principal causa para o incremento destas patologias. A obesidade e a DM são problemas de saúde pública nos países desenvolvidos e a sua incidência tem vindo a aumentar rapidamente entre os homens em idade reprodutiva, contribuindo para o surgimento da subfertilidade e infertilidade nesses indivíduos. A desregulação metabólica e endócrina associada a estes estadios patológicos compromete a função reprodutora masculina, uma vez que o eixo hipotálamo-hipófise-testículo, também conhecido como eixo reprodutivo, é sensível a alterações metabólicas.

O tecido testicular é composto por uma população heterogénea de células somáticas e germinativas. As células germinativas estão dependentes do apoio nutricional fornecido por células de Sertoli, e distúrbios metabólicos podem perturbar essa cooperação metabólica. O metabolismo das células testiculares, em particular o das células de Sertoli, apresenta características únicas, uma vez que estas células são capazes de metabolizar vários substratos (*e.g.*: glucose, ácidos gordos, corpos cetónicos). As células de Sertoli metabolizam preferencialmente a glucose, sendo que a maioria desta é convertida a lactato e não oxidada através do ciclo de Krebs. Os mecanismos que regulam o metabolismo das células de Sertoli são essenciais para a espermatogénese, e este processo metabólico é controlado por vários fatores, entre eles a insulina e as hormonas esteróides sexuais.

Doenças metabólicas, como a DM, apresentam na sua origem resistência à insulina e/ou ausência de insulina, bem como uma incapacidade de as células responderem de forma eficiente à estimulação por esta hormona. Dada a importância da insulina no metabolismo da glucose e o facto de as células de Sertoli expressam receptores específicos para esta hormona, avaliámos o comportamento metabólico das células de Sertoli em situações de privação de insulina. Verificou-se que na ausência de insulina as células de Sertoli alteram o seu metabolismo glicolítico. Nas primeiras horas de cultura observou-se uma diminuição no consumo de glucose que foi depois revertida. Este efeito parece ser devido à modulação da expressão dos transportadores de glucose 1 (GLUT1) e 3 (GLUT3) presentes na membrana dessas células. Já na produção de lactato (o principal substrato para as células germinativas em desenvolvimento) observou-se uma diminuição substancial. Esta alteração terá resultado

da diminuição na expressão da LDH e MCT4. Estes resultados sugerem que as células de Sertoli são afectadas na sua actividade metabólica em condições patológicas associadas à desregulação da insulina, como é o caso da DM.

No entanto, a DM induz uma desregulação endócrina generalizada. Uma consequência directa da DM na função testicular é a inibição da síntese de testosterona (T) e uma eventual desregulação da razão androgénios/estrogénios. Neste trabalho, mostramos que os esteróides sexuais, nomeadamente a 5α-di-hidrotestosterona (um androgénio não aromatizável) e o 17ß-estradiol (E_2), modulam o metabolismo da glucose das células de Sertoli. Células de Sertoli humanas em cultura com 5α-di-hidrotestosterona ou com E_2, observou-se que o piruvato é prontamente consumido nas primeiras horas de cultura. Também observámos que no grupo de células tratadas com 5α-di-hidrotestosterona o consumo de glucose estava aumentado, embora a produção de lactato estivesse diminuída. Para estes resultados podem ter contribuído a diminuição da expressão de enzimas associadas à produção e exportação de lactato, nomeadamente a lactato desidrogenase (LDH) e o transportador de monocarboxilato 4 (MCT4). Estes resultados sugerem que a acção androgénica pode levar a um "desvio" no metabolismo glicolítico para o ciclo de Krebs e a um comprometimento da cooperação metabólica testicular.

Quanto mais severo é o estado de progressão da DM, maior é a redução dos níveis da T. Desta forma, estudámos os efeitos da desregulação dos níveis da T induzidos por diferentes estadios da DM, pré-diabetes e diabetes mellitus tipo 2, no metabolismo glicolítico das células de Sertoli. As células de Sertoli foram expostas a concentrações fisiológicas de T e de E_2 evidenciadas em estadios de pré-diabetes e de diabetes mellitus tipo 2. Os resultados mostraram que o metabolismo glicolítico das células de Sertoli em cultura em condições hormonais similares à pré-diabetes é comprometido, dado que o consumo de glucose e piruvato se encontrava diminuído. Analisámos a expressão e actividades de proteínas envolvidas na via glicolítica e observámos uma diminuição na expressão da enzima fosfofrutoquinase (PFK), que cataliza um dos passos limitantes da via glicolítica, evidenciando um ponto de comprometimento da via glicolítica. Contudo, não se verificaram diferenças significativas na produção de lactato nas células de Sertoli cultivadas nestas condições. Quanto às células cultivadas em condições hormonais similares ao estado de diabetes mellitus tipo 2, verificou-se que o consumo de glucose estava significativamente aumentado no final do tratamento, embora a produção de lactato não tenha acompanhado esse aumento. Na verdade, observámos uma alteração mais acentuada na maquinaria celular associada à produção de lactato em células cultivadas com níveis de T associados à diabetes mellitus tipo 2. Contrariamente à produção de lactato, as células de Sertoli cultivadas nestas condições apresentaram um aumento significativo da produção de alanina, indicando assim uma diminuição do estado redox da célula e consequentemente da capacidade glicolítica. É ainda assim de realçar que as células de Sertoli cultivadas em condições hormonais similares ao estadio de diabetes mellitus tipo 2 exibem mecanismos que promovem o uso de substratos alternativos, como é o caso do glicogénio, que sendo uma "forma armazenada de glucose"

poderá servir de reserva energética para fazer face às necessidades energéticas das células germinativas. Estes resultados sugerem que quanto mais avançado é o estado da doença, mais pronunciados são os efeitos no metabolismo das células de Sertoli.

De facto, quando avaliámos as alterações no metabolismo glicolítico no tecido testicular, evidenciou-se que o estado de pré-diabetes induzido pelo consumo de dietas de alta energia favorece a via glicolítica, devido ao aumento da expressão e actividade de proteínas essenciais que intervêm nessa via metabólica (como é o caso GLUT1, GLUT3 e PFK). Também a expressão de proteínas associadas à produção de lactato, nomeadamente a LDH e MCT4 está aumentada e esta alteração contribuirá para o aumento observado no lactato testicular. No entanto, e apesar da adaptação metabólica evidenciada, os parâmetros espermáticos dos indivíduos pré-diabéticos estavam afectados, o que pode resultar do favorecimento de um ambiente testicular oxidativo. Nessas condições, observou-se uma diminuição da expressão de proteínas envolvidas tanto na manutenção da biogénese mitocondrial, como na activação do sistema de defesa contra as espécies reactivas de oxigénio, nomeadamente do coativador 1 α do receptor γ activado pelo proliferador de peroxissoma e da sirtuína 3. O potencial antioxidante testicular diminuído, assim como, a alteração na respiração mitocondrial testicular contribuíram igualmente para uma deficiente capacidade bioenergética e um aumento do ambiente oxidativo. Verificou-se uma diminuição do conteúdo de DNA mitocondrial e uma desregulação da actividade de complexos da cadeia respiratória mitocondrial, uma vez que se observou um aumento na actividade do complexo I, ao passo que a actvidade do complexo III estava diminuído. Por conseguinte, os níveis testiculares de ATP e ADP encontravam-se reduzidos, contrariamente aos níveis de AMP, evidenciado-se um decréscimo na razão ATP/AMP e, por conseguinte da carga energética testicular. Em simultâneo, o potencial antioxidante testicular total estava diminuído, sendo acompanhado pelo aumento da peroxidação lipídica e oxidação proteica. Já em estadios mais avançados da DM, como é o caso da diabetes mellitus tipo 2, observou-se que o metabolismo glicolítico testicular é seriamente comprometido e que actividade da lactato desidrogenase está severamente diminuída, contribuindo para uma diminuição do conteúdo em lactato neste tecido. Porém, nestas condições destaca-se a adaptação verificada no metabolismo testicular, que promoveu um aumento do conteúdo de glicogénio nos testículos. Estes resultados indicam que a diabetes mellitus tipo 2 induz uma reprogramação metabólica testicular, promovendo vias metabólicas alternativas. No entanto, também neste estadio, os parâmetros espermáticos dos indivíduos que sofriam desta condição estavam comprometidos, sendo que a motilidade e viabilidade estavam acentuadamente diminuídas e o número de espermatozóides com anomalias na morfologia era elevado.

Em conclusão, este trabalho evidencia que as doenças metabólicas, especificamente a DM, podem contribuir para uma diminuição do potencial reprodutivo masculino induzindo alterações profundas no metabolismo celular do testículo, e em particular no metabolismo das células de Sertoli. O processo da espermatogénese é complexo e, do ponto de vista fisiológico, o metabolismo glicolítico é essencial para o sucesso deste evento celular. O

metabolismo da glucose nas células de Sertoli é alvo de vários factores regulatórios e tanto nos estadios iniciais da DM, como nos estadios mais avançados, sofre alterações, sendo que são mais pronunciadas em estadios avançados da doença. De facto, verificou-se que em estadios iniciais da DM o metabolismo testicular tende a adaptar-se de modo a assegurar a produção de lactato para as células germinativas em desenvolvimento. Todavia com a progressão da doença, a produção desse metabolito energético é seriamente comprometida. De facto, estas alterações metabólicas progressivas estão ainda associadas a uma diminuição dos parâmetros espermáticos, que seguramente serão responsáveis pelo declínio da saúde reprodutiva masculina.

Abstract

The survival of the human being lies in a healthy fertility, however, in last decades it has been observed a decline in male fertility. This problem has a particular focus in modern societies, but in the near future will be present in developing countries. External factors associated with lifestyle, such as erroneous eating habits, particularly the excessive intake of high energy diets, have contributed to the increase of metabolic diseases, including obesity and diabetes mellitus (DM). Indeed, the combination of factors such as: changes in the composition of foods, increased consumption of high-energy diets, consumption of foods with high levels of sugar and saturated fats, sedentary lifestyle and the lack of physical activity are the main cause for the increase of this pathology. Obesity and DM are public health problems in developed countries and its incidence has been increasing rapidly among men of reproductive age, contributing to the emergence of subfertility and infertility in these individuals. The metabolic and hormonal dysregulation associated with these pathological stages compromises the male reproductive function, since the hypothalamus-pituitary gonadal axis, also known as reproductive axis, is sensitive to the subtle metabolic disturbances.

Testicular tissue consists of a heterogeneous population of somatic and germ cells, where germ cells are dependent on the nutritional support provided by Sertoli cells and any metabolic disorder may alter this metabolic cooperation. Metabolism of testicular cells, in particular of Sertoli cells, present some unique features. Sertoli cells are able to metabolize various substrates (*e.g.*: glucose, fatty acids, ketone bodies), preferentially metabolizing glucose, being the majority of it converted to lactate and not oxidized via Krebs' cycle. The mechanisms that regulate the metabolism of Sertoli cells are essential for spermatogenesis and this metabolic process is regulated by several factors, among which insulin and sexual steroid hormones play an important role.

Metabolic diseases, such as DM, present in its origin insulin resistance and/or absence, as well as an inability of cells to efficiently respond to insulin stimulation. Given the relevance of this hormone on glucose metabolism and the fact that Sertoli cells express the specific receptors for insulin, we evaluated the metabolic behavior of Sertoli cells under insulin deprivation conditions. In these circumstances, Sertoli cells altered their glycolytic metabolism, decreasing the rate of lactate production through the modulation of the expression of proteins associated with the production and export of lactate. This suggests that Sertoli cells are affected in their metabolic activity under specific pathological conditions associated with insulin deregulation, such is the case of DM.

DM induces a generalized endocrine disruption. A direct consequence of DM on testicular function is the inhibition of the synthesis of testosterone (T) and the more severe is the state of DM, the greater the reduction in levels of T. In this work, we showed that the sex steroids, particularly testosterone (and its non aromatizable metabolite 5α-dihydrotestosterone) and 17ß-estradiol, modulate the glycolytic metabolism of Sertoli cells, favoring the increase of

glucose consumption, although the production of lactate is not promoted. In fact, lactate production, which is the primary substrate of developing germ cells, is diminished by the androgenic action.
We further studied the effects of T deficiency induced by different stages of DM, pre-diabetes and type 2 diabetes mellitus, in the glycolytic metabolism of Sertoli cells. Our results showed that the more advanced is the state of the disease, the more the glycolytic pathway is compromised. Interestingly, Sertoli cells cultured under T conditions similar to those of type 2 diabetes mellitus stage are able to adopt alternative mechanisms that promote the use of alternative substrates, such as glycogen.
At testicular level, it was evidenced that the pre-diabetic state induced by high-energy diets consumption also alters the glycolytic metabolism. Under these conditions, the glycolytic pathway is favored, given the increased expression and activity of essential proteins involved in this metabolic pathway. The expression of proteins associated with the production of lactate is also increased, which may have contributed to the increase in the testicular lactate content. However, and despite the metabolic adaptation observed, the reproductive parameters were affected, which may result from the favoring of a high oxidative environment. In fact, in those conditions, we observed a significant decrease in the expression of proteins involved not only in the maintenance of mitochondrial biogenesis, as in the activation of the reactive oxygen species defense system. The decreased testicular antioxidant potential, as well as the altered mitochondrial respiratory function contributed to a deficient bioenergetic capacity and augmented oxidative environment. In more advanced states of disease, as is the case of type 2 diabetes mellitus, testicular glycolytic metabolism was seriously compromised. Lactate dehydrogenase activity was severely diminished contributing to lower testicular content of lactate. Moreover, in those conditions there seems to be an adaptation of the testicular metabolism, reflected in the content of glycogen in the testes, which was increased. These results implied a testicular metabolic reprogramming under type 2 diabetes mellitus conditions, which promoted alternative metabolic pathways. However, the sperm parameters of the individuals with type 2 diabetes mellitus were seriously compromised, since motility and viability were substancially decreased and the number of sperm with abnormal morphology was increased.
In conclusion, this study showed that metabolic diseases, particularly DM, contribute to a decrease in male reproductive potential by promoting profound alterations in testicular cellular metabolism, and particularly in the metabolism of Sertoli cells. Spermatogenesis is a complex process and the glycolytic metabolism is pivotal for the success of this cellular event. Glucose metabolism in Sertoli cells is targeted by numerous regulatory factors and both the initial and the advanced stages of DM the metabolism of glucose is altered in these cells. Furthermore, the more pronounced effects were observed in the most advanced stages of DM. In fact, we observed that, in the prodromal stage of DM, testicular metabolism tends to adapt in order to ensure an adequate production of lactate for developing germ cells. However, in more advanced stages of that disease, lactate production is seriously

compromised. Moreover, these metabolic changes were associated with a decline in the reproductive parameters, that may lead to infertility, and that surely will be accountable for the decline in male reproductive health.

Keywords

Male fertility; Pre-diabetes; Diabetes mellitus; Type 2 Diabetes mellitus; Testicular metabolism, Sertoli cells, High-energy diets.

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List of Abbreviations

^{1}H-NMR	proton nuclear magnetic resonance
5α-DHT	5α-dyhidrotestosterone
a.u.	arbitrary units
ADP	adenosine diphosphate
AEC	adenylate energy charge
ALT	alanine aminotransferase
ALT2	alanine aminotransferase 2
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	adenosine triphosphate
AUC_g	area under the curve for glucose tolerance test
AUC_{GTT}	area under the curve for the glucose tolerance test
AUC_{ITT}	area under the curve for the insulin tolerance test
bFGF	basic fibroblast growth factor
BMI	body mass index
BPA	bisphenol A
BSA	bovine serum albumine
BTB	blood-testis barrier
Ck-18	cytokeratin-18
DCPIP	dichlorophenolindophenol
DM	diabetes mellitus
DNPH	2,4- dinitrophenylhydrazine
dNTPs	deoxynucleotide triphosphates
E_2	17ß-estradiol
EGF	epidermal growth factor
ERα	estrogen receptor α
ETC	electron transport chain
FRAP	ferric reducing antioxidant power
FSH	follicle-stimulating hormone
G-6-P	glucose-6-phosphate
GK	Goto-Kakizaki
GLUT1	glucose transporter 1
GLUT2	glucose transporter 2
GLUT3	glucose transporter 3
GLUT8	glucose transporter 8
GLUTs	glucose transporters
GnRH	gonadotropin-releasing hormone

GSK-3	glycogen synthase kinase 3
GYS1	muscle glycogen synthase
HbA1c	glycated hemoglobin
HBSS	Hanks' balanced salt solution
HED	high-energy diet
HPT	hypothalamus-pituitary testicles
hSCs	human Sertoli cells
IFG	impaired fasting glucose
IGF-I	insulin growth factor I
IGT	impaired glucose tolerance
IP	intraperitoneal
LCs	Leydig cells
LDH	lactate dehydrogenase
LDHA	lactate dehydrogenase A
LDHC	lactate dehydrogenase C
LH	luteinizing hormone
MCT4	monocarboxylate transporter 4
MCTs	monocarboxylate transporters
M-MLV RT	moloney murine leukemia virus reverse transcriptase
mtDNA	mitochondrial DNA
mtND1	gene – mitochondrial ND1 gene
NADPH	nicotinamide adenine dinucleotide phosphate
NRF-1	nuclear respiratory factor 1
NRF-2	nuclear respiratory factor 2
n-STZ	neonatal-streptozotocin
OXPHOS	oxidative phosphorylation system
PFK	phosphofructokinase
PFK1	phosphofructokinase 1
PGC-1α	peroxisome proliferator-activated receptor γ coactivator 1 α
PI3K	phosphatidylinositol 3-kinase
PreD	pre-diabetes
PUFAs	polyunsaturated fatty acids
PYG	glycogen phosphorylase
PYGB	brain-type glycogen phosphorylase
PYGL	liver-type glycogen phosphorylase
qPCR	quantitative real-time PCR
RNA	ribonucleic acid
RNAt	total ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction

SCs	Sertoli cells
SIRT3	sirtuin 3
SP	statistical power
STF	seminiferous tubules fluid
STZ	streptozotocin
T	testosterone
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive species
TCA	tricarboxylic
TFAM	mitochondrial transcription factor A
TIF	testicular interstitial fluid
TPTZ	2,4,6-tripyridyl-s-triazine
UDP-glucose	uridine diphosphoglucose
WAT	white adipose tissue
WHO	World Health Organization
ß2MG	beta-2-microglobulin gene

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Chapter 1

Introduction

Considerations about the testicular environment

Metabolic regulation is important for spermatogenesis

High-energy diets: a threat for male fertility?

Diabetes mellitus and male fertility

Considerations about the testicular environment

Testicular anatomy and histology

Morphology and structure of Sertoli cells

Seminiferous epithelium environment

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M. G. Alves, ***L. Rato***, *A. C. Moreira and P. F. Oliveira. Capítulo 6: A Célula de Sertoli: Fisiologia, Estrutura e Função in Fardilha, M., editor. Reprodução Masculina, ACR Publishing. (2015). In press*

Testicular anatomy and histology

Testicles are responsible for two major tasks: synthesis and release of sexual steroids hormones, mainly testosterone (T), and formation of haploid germ cells, sperm. These functions are primarily regulated by the reproductive axis (hypothalamus-pituitary-testis-axis, HPT-axis). Pituitary gonadotropins release is stimulated by gonadotropin-releasing hormone (GnRH) , with luteinizing hormone (LH) acting on the T-producing Leydig cells (LCs) located in the *interstitium*, and follicle stimulating hormone (FSH) acting on Sertoli cells (SCs) in the seminiferous tubules [1, 2]. Nevertheless, a chain of complex local interactions involving the various testicular cell types such as germ, Sertoli, peritubular and Leydig cells are responsible for the spermatogenesis control [2, 3]. Spermatogenesis is the process by which immature germ cells undergo division, differentiation, and meiosis to give rise to haploid elongated spermatids. This process takes place within seminiferous tubules, the functional unities of the testis, through close association of germ cells with epithelial somatic cells, the SCs. SCs play a central role on the development of functional testis, and consequently on the expression of a male phenotype [4, 5]. They influence testis formation in the embryo and spermatogenesis in the adult, by regulating the surrounding environment of the developing germ cells [1]. Within the seminiferous tubules, the SCs reside on the basement membrane, under which are the lymphatic endothelium and the peritubular myoid cells [6]. SCs face the lumen of the seminiferous tubule, providing structural support and creating an immunologically protected space for germ cells [7]. Developing germ cells form intimate associations with SCs and at a given moment various germ cell types are in contact with one Sertoli cell. The various generations of germ cells are not randomly distributed within the seminiferous epithelium, but are arranged in strictly defined cellular associations [5, 8]. The cell-to-cell interactions, not only between SCs and specific germ cells but also between SCs, are essential in the regulation of mammalian spermatogenesis [5, 9]. These direct intercellular contacts are accomplished via the establishment of different types of junctions. Adherent, tight and gap junctions are implicated in cell adhesion [10], in the regulation of paracellular diffusion, the establishment and maintenance of cell polarity [11] and intercellular communication processes (through the establishment of intercellular plasma membrane coupling channels) [12] that occur in the seminiferous epithelium. Germ cell development relies on a highly coordinated interaction with SCs and they can communicate directly via ligand/receptor-mediated interactions or paracrine factors [5, 13] and through intercellular communication channels, consisting of gap junctions and their constitutive proteins, the connexins [12]. The production and secretion of many Sertoli cell proteins and factors involved in germ cell development occur in a stage-dependent manner, reflecting the ability of the Sertoli cell to adapt to the changing needs of the germ cell [5]. Without the physical and metabolic support of the SCs, germ-cell differentiation, meiosis and transformation into spermatozoa will not occur [11, 14]. The number of SCs determine the number of germ cells that can be supported through spermatogenesis and numerically determine the extent of sperm production since each Sertoli cell has a fixed capacity for the number of germ cells that it can support [14,

15]. Hence, a well-functioning Sertoli cell (Figure 1.1) provides the developing germ cells with the appropriate nutrients, energy sources, hormones, and growth factors as well as protecting them from harmful agents and from the host's own immune system [15]. SCs also control the composition of the seminiferous tubules fluid (STF), the physico-chemical *milieu* where spermatogenesis occurs.

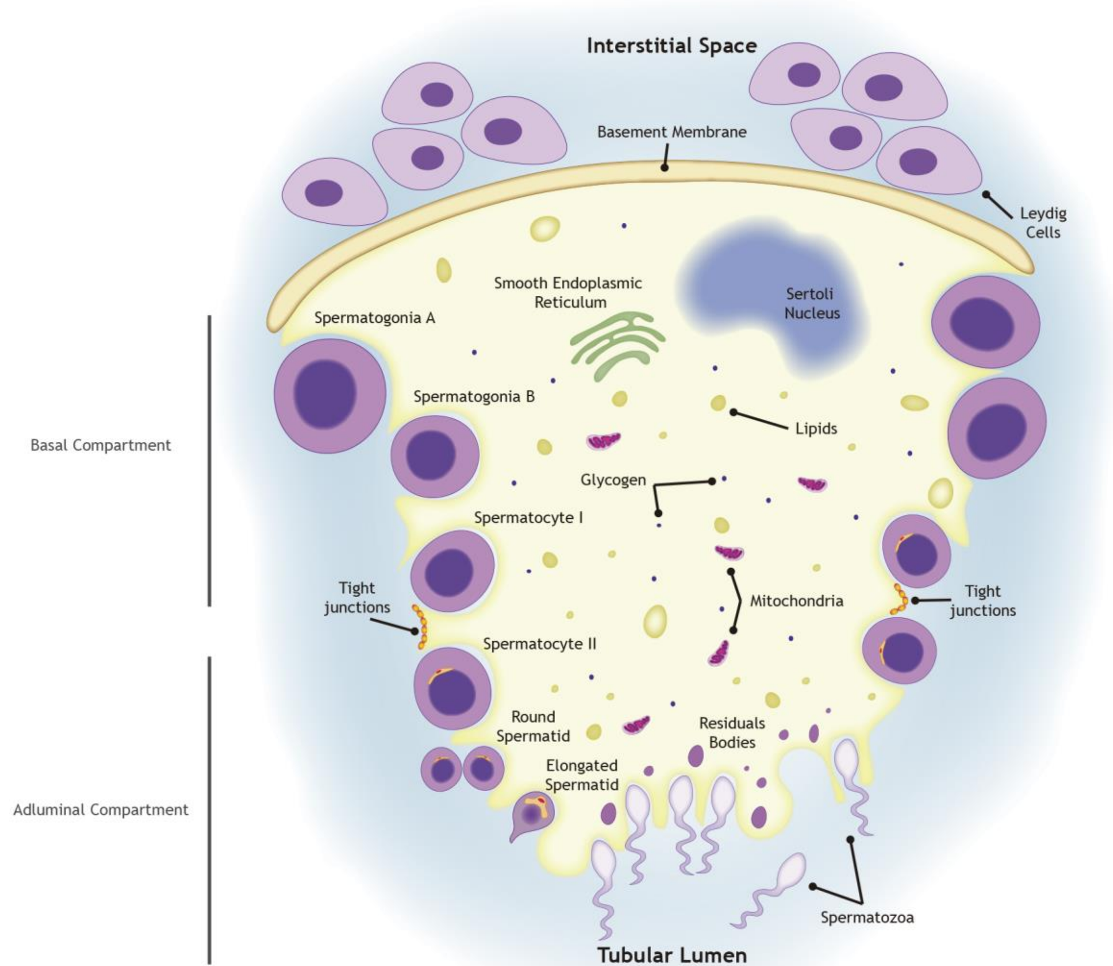

Figure 1.1. Schematic illustration of the Sertoli cells (SCs) and spermatogenesis. The seminiferous epithelium is composed by SCs and different subtypes of developing germ cells. Leydig cells, which produce testosterone in the presence of luteinizing hormone and the blood vessels, are located at the interstitial space. Male gametes (spermatozoa) are formed in the seminiferous tubules of the testis in a complex process, known as spermatogenesis, the cellular division and transformation that produces male haploid germ cells from diploid spermatogonial stem cells. This process is usually described by timed and highly organized "stages" or "phases" that are essential for continuous sperm production, which is dependent upon several intrinsic (Sertoli and germ cells), extrinsic (hormonal, among others), as well as species-specific factors. The supporting SCs adhere to the basement membrane where spermatogonia are also adherent. Then, spermatogonia type A divide and develop into spermatogonia type B, which enter meiotic prophase and differentiate into primary spermatocytes (Spermatocyte I) that separate the homologous pairs of chromosomes in meiosis I (reduction division) to form the haploid secondary spermatocytes (Spermatocyte II). The meiosis II yields four equalized spermatids that migrate toward the lumen where fully formed spermatozoa are finally released.

Morphology and structure of Sertoli cells

Differentiated SCs are structural elements of the seminiferous epithelium, physically supporting the development of spermatogenesis and regulating the flow of nutrients, growth factors and other substances to male germ cells. In mammals, at the time of puberty, SCs suffer a profound alteration on their morphology and function, becoming biochemical and morphologically distinct from the undifferentiated cells. SCs present a columnar shape, which extends from the base of seminiferous tubules to the lumen. These cells are adhered to the basal lamina of the seminiferous tubules, a fibrous structure composed of various extracellular matrix proteins (such as laminin, collagen and heparin sulfate) that maintain the structural integrity of these tubules (Figure 1.1) [16]. In mammals, the size of SCs may vary from 2000 to 7000 μm^3 [16] and the percentage of these cells in the entire seminiferous epithelium also vary according to the species, from approximately 15% in mice to 40% in humans [17]. Images from conventional microscopy show that SCs present a clear cytoplasm with prolonged extensions that surround germ cells. In most of species the nucleus of SCs is located at the basal portion of the cytoplasm presenting an irregular shape and large dimensions (up to 850 μm^3), but these characteristics may depend on the stage of the seminiferous tubule cycle [17, 18] and on the age of development of the individual [19, 20]. The nuclear envelope is characterized by deep invaginations that are associated with an accumulation of vimentin intermediate filaments. Another characteristic of the nucleus is the large dimension nucleolus with a three-partite structure that can easily be recognized in the nucleoplasm, usually with two chromocenters associated at diametrically opposed sides in which the centromeric regions of the chromosomes are clustered [21, 22]. Electron microscopy images show that these cells also contain a sparse quantity of rough endoplasmic reticulum, while the smooth endoplasmic reticulum is abundant and is associated with tight junctions in the basal portion of the SCs and ectoplasmic specializations, being part of the junctional complexes established between SCs and germ cells. The smooth endoplasmic reticulum is organized into cisterns that involve lipid droplets and can be found near to mitochondria suggesting that are involved in the metabolism of lipids or steroids [23]. Lipid droplets and glycogen particles are also widely present in the cytoplasm of SCs [24] and their quantities vary according to the spermatogenesis stage and between species [23]. The presence of lipid droplets has been associated with the ability of SCs to recycle lipids from the breakdown of germ cell degeneration and from residual body phagocytosis [25], whereas the amount of glycogen present in these cells is stage and species dependent and an increase of glycogen storage has been reported in several pathological conditions [26, 27].

SCs are pivotal for the regulation of spermatogenesis and for the establishment of the different rates of spermatozoa production [2]. Adjacent SCs establish between them tight junctions creating a unique environment within the tubular compartment where meiotic and post-meiotic steps of the spermatogenesis occur. The establishment of this barrier, often known as “blood-testis barrier” (BTB), allows the formation of specific intratubular fluid that is dependent on the function of SCs. These cells control the composition of adluminal

compartment and developing germ cells are dependent on the secretion and/or selective passage of nutrients and other factors by SCs. In fact, functional BTB: (a) prevents the entry of molecules and substances into the adluminal compartment of the seminiferous tubules; (b) limits the movement of immune cells of the immune system and regulating the levels of cytokines in the seminiferous epithelium; (c) acts as physiologic barrier, since it contains physiological transporters and channels in the apical and basolateral membranes that are highly dynamic and responsible for the formation of a specific microenvironment. However this barrier is sufficiently "permeable" to allow the migration of developing germ cells throughout the seminiferous epithelium. In fact, BTB is restructured in specific stages of spermatogenic cycle to allow the migration of germ cells, in such a well-coordinated process that even immune privilege is maintained [28]. The modification of proteins associated to tight junctions (occludins, claudins and JAM proteins) and to the establishment of BTB is on the basis of the permeability barrier, which is observed in several pathological conditions associated to the male subfertility/infertility. The seminiferous tubules consist of a complex stratified epithelium composed by two cell types: SCs and germ cells. SCs approximately occupy 17-19% of the seminiferous epithelium in adult rats [17] and exhibit cytoplasmic extensions that allows them to sustain a vast number of germ cells in development. Each cell can support between 30 to 50 germ cells in various stages of development [28].

Seminiferous epithelium environment

STF serves as a mean of transport for spermatozoa, and also allows keeping an appropriate microenvironment within tubules required for the normal occurrence of spermatogenesis. STF starts to be produced by SCs when they switch to the maturational stage [4, 15, 29]. Its composition is controlled by SCs, which establish the physicochemical *milieu* where spermatogenesis occurs. It has been shown that the composition of the fluid within the seminiferous tubules is very stable due to the existence of the BTB [30], supporting the fundamental relevance of the STF composition. This luminal *milieu* is markedly distinct from the plasma and the testicular interstitial fluid (TIF) and is critical to the occurrence of spermatogenesis [31], since meiosis can only be completed after the fluid secretion has been established [32]. Several reports have attempted to elucidate about the formation and composition of STF, but unfortunately some of them lack in information concerning the origin and composition of STF. Recently, the knowledge on the mechanisms involved in the secretion, composition and regulation of SFT has been reviewed [29]. First reports from Tuck and collaborators [33] postulated that SCs were responsible for fluid secretion in the seminiferous tubules. These authors, using a variation of the micropuncture technique, suggested that the luminal fluid is composed by a K^+-rich solution [33]. Later Jenkins and collaborators [34] observed the similar results in their study. Clulow and Jones [35] determined a most suitable approach for defining the composition of the secretions of the seminiferous epithelium and described a fluid rich in Na^+ and Cl^- content, with a K^+

concentration of at least twice that of blood concentration. These authors suggested that this fluid is the main source of the luminal solutes in the extratesticular ducts.

Another important feature in seminiferous *milieu* is the control of its pH. Recent reports have elucidated the role of membrane transporters, both in controlling the STF pH, as in the control of the intracellular pH of SCs [36, 37]. This parameter is kept by intracellular buffers and the balancing between the elimination and production of protons [38]. SCs express various types of ion membrane transporters directly involved on the movement of basic and acidic particles across the membrane [39]. The involvement of such transporters in the establishment of the STF is not yet completely disclosed, so it is essential further knowledge on their functioning, regulation and in the mechanisms responsible for determining the osmolarity and pH of STF [29]. Furthermore, in the formation of STF, it should be taken into account the other functions of this fluid: nourishment of germ cells, transport of secretion products and transport of the newly formed sperm towards the epididymis [40].

Metabolic regulation is important for spermatogenesis

The central role of Sertoli cells

The metabolic needs of germ cells

Sertoli cell metabolism

Regulators of Sertoli cell metabolism

Metabolism and reproduction

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The central role of Sertoli cells

A decline in male reproductive health has been observed over the last few decades in European men. Studies from Western Europe have identified a high prevalence of low sperm counts in young men (<40 years), demonstrating the impact of environmental and lifestyle factors on spermatogenesis [41-44]. Several factors have been suggested to contribute to this problem, including current lifestyle in industrialized countries (diet, smoking, alcohol, drugs) and exposure to environmental chemicals, which can negatively affect testicular function to a greater degree than genetic factors [45-50]. Furthermore, systemic diseases can affect the reproductive axis at multiple levels, directly or indirectly, and impair spermatogenesis irreversibly [51-53]. The regulation of these metabolic processes is crucial and could have a direct influence on male fertility. Modulation of metabolic pathways in testicular somatic cells, especially SCs, is likely to be determined by multiple elements including metabolic substrate availability and the action of hormones and other endogenous or exogenous factors that will have a synergistic contribution to the progression of spermatogenesis.

The BTB is one of the tightest blood–tissue barriers in mammalian tissues and the central structural element in testicular physiology [54-56], responsible for conferring polarity to SCs [56]. When the BTB is dysfunctional, germ cell differentiation is arrested [57]. Generation and maintenance of the BTB is assured by somatic SCs, the sustentacular cells of the seminiferous tubules [58], and its molecular composition has long been a matter of debate [28, 56, 59-61]. The BTB is composed of specialized junctions between adjacent SCs, located near the basement membrane, which include tight junctions, basal ectoplasmic specializations, basal tubulobulbar complex gap junctions and desmosome-like junctions [28, 60]. Despite the complex composition of the BTB, it undergoes highly dynamic restructuring at specific stages of the spermatogenic cycle to allow developing germ cells to cross the BTB into the adluminal compartment [62]. Throughout this process, germ cells continue to be tightly anchored to SCs via the anchoring junctions mentioned above [63]. Opening and closing of the BTB is such a well-coordinated process that even immune privilege is maintained [56]. Endothelial and peritubular myoid cells (which are found outside of the seminiferous epithelium) also contribute to the selective ability of the BTB, by regulating the access of substances, including xenobiotic and vitamin A–related compounds, into seminiferous tubules [64, 65]. These cells appear to be involved, together with SCs, in the metabolism and transport of retinoids into the tubules towards the germ cells [66]. The compartmentalization of retinoic acid metabolism within this epithelium seems to be essential to spermatogonia proliferation and spermatogenesis [67, 68]. Mammalian spermatogenesis is a continuum of cellular differentiation with three main stages: mitotic spermatogonial proliferation and differentiation; meiotic phase; and spermiogenesis [69]. This is a well-coordinated process dependent on SCs and regulated by the hypothalamus–pituitary–testis (HPT) axis. Spermatogonial stem cells, which lie at the basement membrane, replicate mitotically to both guarantee the germ cell line (spermatogonia A), and give rise to new populations (spermatogonia B) committed to differentiate and move along the seminiferous epithelium

[60, 69]. Spermatogonia B differentiate into primary spermatocytes and then, after crossing the BTB, undergo the first division of meiosis yielding secondary spermatocytes. Round spermatids are produced through the second meiotic division. Once spermatids are formed, cell division stops and spermiogenesis starts giving rise to elongated spermatids (Figure 1.1). This process culminates in the release of elongated spermatids into the lumen of the tubule as immature spermatozoa (in a process called spermiation) [69].

The metabolic needs of germ cells

Germ cells are subjected to a number of different conditions during their development within the seminiferous tubules. SCs perform a range of functions from physical support and immunoprotection, to the supplying of nutrients and other factors in order to achieve successful spermatogenesis [29, 37, 70]. Furthermore, during this multifaceted process, SCs are targeted by external and internal adverse conditions, such as environmental factors [47], hormonal deregulation [47], diseases [51], and oxidative stress [71], which might impair maintenance of the appropriate environment for proper development of germ cells. Germ cells have peculiar nutritional requirements during spermatogenesis, switching their metabolic profile throughout development [72]. Why this happens is unclear, although it is noteworthy that testicles are tightly compartmentalized organs, which might restrict the availability of essential substances for germ cell energy metabolism [73]. Furthermore, testicles have been reported to be a naturally oxygen-deprived organs [74]. Together, this may explain why germ cells utilize different metabolic pathways for energy production in their various developmental stages [72]. Glycolysis has been highly conserved among species throughout evolution; however, many glycolytic enzymes have testis-specific isoforms, expressed specifically or predominantly in spermatogenic cells (often during the postmeiotic phase) [75]. Germ cells are strictly dependent on carbohydrate metabolism, including both aerobic and anaerobic pathways [72]. Spermatogonia, which lie in the basal compartment of the BTB, are supplied with nutrients from blood components and use glucose as fuel for adenosine triphosphate (ATP) production [76]. Spermatocytes are intermediate developing germ cells that may also depend on glycolysis, although the utilization of lactate by cells at these stages of development has also been reported, especially those that lie closer to the adluminal compartment [72]. Although they express all enzymes of the glycolytic pathway, mature germ cells are dependent on lactate, present in extracellular medium and supplied by SCs (Figure 1.2) [76]. It has been reported that intratesticular infusion of lactate into adult cryptorchidic rat testis improves spermatogenesis [77]. Lactate is also responsible for ribonucleic acid (RNA) and protein synthesis stimulation in spermatids [78] and exerts an anti-apoptotic effect on germ cells [79]. The glycolytic potential in spermatids is lower than in germ cells at earlier developmental stages. Indeed, ATP levels in spermatids decrease in response to glucose metabolism [80]. However, both glycolytic and gluconeogenic pathways may be functional in round spermatids, owing to metabolic recycling of lactate to glucose-6-phosphate [81]. Of all the germ cells, spermatozoa exhibit the highest glycolytic activity and

the lowest tricarboxylic acid (TCA) cycle activity, using only glucose or fructose for their energy metabolism [72]. In addition, energy production in spermatozoa is compartmentalized so that mitochondria and oxidative phosphorylation are restricted to the midpiece, while glycolysis occurs in the principal piece [82]. The source of energy for the acrosome reaction is unclear [75]. The pentose phosphate pathway is also active in germ cells, though significantly more active in spermatocytes than in spermatids, as indicated by the modulation of glucose-6-phosphate dehydrogenase activity in these cells [72]. This pathway is required for the biosynthesis of nucleotides for RNA and the production of nicotinamide adenine dinucleotide phosphate (NADPH) and ribose 5-phosphate [72]. Regarding lipid metabolism, several authors have proposed that there is effective transport of long polyenes from SCs to germ cells, although it remains to be fully proved [83-85]. Nevertheless, during their journey through the epididymis, rat spermatozoa undergo considerable lipid remodelling and pachytene spermatocytes are known to actively metabolize fatty acids [86]. The energy production capacity of the germinal cell line, which depends on the regular provision of available fuel by the somatic SCs, remains a matter of debate. It is certain, however, that the versatility of germ cells in utilizing distinct energy sources at different stages is useless in the presence of nonfunctional SCs, leading to unsuccessful spermatogenesis.

Sertoli cell metabolism

The mechanisms that regulate Sertoli cell metabolism are central to the maintenance of spermatogenesis and male fertility. Carbohydrate metabolism in SCs has some unique characteristics. For example, only 25% of the pyruvate produced from glucose in these cells is oxidized via the TCA cycle [87]. Furthermore, Robinson and Fritz [88] showed that cultured SCs convert the majority of glucose into lactate, which is then secreted. It has also been reported that *in vitro*, the pentose phosphate pathway (determined by the rate of NADPH oxidation) does not operate at its maximum rate in SCs [87, 88]. Finally, exogenous pyruvate is oxidized at very low concentrations by these cells during incubation with glucose [88]. SCs are the major source of lactate in the testes, and several mechanisms are known to modulate production of this metabolite (Figure 1.2). SCs produce lactate primarily from glucose, and the rate-limiting step is the membrane passage of glucose from the extracellular space, via specific glucose transporters (GLUTs) [89]. Four GLUTs (GLUT1, GLUT2, GLUT3 and GLUT8) have been identified in SCs to date [90-93]. However, GLUT8 is not expected to be involved in glucose transport from the extracellular *milieu*, since it has not been identified in the plasma membrane of SCs, but rather in the endoplasmic reticulum membrane, and thus its role in glucose uptake from extracellular space can be excluded [94, 95]. Lactate dehydrogenase (LDH) also has a crucial role in providing lactate to developing germ cells. The export of lactate from SCs by specific monocarboxylate transporters (MCTs) is responsible for improved lactate supply to germ cells (Figure 1.2) [96]. SCs have a high glycolytic activity that can be adapted to conditions of glucose deprivation, ensuring an adequate lactate concentration in

the microenvironment where germ cells develop even in extreme conditions (when glucose levels are low or in the complete absence of glucose) [97]. In such conditions, SCs adjust their metabolism by activating specific signal transduction pathways and molecules, such as AMP-activated protein kinase (AMPK), a key mediator in cellular energy homeostasis [98]. AMPK is a serine–threonine kinase that restores cellular ATP levels by switching on catabolic pathways and switching off anabolic pathways [99]. Activation of AMPK increases lactate production via increased glucose uptake, and increased GLUT1 and MCT4 expression [98]. This energy sensor seems to be the main activator when SCs are under stressful conditions [100, 101]. Nevertheless, other metabolic sensors cannot be excluded [102] Despite being an energy substrate, glucose is not the main metabolite used for ATP synthesis in SCs, which require high energy levels to function correctly [97]. SCs can maintain their viability in culture in the complete absence of glucose, still producing ATP and lactate via metabolism of lipids [25], amino acids and even glycogen [24, 97, 103]. Xiong and collaborators [25] showed that SCs preferentially use lipids as an energy source. Thus, lipid β-oxidation seems to be the main metabolic pathway used by SCs to produce energy. Although SCs maintain ATP production when glycolysis is blocked, ATP synthesis decreases significantly if β-oxidation is blocked [25]. Interestingly (and paradoxically to their protecting and nourishing role), SCs can induce apoptosis of germ cells [104], phagocytose apoptotic spermatogenic cells, and are also responsible for endocytosis and degradation of residual bodies, converting them into lipids that are further metabolized to produce ATP [25]. After engulfment of apoptotic germ cells, the expression of long-chain acyl-CoA dehydrogenase (responsible for the catabolism of long-chain fatty acids) increases significantly in mitochondria of SCs (Figure 1.2) [25]. Given that under normal physiological conditions >75% of spermatogenic cells undergo apoptosis [25], and the lipid content of residual bodies is recycled by SCs, there is an enormous quantity of energy reserves available to meet the energetic needs of the multifaceted SCs. SCs can also use amino acids for energy production; oxidation of glutamine and leucine provides most of the energy required by these cells [105]. Other amino acids, such as alanine and valine, also have an important role in Sertoli cell metabolism [105]. Kaiser and collaborators [105] suggested that low amounts of acetyl-CoA arising from glucose could modulate the oxidation of alanine and valine to CO_2, by competing with the acetyl-CoA derived from these amino acids. Glucose metabolism can also stimulate the conversion of valine into lipids [105]. Glutamine inhibits the oxidation of leucine, valine, and alanine, but does not alter the conversion of these amino acids into lipids. Glutamine also inhibits the incorporation of alanine into proteins [105]. Alanine is the main glucogenic amino acid, since it can be converted to pyruvate that can be used as a substrate by SCs for several biochemical pathways, including the TCA cycle and gluconeogenesis. The relationship between the quantities of alanine produced and pyruvate consumed reflects the NADH:NAD^+ ratio and the cytosolic redox cell status [96].

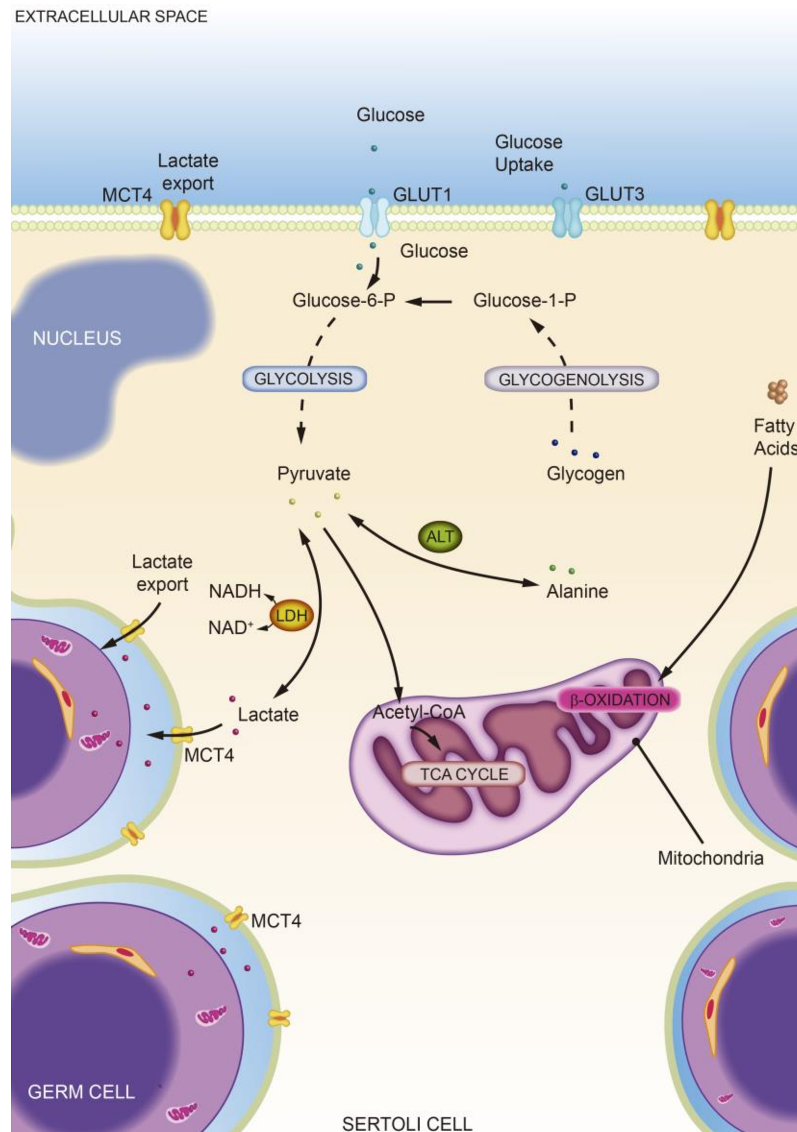

Figure 1.2. Schematic illustration of the glycolytic metabolism of Sertoli cells (SCs). The SCs are capable of consuming a variety of fuels including glucose, lactate and fatty acids. Nevertheless, SCs actively metabolize glucose being the majority of it converted to lactate. The extracellular lactate and pyruvate are transported via the family of proton-linked plasma membrane transporters known as the monocarboxylate transporters (MCTs), while glucose is imported via the family of membrane proteins called glucose transporters (GLUTs). Once glucose enters the glycolytic pathway, it is decomposed to pyruvate which can a) be converted into lactate via lactate dehydrogenase (LDH) b) be converted into alanine via alanine transaminase or c) be transported to the mitochondrial matrix, oxidized and decarboxylated by pyruvate dehydrogenase, forming the two carbon intermediate Acetyl-CoA, which can enter the TCA cycle. The oxidation of these substrates is coupled with ADP phosphorylation, via the electron transport chain to form ATP. Abbreviations: GLUT, glucose transporter; ALT, alanine aminotransferase; LDH, Lactate dehydrogenase; MCT, monocarboxylate transporter; TCA, tricarboxylic acid.

Regulators of Sertoli cell metabolism

To date, several factors that regulate Sertoli cell metabolism and lactate production have been identified (Table 1.1): FSH [106], insulin [106], insulin growth factor-I (IGF-I) [106], epidermal growth factor (EGF) [107], paracrine factor P-Mod-S [108], tri-iodothyronine [109],

basic fibroblast growth factor (bFGF)[110], cytokines [111], arachidonic acid [112], carnitine [113], AMPK [98], and sex steroid hormones [96]. Gumma and collaborators [114] demonstrated that FSH and insulin can affect lipid metabolism of SCs by stimulating lipid esterification. FSH and IGF-I also stimulate lactate production by SCs, probably at the enzymatic level or via glucose transport regulation [106]. FSH and interleukin 1ß exert positive effects on glucose uptake, regulating glucose transporter activation or translocation in rat SCs [111]. bFGF has been shown to augment lactate production through glucose uptake, via increasing GLUT1 expression and LDHA activity [115]. Lactate production is also induced by EGF in cultured SCs [107]. Gallardo and collaborators [98] showed that AMPK favors lactate production via an increase in GLUT1 expression and the lactate exporter MCT4. Similarly, carnitine has been described to be a positive metabolic modulator of Sertoli cell metabolism. In vitro supplementation with carnitine increased the production of both lactate and pyruvate, activity of LDH and hexose transport [113]. It has also been suggested that arachidonic acid regulates lactate production by SCs, stimulating glucose uptake, LDH activity and increasing LDHA mRNA levels [112]. The paracrine factor P-Mod-S appears to stimulate lactate production by SCs at various stages of pubertal development [108]. Sex steroid hormones (androgens and estrogens) also regulate Sertoli cell metabolism. Androgens are essential for the maintenance of pivotal mechanisms required for fertility [116], although few studies have reported relevant findings regarding the regulation of lactate production and metabolite-related genes by these hormones [96, 117-119]. Recent findings, however, have shown that androgen stimulation of SCs can alter transcription of a number of metabolism-related genes [96, 120]. We have described that 5α-dihydrotestosterone (5α-DHT) and 17ß-estradiol (E_2) regulate glucose uptake and lactate production in SCs from rats [96]. Moreover, 5α-DHT increased glucose uptake despite decreasing lactate synthesis, suggesting that this androgen may reduce lactate production (via LDH), or transport of lactate to the extracellular medium (via MCTs) [96]. 5α-DHT drives SCs to achieve an efficient metabolic status, redirecting glucose metabolism to the TCA cycle [96]. Results obtained in monkey epididymis (*Macaca mullata*) are in accordance with this hypothesis [121]. Moreover, 5α-DHT was reported to stimulate succinate dehydrogenase and malate dehydrogenase activity in the epididymis of castrated animals [121]. T is also involved in polyunsaturated fatty acid biosynthesis by SCs, modulating the activity of Δ5 and Δ6 desaturases [119]. Experiments involving the selective ablation of the androgen receptor in mouse SCs revealed that androgens are essential to regulate glycerol-3-phosphate dehydrogenase expression [122]. Analogously, it has been described that in the lizard (*Hemidactylus flaviviridis*), E_2 and 5α-DHT markedly suppress lactate production by SCs in a dose-dependent and time-dependent manner [118].

Table 1.1. Factors regulating Sertoli cell metabolism and lactate production. +, increase; -, decrease; /, no effect. Abbreviations: GLUT, glucose transporter; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; ND, not determined.

Factor	Expression				Lactate production
	GLUT1	GLUT3	MCT4	LDH	
Folicule-stimulating hormone	+ [91]	/ [91]	nd	+ [111]	+ [91]
Insulin	nd	nd	nd	nd	+ [106]
Insulingrowth factor-I	nd	nd	nd	nd	+ [106]
Epidermal growth factor	nd	nd	nd	nd	+ [107]
Paracrine factor P-Mod-S	nd	nd	nd	nd	+ [108]
Tri-iodothyronine	nd	nd	nd	nd	- [109]
Basic fibroblast growth factor	+ [91, 115]	/ [91, 115]	nd	+ [115]	+ [91, 115]
Cytokines (IL1-β)	+ [91]	/ [91, 111]	nd	+ [111]	+ [91]
Arachidonic acid	nd	nd	nd	+ [112]	+ [112]
Carnitine	+ [123]	nd	nd	+ [113]	+ [123]
AMP-activated protein kinase	+ [98]	- [98]	+ [98]	nd	+ [98]
5α-dihydrotestosterone	nd	nd	/ [96]	- [96]	- [96]
17β-estradiol	nd	nd	nd	- [96]	/ [96]

Metabolism and reproduction

Normal reproductive function requires an adequate nutritional intake. Conversely, extreme conditions, such as caloric deprivation with weight loss, excessive food intake and obesity, are deleterious for reproductive function. Recent data point toward the possible biochemical links between metabolism and reproduction [99, 124, 125]. Although this is an issue that has received some attention, further elucidation is required. There is a growing awareness that sex steroid hormones participate in the homeostasis of energy balance and that reproductive activity and energy metabolism are intimately related [125, 126]. The GnRH pulse generator is exceptionally sensitive to energetic deficits, environmental contaminants and extreme exercise [127-129]. In men, brief periods of fasting cause suppression of GnRH pulses, thus decreasing LH levels and consequently T levels by downregulating the reproductive axis and therefore affecting male reproductive function [127]. The HPT axis is downregulated by minor energetic disturbances in men, as demonstrated by Trumble and collaborators [127]. Their study revealed that LH and T levels of young men were decreased, and T clearance rates increased, after an evening of fasting [127]. During extreme exercise, T is decreased by about 55% and testicular oxidative stress is enhanced [130]. Disturbances of the reproductive axis will severely affect Sertoli cell functions, since spermatogenesis is highly dependent upon both gonadotropic and androgen action [15]. Androgens and estrogens also seem to have an

important role in the control of metabolic disorders. In men, E_2 at physiological levels favors insulin sensitivity [131]. E_2 is a product of T aromatization catalyzed by the aromatase enzyme complex [132], and thus, a deficiency of aromatase or estrogen receptor α (ERα) leads to insulin resistance and glucose intolerance [133, 134]. Indeed, T aromatization to E_2 acting on ERα is essential for energy homeostasis in men [135]. Energy retention in men can lead to the development of several chronic diseases, and it has been proposed that current lifestyle trends in developed countries has led to the increased incidence of multiple clinical symptoms, together termed as metabolic syndrome [136]. Increased energy retention promotes adipogenesis, aromatase activity and consequent irreversible conversion of T into E_2, resulting in decreased T and elevated estrogen levels and directing male physiology to a hypogonadal state [137, 138]. Obesity and insulin resistance have been associated with hypogonadism [139]. Furthermore, reduced T levels promote estrogen receptor β expression, suppressing GLUT4 expression and resulting in impaired glucose homeostasis and insulin resistance [137]. Disruption of the molecular and cellular mechanisms of reproduction may affect SCs, since they are the main target of both sex steroid hormones and FSH [15]. Recently, Robeva and collaborators [140] have shown that obese men with metabolic syndrome have impaired Sertoli cell function and spermatogenesis. Sperm counts and sperm quality may be also affected [141]. Fertility is also affected by nutrition and the availability of energy reserves. However the cellular and molecular mechanisms that link energy stores and reproduction, and the signals that mediate these processes, are not entirely understood. Metabolism-associated hormones, such as insulin and thyroid hormone, play crucial roles in the relationship between metabolism and reproduction [124]. Tri-iodothyronine induces membrane hyperpolarization in SCs stimulating amino acid accumulation in immature rat testes [142]. The interaction between tri-iodothyronine and neuropeptides is essential for the integration of metabolism and reproduction. Three adipokines (leptin, resistin and adiponectin) have also been associated with the link between energy reserves and reproductive function. Leptin, an adipocyte-derived hormone, is pivotal in the regulation of both neuroendocrine function and fertility, stimulating GnRH secretion, gonadotropin secretion (FSH and LH) and rescuing impaired sexual function of leptin-deficient mice [143]. These leptin-deficient mice are reproductively incompetent, but exogenous administration of leptin can reverse the situation [124]. Resistin, originally described as a factor that impairs insulin sensitivity and glucose tolerance [144], increases both basal T levels and T levels after human chorionic gonadotropin stimulation in a dose-dependent manner [145]. Adiponectin also influences the neuroendocrine axis, possibly through direct actions on the pituitary gland [146]. These adipokines are involved in the control of reproductive functions at the HPT axis level and may act through the AMPK system, which could be one of the signalling pathways controlling the interactions between energy balance and reproduction.

High-energy diets: a threat for male fertility?

The consumption of high-energy diets and the risks for male fertility

High-energy diets intake disturbs whole body metabolism and the normal function of male reproductive axis

High-energy diets alter testicular metabolism

High-energy diets consumption favors testicular oxidative stress

Intake of high-energy diets decreases semen quality

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The consumption of high-energy diets and the risks for male fertility

Most developed countries have witnessed an unprecedented increase of obesity rates in the last decades. Obesity is a global health problem that is reaching epidemic proportions. According to the World Health Organization (WHO), in 2008, nearly 2 billion adults were classified as overweight or obese [147]. Nevertheless, these numbers may double in the near future [147]. Overweight and obesity are characterized by an excess of fat and are frequently defined by the ratio between weight and the square of the height, the so-called body mass index (BMI). The WHO considers individuals with a BMI over 25 kg/m^2 as overweight, and as obese when the BMI is over 30 kg/m^2 [147]. Obesity is closely related to disordered eating habits, particularly the over-intake of high-energy diets (HED). Indeed, combination of the following factors are the primary causes for this problem: changes in food composition and diet, such as increased consumption of HED, highly palatable, nutrient-poor foods with high levels of sugar and saturated fats; sedentary lifestyle and lack of physical activity [147]. In parallel with this, numbers have shown that total fertility rates have decreased 2% in developed countries, reaching the lowest values ever reported [148]. The prevalence of infertility in a couple is one in seven, in many countries, with the male factor being a major contributor [47]. Alarmingly, the increase of HED consumption is concomitant with the increased incidence of obesity in men of reproductive age [149]. Moreover, several reports estimate that fertility rates will continue to decrease in countries where obesity is prevalent [150, 151]. Reduced fertility has been recognized as one of the negative health consequences of obesity promoted by dietary lifestyle [149, 152-155]. The high caloric load induced by HED intake disrupts the male reproductive function at either central and/or gonadal levels (Figure 1.3). The male reproductive process is under control of a neurohormonal network operating in the HPT axis [156]. The signals derived from this system are closely linked to other functions, such as metabolism of testicular cells [111, 157]. In this regard, we must highlight the metabolic cooperation established between testicular cells that is very sensitive to hormonal control [158]. In fact, the multifaceted roles of the metabolic regulatory signals make the reproductive process highly dependent and sensitive to fluctuations in hormonal levels and overall energy metabolism [124, 125, 127]. Additionally, the effects of HED consumption on physical and molecular structure of germ cell line and mature sperm deserve special attention. There is growing awareness that lifestyle habits affect male fertility. It may seem a paradox but as life expectancy increases, sperm quality declines. Increasing evidences suggest that dietary habits based on HED consumption are not compatible with a healthy male reproductive function. In support of this, paternal obesity may also compromise offspring reproductive health and thus may have significant implications for the transgenerational amplification of subfertility [159]. Moreover, disordered eating habits appear to be common in developed and developing countries rendering the consumption of HED a hot topic to

discuss. Thus, we aim to present an up-to-date review on how HED consumption affects testicular physiology and may lead to lower semen quality and infertility.

High-energy diets intake disturbs whole body metabolism and the normal function of male reproductive axis

The normal reproductive function depends on an adequate nutritional state. Therefore, it is expectable that excessive food intake affects reproductive function. Recent data showed that metabolism and reproduction are closely connected [124, 125]. The current eating behaviors of developed societies are focused on high-energy intake, with high quantities of unhealthy fats, which in turn favors the accumulation of white adipose tissue (WAT). Additionally, the high levels of obesogenic toxicants present in HED also accelerate adiposity, and contribute to the increased storage of fat-soluble toxicants in the body [160, 161]. Since WAT is intimately associated with energy storage, excessive high energy intake results in increased energy retention [162]. Thereafter, the unbalanced consumption of high-energy food disrupts the molecular mechanisms responsible for the control of appetite and energy intake [163]. These processes are mediated by gut and adipose hormones. Gut and adipose tissues are ideally situated to sense energy status and some gut and adipose-derived hormones have been recognized as regulators of reproduction-related events (via HPT axis) [163]. Hence, a disruption of the endocrine activity of these tissues may affect the reproductive axis function and compromise male fertility. The HPT axis regulates the spermatogenic process, involving the interplay between hypothalamus, pituitary and testicles. Specialized neurons in hypothalamus release the GnRH, in a pulsatile manner, into the hypophyseal-portal circulation. Then, GnRH arrives at the anterior pituitary gonadotroph cells that release the two pituitary hormones, LH and FSH, which are the functional link between the brain and the testicles [156]. FSH primarily target SCs, which produce several proteins and factors (*e.g.* androgen-binding protein [164], transferrin [165], plasminogen activator [166], glycoproteins [167], sulphoproteins [168], myo-inositol [169] and sertolin [170]) that play important roles on germ cell development. They are responsible for the transport of water from the interstitial space into the lumen, which serves as the vehicle for moving sperm from the testis to the epididymis [171], and they also control the pH of the seminiferous fluid [36, 37]. On the other hand, LH acts on LCs, stimulating T production. T can also be irreversibly converted by aromatase P450 complex into E_2 in LCs [172]. LCs also produce other factors, such as insulin-like protein 3 and oxytocin, which influence the spermatogenic event [173]. However, the hormonal control of germ cell development is more complex than this classical view, with a tight interaction between central nervous system, gastrointestinal system and adipose tissue supporting spermatogenesis [163]. Nevertheless, studies illustrate that the HPT axis is very sensible and responds to subtle energetic disturbances [127] and its dysfunction leads to impaired fertility [174]. Although the understanding of the relation between gut and adipose-derived hormones with reproductive axis has increased, most of the cellular and molecular

mechanisms that mediate these processes remain to be fully elucidated. Leptin is one of the most relevant adipose-derived hormones whose production is proportional to body fat mass. Studies have highlighted the importance of this adipokine in male reproduction, since leptin acts at multiple levels on the HPT axis, modulating not only the firing rate of gonadotropins, but also by regulating the synthesis of steroids within testicles through a direct interaction with its receptors present in LCs [175, 176]. On one hand, the excess of leptin has been associated with abnormal spermatogenesis and infertility, probably due to inadequate gonadotropin support of spermatogenic event, ending-up in lower sperm quality. On the other hand, leptin resistance, enhanced by HED overconsumption, contributes to dysfunctional HPT axis and impairment of LCs, which lead to development of androgen deficiency [177]. Moreover leptin deficiency may also lead to a decline in sperm quality, since it has been reported that *in vitro* administration of physiological doses of leptin stimulates sperm motility [178]. Analogously to leptin, ghrelin also plays a relevant role in male fertility since it modulates reproductive events throughout the HPT axis [179, 180]. For instance, it has been reported that ghrelin decreases the firing rate of GnRH [181]. Moreover, ghrelin is able to directly act on testicular parenchyma, since intratesticular injection of this hormone was reported to inhibit LCs proliferation and decrease mRNA levels of stem cell factor, which acts as a survival factor for germ cells [182]. However, these outcomes may differ from physiologic conditions since ghrelin levels are known to change according to the whole body energetic state [183]. In this sense, overconsumption of HED is responsible for a decrease in ghrelin levels, which contributes to an increase of food intake, enhancing adiposity and the obesity-induced dysfunctional secretion of gut, adipose and pancreas-derived hormones. Noteworthy, obesity-induced insulin resistance accounts for reduced gonadotropin secretion at hypothalamic-pituitary level and consequently reduced testicular T production [135, 184]. It has been highlighted that glucose is also essential for T production, since LCs cannot produce T in the absence of this hexose [185]. Thus, deregulated glucose metabolism, as occurs in individuals consuming HED, may impair T production and arrest spermatogenesis. Additionally, consumption of high saturated fats, such as palmitic acid and stearic acid, may compromise T levels by inducing apoptosis in LCs [186]. Increased adiposity resultant from HED consumption also favors T/E_2 imbalance, due to increased aromatase activity with a consequent stimulation of E_2 production and increased whole body levels of that hormone [187]. E_2 acts at hypothalamic-pituitary level through a negative feedback, inhibiting the release of GnRH, as well as of FSH and LH. Therefore, the increase in E_2 levels will compromise all downstream events of the HPT axis and spermatogenesis (Figure 1.3). In sum, the intake of HED is responsible for alterations in whole body metabolism that alter the normal functioning of the male reproductive axis, impairing male fertility.

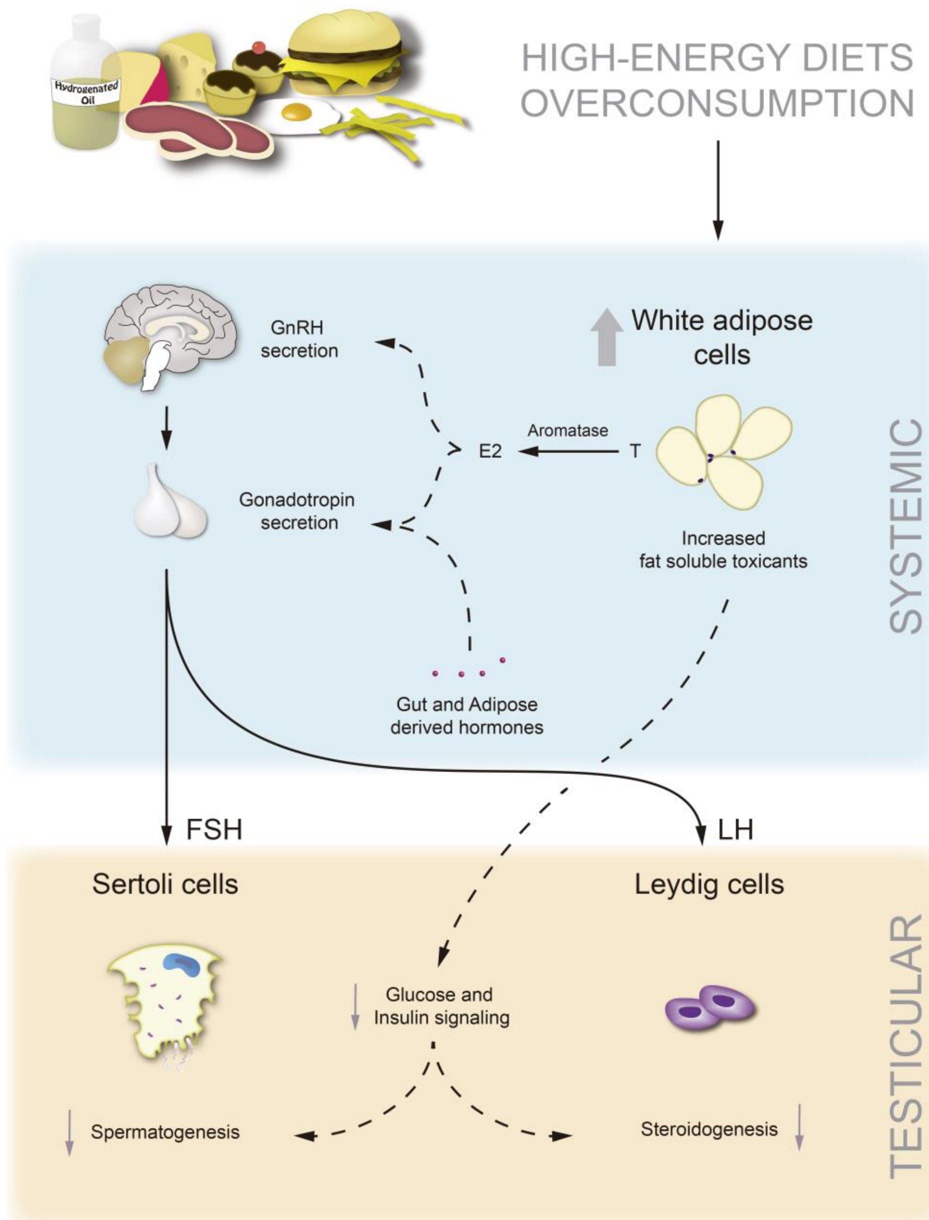

Figure 1.3. Effect of high-energy diets (HED) overconsumption in the male reproductive axis. Spermatogenesis is tightly controlled by two gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which functionally link brain and testicles. FSH acts on Sertoli cells, whereas LH acts on Leydig cells. The overconsumption of HED exerts inhibitory effects on the male reproductive axis. For instance, the increased adiposity resultant from overconsumption of HED increases the systemic levels of 17β-estradiol (E_2), due to increased aromatase activity in the adipocytes. The increased whole body levels of E_2 act on hypothalamic-pituitary through a negative feedback, thus inhibiting GnRH and pituitary gonadotropins. Gut and adipose-derived hormones also modulate the firing rate of gonadotropin secretion. The increased storage of fat soluble toxicants also impairs testicular function by disrupting both steroidogenesis and spermatogenesis. Solid lines represent stimulatory effects; dashed lines represent inhibitory effects.

High-energy diets alter testicular metabolism

Testicular metabolism is essential for the normal occurrence of spermatogenesis and shows specific characteristics. If on one hand glycolytic metabolism is essential for the production of energy sources (*e.g.* lactate) that are determinant for germ cells' fate [96], on the other

hand lipids are pivotal, functioning as "fuel" for SCs, and are also used in membrane remodeling of developing germ cells [83, 188, 189]. Germ cells present high levels of polyunsaturated fatty acids (PUFAs), which are crucial to membrane fluidity and flexibility and thus for fertilization. Nevertheless, these cells are unable to synthesize PUFAs, being dependent on their uptake [83]. This process is likely driven by SCs, since these cells show a highly efficient conversion of 18-carbon into 22- and 24-carbon PUFAs [190, 191] and express the enzymes necessary for this metabolic process at high levels, including Δ5-/Δ6-desaturases and fatty acid elongases [192]. Developing germ cells are connected to SCs and take up PUFAs to incorporate them into phospholipids, via lysophosphatidic acid acyltransferase 3 [193]. However, unhealthy fats may disrupt these mechanisms, since *trans* fatty acids decrease the activity of Δ5-/Δ6-desaturases, potentially limiting the incorporation of long chain PUFAs into sperm membranes. This alteration affects the spermatogenic process and contributes to impairment of sperm parameters and consequently altered sperm functionality (Figure 1.4), being consistent with the highest levels of saturated fatty acids reported in sperm of asthenozoospermic and oligospermic individuals [194, 195]. Additionally, Stroud and collaborators [196] reported that Δ6-desaturase knockout animals were infertile, showing complete absence of normal spermatozoa or elongated spermatids in the seminiferous tubules. This event was caused by major alterations in testicular fatty acid metabolism that resulted in impaired spermatid elongation. Alterations in whole body metabolism caused by HED include disruption of the HPT axis, which is expected to lead to changes in signaling pathways that regulate the metabolism of SCs. In addition to alterations in whole body metabolic status, testicular glucose metabolism may also be affected by HED intake and/or chemicals (*e.g.* BPA) present in HED [197]. Indeed, it has been recently demonstrated that BPA exposure significantly compromises testicular insulin signaling by downregulating insulin levels and insulin signaling molecules (such as insulin receptor substrate 1, insulin receptor substrate 2 and phosphatidylinositol 3 kinase) [197]. Testicular glucose transport is also affected by BPA, since this compound not only decreases GLUT2 and GLUT8 protein levels, but also interacts directly with these transporters, blocking the movement of glucose by binding to the intracellular pore region of GLUTs [197]. These evidences strongly suggest that testicular glycolytic metabolism may be compromised by HED overconsumption. As developing germ cells almost exclusively rely on lactate for their energy metabolism and SCs are responsible for the lactate production in testicular *milieu*, the impairment of glycolytic metabolism may result in deficient germ cells nurture. Nevertheless, SCs display a "metabolic flexibility", as they maintain their viability in culture in the complete absence of glucose, still producing energy and lactate via other energy sources [87, 105, 123].

In sum, testicles are able to develop adaptive mechanisms to ensure an adequate microenvironment for germ cells development. However, disproportionate consumption of HED enables the increase of fatty acid supply within testicular *milieu*, compromising key testicular metabolic mechanisms (Figure 1.4) that ultimately compromise germ cells fate.

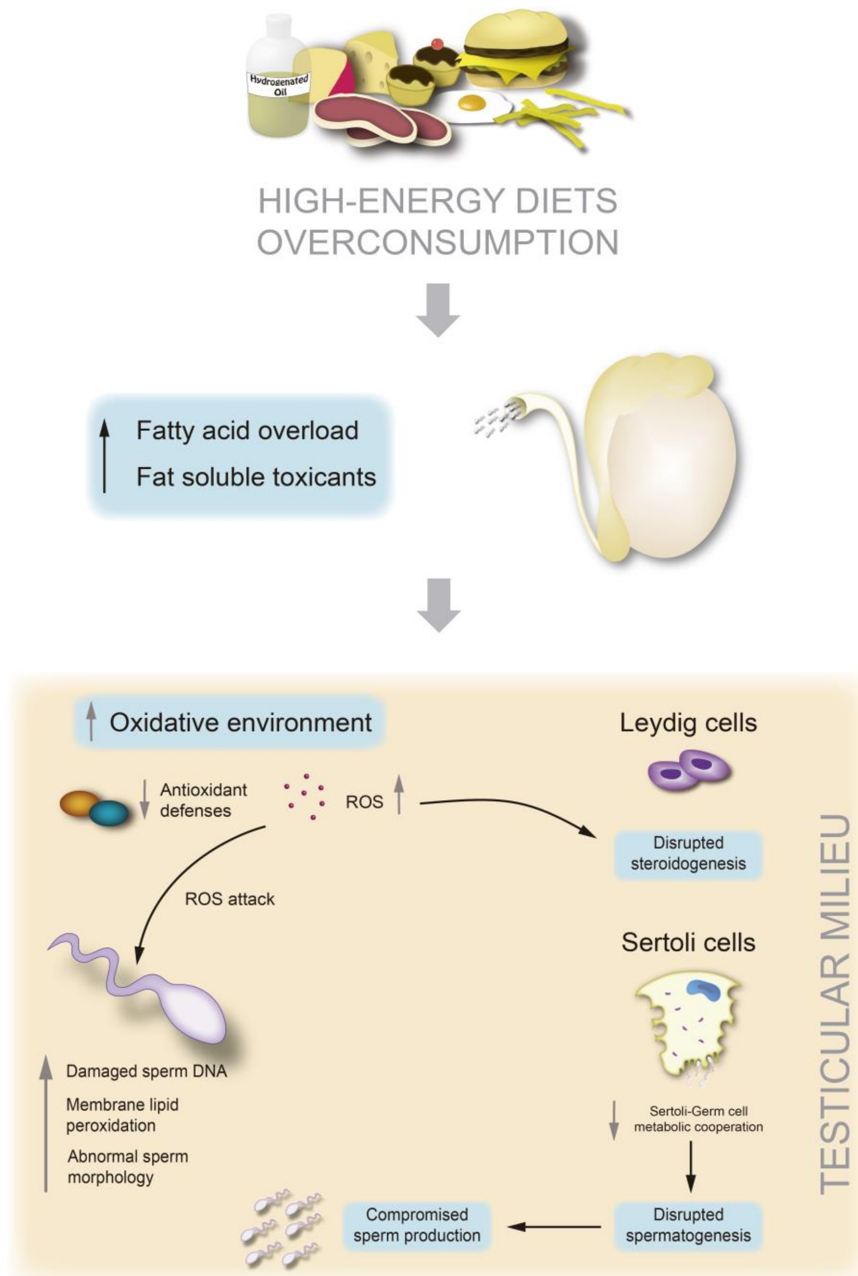

Figure 1.4. Effect of high-energy diets (HED) overconsumption in testicles. Overconsumption of HED leads to fatty acids accumulation and fat soluble toxicants within the testicular environment resulting in adverse reproductive outcomes such as impaired steroidogenesis and consequent decreased testosterone synthesis by Leydig cells. Testicular metabolism is also disrupted by fatty acid overload. Consumption of HED also impairs testicular metabolic cooperation between Sertoli cells (SCs) and germ cells (GCs). Disrupted lipid metabolism of SCs influence GCs membrane remodeling and structure. Glycolytic metabolism may also be affected compromising GCs fate. Another side effect of HED intake is its contribution for increased oxidative environment through the overproduction of reactive oxygen species (ROS). ROS can easily target testicular lipids and proteins, as well as sperm plasma membrane, consequently, sperm integrity and sperm membrane-related events will be compromised. Altogether the effects of HED overconsumption on testicles ultimately compromise sperm quality leading to decreased reproductive performance and male infertility.

High-energy diets consumption favors testicular oxidative stress

One side effect of high-energy intake might be its contribution to enhance oxidative stress within the testicular environment [198]. Oxidative stress occurs due to uncontrolled and excessive reactive oxygen species (ROS) production and an inefficient ROS scavenging system. Literature has highlighted oxidative stress as a cause for male infertility [199]. In fact, evidence showed that infertile men present high levels of oxidative DNA damage in spermatozoa, with particularly high levels of 8-hydroxy-20-deoxyguanosine, the major oxidized base adduct formed when DNA is targeted by ROS [200]. The heterogeneous population of somatic and germ cells of the testicular tissue presents a high variety of biomolecules highly vulnerable to ROS damage. Additionally, spermatogenesis is a continuum proliferation and differentiation process with high rates of mitochondrial oxygen consumption by the germinal epithelium and a concomitant elevated production of ROS. This outcome may be even more exacerbated by lipid accumulation, resulting from the increased fatty acid supply from HED intake, within the testicular *milieu* [201]. Consequently, more fatty acids will be transported into the mitochondria and oxidized. Although the rate of ß-oxidation increases in response to high-energy intake [202], testicular mitochondria may not be able to oxidize all fatty acids and this energy overload may lead to mitochondrial stress thus disrupting the normal functioning of testicular electron transport chain (ETC). Under stress conditions, mitochondria are prone to trigger a cascade of oxidative damages, since these organelles are major producers of ROS. This is of high relevance since mitochondria are essential for fully functional sperm [72].

Associated with pro-oxidative conditions is a decrease in mitochondrial DNA (mtDNA) content. mtDNA encodes core proteins essential to the efficiency of oxidative phosphorylation. Since mtDNA is highly susceptible to oxidative stress, partly due to its localization, mutations in mtDNA exacerbate the effects of dysfunctional ETC, contributing to more and more ROS production. These evidences support the hypothesis that lipid accumulation resulting from HED consumption may induce testicular mitochondrial ETC dysfunction, which is closely associated to oxidative stress. Both testicular lipids and proteins are highly sensitive to free radical attack and plasma membrane of germ cells is one of its “preferred targets”. This vulnerability results from superabundance of PUFAs, namely docosahexaenoic acid, which is the major constituent of human spermatozoa fatty acids. These unsaturated fatty acids are particularly prone to free radical attack due to the conjugated nature of the double bonds adjacent to methylene group (C-H), making the methylene bond weaker and consequently the hydrogen more susceptible to abstraction. The new carbon centered-radical formed in this process is stabilized by conjugated diene, which then combines with oxygen to origin a lipid peroxyl radical. This peroxyl radical is able of subtracting a hydrogen atom from another polyunsaturated fatty acid and triggering a lipid peroxidation cascade [203]. Consequently, sperm membrane is damaged, motility is lost, as well as the ability to undergo capacitation and fuse with the oocyte [198]. Similarly to what happens with membrane lipids, nuclear DNA is also targeted by ROS. High levels of both intracellular and mitochondrial ROS were reported

in spermatozoa from high-fat fed mice, with a concomitant increase in the levels of sperm DNA damage [198], linking HED consumption with poor sperm DNA integrity.

Spermatozoa are highly susceptible to oxidative injury, since they are relatively deficient in ROS-scavenging enzymes. So, they are particularly dependent on the antioxidant protection that exists within the testicular *milieu* and throughout the male reproductive tract [204]. Taking into account the previously reported association between HED consumption and sperm DNA damage [198], it is suggested that HED consumption also affects testicular antioxidant defense system (Figure 1.4). This contributes to a decrease of the testicular antioxidant defenses, which may be associated to a deficient mitochondrial function, compromising testicular bioenergetic capacity and ultimately contributing for declined sperm quality.

Intake of high-energy diets decreases semen quality

Emerging evidence demonstrate that poor nutritional food intake, particularly of unhealthy fats, negatively influence semen parameters and fertility [152, 153, 198, 205]. Attaman and collaborators [152] reported a negative correlation between saturated fat intake and both sperm count and sperm concentration. The presence of a negative correlation between semen parameters and dietary fats was also confirmed by Mendiola and collaborators [205]. They described that the intake of processed meat, a source of saturated fats, is associated with poor semen quality. In line with those reports, Jensen and collaborators [153] have demonstrated the effects of saturated fats in semen parameters of Danish men. Those authors found lower sperm concentration in men with high intake of saturated fats. All these reports are unanimous concerning the deleterious effects of HED intake in sperm parameters. In fact, the association between HED and decreased sperm quality was proposed to be driven by the intake of unhealthy fats present in these diets (Figure 1.4).

Interestingly, Gaskins and collaborators [206] did not report major alterations in sperm parameters of men consuming HED, which somehow contradicts the observations by other authors. The homogeneity of the study population (young healthy, college men) is a great contributor to unaltered sperm parameters. Moreover, the authors also detailed information on a variety of lifestyle factors, which may have contributed to these results. Indeed, human studies present several limitations since external factors (*e.g.* smoking, alcohol, drugs, environmental toxicants) and pathological conditions easily disturb human spermatogenesis and are limiting aspects when interpreting the results concerning sperm production. Another aspect is the limited type of studies that can be performed in humans. Most do not allow an in depth study of the molecular mechanisms that lead to alterations in reproductive parameters. Fortunately, the use of animal models allows a more detailed analysis of how unhealthy fats present in HED affect male fertility and sperm quality. They allow an in-depth identification of mechanisms responsible for altered testicular metabolism, disruption of spermatogenesis and changes in sperm membrane structure. Interestingly, data obtained with animal models are in accordance with the observations reported in the human studies referred above [198, 207], illustrating that consumption of HED is associated with declined

sperm parameters. For instance, Bakos and collaborators [198] found that sperm parameters from high-fat fed animals were significantly impaired. These rats presented decreased sperm motility and sperm capacitation, as well as a lower sperm binding capacity to oocytes. Concomitantly, Fernandez and collaborators [208] found that long-term high-fat intake resulted in decreased sperm motility with reduced fertility potential. The authors suggested that higher consumption of HED raises testicular fatty acid and cholesterol contents, altering sperm plasma membrane composition and consequently sperm membrane-related events [209]. This is consistent with a recent report by Lancellotti and collaborators [210] in which a decreased sperm functionality was described in hypercholesterolemic rabbits. Cholesterol is a constituent of sperm membranes but its increased levels compromise membrane structure and fluidity. In the case of sperm, this phenomenon originates a less functional spermatozoon. In fact, even a slight increase on cholesterol dietary levels may induce alterations in the cholesterol content of sperm membranes, resulting in altered acrosome reaction and capacitation state [210]. Additionally, high cholesterol levels are also associated with decreased sperm production. Hypercholesterolemia compromises both SCs and LCs secretory functions, which are known to be a prerequisite for initiation and maintenance of spermatogenesis [211]. Altogether these data lead us to conclude that HED impairs sperm membrane structure, compromising sperm membrane-related events, which are pivotal for fully functional sperm.

Noteworthy, the effects of HED observed on sperm parameters point toward the type of fat consumed as the major cause for declined fertility. HED frequently contains high amounts of saturated and *trans* fatty acids [206, 212]. *Trans* fatty acids are unsaturated fatty acids with at least one double bond in the *trans* configuration, instead of the physiological *cis* configuration. They are easily found in foods containing partially hydrogenated vegetable oils used in margarines and commercially prepared foods. Importantly, consumption of *trans* fatty acids compromises male fertility, since a close relationship between their consumption and decreased sperm parameters has been consistently reported [154, 155]. As these fats cannot be endogenously synthesized, its excessive consumption contributes to fat accumulation within the testicular environment [201, 213]. Fatty acids accumulate in testicular cells by two distinct processes: passive diffusion through the lipid bilayer and/or protein facilitated transport mediated by CD36 glycoprotein [214, 215], which is widely expressed in SCs [188]. Indeed, the increased fatty acid content within the testicular *milieu* contributes to altered testicular lipid metabolism, disrupting the reproductive events that depend on it. The following evidences have supported this hypothesis: (1) male rodents fed with hydrogenated oils instead of non-hydrogenated vegetable oils accumulate *trans* fatty acids in the testis [201]; (2) the accumulated fat in testicular environment resulted in adverse male reproductive outcomes, such as decreased sperm parameters, decreased T levels and even arrested spermatogenesis and testicular degeneration [201, 213]. In addition, hydrogenated fats and specific fatty acid isomers affect the activity of desaturases, elongases and may

disrupt *de novo* lipogenesis that is highly active in cells of the seminiferous epithelium, particularly in SCs.

Diabetes mellitus and Male Infertility

Diabetes mellitus at a glance

Diabetes related male infertility/subfertility

Role of insulin dysfunction in male fertility

Molecular mechanisms of testicular glucose metabolism in diabetic conditions

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Diabetes mellitus at a glance

Diabetes mellitus (DM) is a chronic, metabolic disease characterized by hyperglycemia that can result from defects in insulin secretion and/or insulin action [216]. Moreover, there is a severe alteration in carbohydrate, lipid and protein metabolism [216] that causes several systemic complications and co-morbidities such as renal failure or hypertension [217, 218]. Therefore, the origin of the DM-induced dysfunctions is a result of multifactorial causes that cannot be disregarded and highly complicates the study of this disease. The vast majority of the diagnosed DM cases are classified as type 1 diabetes mellitus (T1DM) or type 2 diabetes mellitus (T2DM). In the first category, there is an absolute deficiency of insulin secretion, while in the second the cause can be a combination of insulin resistance and insufficient insulin secretion [216]. Noteworthy, in T2DM the clinical symptoms are frequently detected only in an advanced phase of the disease, allowing the progression of functional changes in cells and tissues that may not be reverted even when a correct therapy is achieved [219]. One must also note that although the glycemic management in diabetic patients is crucial to reduce the development of several complications, it does not eliminate all the undesirable effects [220, 221]. Recent reports have questioned the safety of insulin [222] since some of the most important side-effects that diabetic patients suffer are related to the occurrence of hypoglycemic events, even if they occur transiently [223, 224]. Nevertheless, insulin remains the most powerful antihyperglycemic agent available although it is often combined with other antihyperglycemic agents to achieve euglycemia in diabetic patients. Noteworthy, recent studies reported that adults with DM can have a poor glycemic control due to intentionally missed insulin therapy [225] thus evidencing that clinical studies can, sometimes, be misled by this factor.

The complexity of DM diagnostic, especially in obese patients, led the researchers to establish an intermediate state, often called as pre-diabetes, where the patients present mild glycaemia that does not meet the established criteria for DM. Nevertheless pre-diabetic patients have important metabolic alterations that increase the risk for T2DM development [226] and associated complications. Besides mild glycaemia, pre-diabetic individuals also present impaired glucose tolerance and insulin secretion as well as relative insulin insensitivity [226, 227]. Thus, special care must be taken in account when discussing the overall effects of DM in any relevant physiological condition because the spectrum of development stages of this disease is very complex [228]. It is also well known that DM alters the HPT axis, which is responsible for some of the problems related to DM, such as impotence [229, 230]. Earlier studies reported elevated pituitary gonadotropins in diabetic rats [231] and altered levels of LH and FSH [232, 233]. Moreover, diabetic rats have abnormal sexual steroids feedback in the hypothalamic-pituitary axis described as a consequence of abnormal steroid transport or reduced pituitary sensitivity [234, 235]. Several reports also demonstrated that DM is associated with hormonal deregulation, particularly of sex steroids hormones [236-238].

In summary, DM is a metabolic disease that is growing to epidemic proportions. Besides, there are several co-morbidities that highly increase the complexity of the disease. The human and economic costs of such a wide spectrum disease are beyond estimations, making DM a preferential spotlight for researchers all over the world.

Diabetes-related male subfertility/infertility

a) Clinical Data

Recently, a high prevalence (51%) of subfertility was reported among patients with DM [239]. This led to the perception that DM is responsible for inducing subtle but crucial changes in sperm quality and function [240]. It is well known that in men DM is responsible for important sexual disorders such as decreased libido and impotence [241]. Erectile dysfunction (ED) is also very prevalent in diabetic men [242], but it ultimately depends on the male age, the duration of the disease and the level of blood glucose levels control. In a population-based cohort study, there was an increase of ED in older men with DM [243] and it has been reported that the glycemic control is correlated with ED [244]. Ejaculation disturbance is another factor that has been long identified in men with DM, especially retrograde ejaculation [245, 246], a condition where semen passes backwards into the bladder.
Diabetic male reproductive capacity is undoubtedly affect by ED and retrograde ejaculation, but when studying sperm parameters and sperm quality markers, the literature shows some conflicting results. In the 70's a study involving 25 diabetic individuals and 24 control individuals (16-22 years old), showed that T1DM juveniles presented lower sperm values and significant differences in sperm motility and morphology [247]. A few years later, other study compared the ejaculated of 65 diabetic and 77 control men and it was reported a negative effect of DM in the ejaculated. The parameters mostly affected were, in decreased order, sperm motility, morphology, volume and count [248]. Slightly different results were reported in a 1984 study, where T1DM adolescents presented a minor, non-significant, decrease in sperm count relatively to control individuals [249]. The semen from these T1DM adolescents had lower volume and motility, as well as altered morphology, and presented significantly higher fructose and glucose levels, evidencing that an ineffective metabolic control can be deleterious and/or responsible for the observed alterations in the semen [249]. Later, a study in testicular biopsies from impotent men with DM, concluded that the testes of impotent diabetic men presented ultrastructural lesions in apical Sertoli cell cytoplasm and morphological changes in the interstitial compartment, thus suggesting spermatogenic disruption and subfertility or infertility problems [250]. Another work reported that T1DM and T2DM subjects had a significant increase in sperm count and concentration, and a significant decrease in sperm motility and semen volume [77]. Interestingly, the sperm morphology and the quality of sperm motility remained unaffected [251]. Spermatozoa motility of T1DM patients was also evaluated by Niven and collaborators using a computerized image analysis

system [252]. They reported no correlations between sperm motility and age, age of onset of DM and duration of DM. Nonetheless, when comparing the diabetic group with control subjects, they concluded that several parameters related to sperm motility, such as track speed, path velocity, progressive velocity, and lateral head displacement, were not altered. On the other hand, other sperm motility related parameters, such as linearity and linear index that analyze the straightness of swimming, were significantly greater in diabetic men [252]. The controversy about the real impact of DM in male reproductive health continued, and, in 2002, a sperm cryopreservation study of patients with several diseases, including DM, reported that only the sperm from diabetic men presented significant differences in sperm parameters, namely in sperm counts [253]. More recently, a study with both T1DM and T2DM patients reported that diabetic men may present normal semen parameters, or only a significant decrease in semen volume, but they have a higher level of damage in sperm nuclear and mitochondrial DNA than control individuals [254].

There are apparently contradictory results concerning sperm motility and other male reproductive parameters when studying male diabetics. In fact, the conflicting nature of the existing data should be clarified. The fact that most of these studies are more focused on the clinical significance, rather than the molecular mechanisms behind DM-induced alterations, may explain at some extend the lack of consensus. For instance, the conventional semen analysis that was used in several of these studies, has been recognized by some authors, as very limited in the determination of the fertility status [255]. Moreover, there are several factors very difficult to control such as the duration of the disease, the glycemic control, the type of treatment as well as all the comorbidities associated to DM that mask the outcomes of the works related with DM and male fertility. Molecular studies with sperm from diabetics are still scarce and most of the mechanisms through which sperm manage to attain their energy metabolism in diabetic men remain to be disclosed. Therefore, there is an urgent need for more studies focused on the molecular mechanisms beyond glucose transport in sperm of diabetic men. However, there is a more profound lack of literature concerning the effect of DM in the functioning of testicular cells. It is expected that the metabolic cooperation between testicular cells may be compromised. To surpass these issues, as experimentation in humans has great restrictions and in most cases the tested hypotheses are impossible to assess in human material, the use of animal models that allow a tight control over experimental conditions are of extreme importance.

b) Animal Models data

Interestingly, data from animal models are successful in demonstrating that DM impairs male fertility and it is responsible for alterations in the reproductive health of individuals. As expected, there are more studies with animal models than with clinical data concerning DM-related male infertility. Since the 70's there are studies reporting a reduced fertility in male diabetic rats [256] and in the early 80's several reports using BB Wistar rats, a strain that spontaneously develop DM, described gonadal dysfunction in these animals [257, 258]. A few

years later the first study with a DM rat model induced by streptozotocin (STZ) and insulin therapy was done, reporting that STZ-treated rats presented decreased sperm counts and motility and that insulin treatment was able to restore these parameters [259]. This study evidenced that glycemic control may play a key role in reducing DM-related subfertility or infertility problems. Concomitant with that hypothesis, it was reported in the early 90's that long-term DM with sustained uncontrolled hyperglycemia is responsible for testicular dysfunction, resulting in decreased fertility potential [260]. Later it was described that STZ-induced DM male rats have altered sex behavior and diminished reproductive organ weight, testicular sperm content, epididymal sperm content, as well as sperm motility [261]. Studies on STZ-treated diabetic rats also showed that DM may cause regression of epididymis, leading to a decrease in caput weight, corpus, and caudal regions. Those studies also described atrophic changes in the caput, corpus, and caudal epididymis that resulted in voiding of spermatozoa from the epididymal lumen [262]. Insulin treatment was able to prevent some of these deleterious effects but only on certain epididymal regions [262]. Additionally, others studied the fertilization ability of spermatozoa from male STZ-induced diabetics and reported that these animals had a significant reproductive dysfunction that resulted from a decrease in the reproductive organ weights and in sperm counts, though not compromising sperm fertilizing ability using in utero insemination [263]. Also, a study comparing T1DM and T2DM using STZ-treated and Goto-Kakizaki (GK) rats showed that hyperglycemia had an adverse effect in sperm concentration and motility [264]. More recently, Kim and Moley [265] studied diabetic male mice sperm quality and fertilization capacity as well as subsequent embryo development. They concluded that DM decreased sperm concentration and motility and may cause male subfertility by altering steroidogenesis [265]. Others reported that the offspring of diabetic female rats, presented altered testicular parameters during fetal life, which can affect reproductive health during post puberty [266]. The offspring males presented increased number of seminiferous tubules besides thickness of the testicular capsule and reduced number of Leydig, Sertoli and spermatogonia cells [266].

Although there are contradictory results concerning sperm motility and other male reproductive parameters in diabetic men, it seems more consensual that DM rat models present significant alterations that end-up in reduced male reproductive health (see Table 1.2 for details). Nevertheless most of the works are not actually focused on the molecular mechanisms beyond glucose deregulation. The pathological alterations induced throughout the male reproductive tract of rodents are diverse in nature and although the histological changes are evident, most of the biochemical changes remain unknown. Thus, it is imperative to disclose the molecular mechanisms behind testicular cells metabolic dysfunction caused by DM to identify key points for possible therapeutic interventions.

Table 1.2. Summary of the main studies reporting diabetes-related reproductive effects. Legend: BB Rat – BioBreeding genetic rodent model; GK Rats – Goto-Kakizaki genetic rodent model; STZ rat – Streptozotocin-induced rodent model; ALX rat – Alloxan-induced rodent model. T1D – type 1 diabetes; T2D – type 2 diabetes; ↑ increase; ↓ decrease; superscript numbers are references as indicated in references section.

	Model		Type of Diabetes	Reproductive Effects
Animal Studies	Genetic Models	BB Rat	T1DM	↓ Testis weight [260]
				Severe germ-cell depletion [258]
				↓ Serum testosterone [258, 260]
				Disruption of seminiferous tubular morphology [258, 260]
				Sertoli-cell vacuolization [258]
				↓ Sperm production [260]
				↓ Fertility [257, 260]
		GK Rat	T2DM	Decreased sperm production [264]
	Chemically-Induced Models	STZ Rat	T1DM	↓ Testis weight [259, 263]
				Disruption of epididymis morphology and density [262]
				↓ LH, FSH and testosterone serum levels [259, 261, 263]
				↓ Sperm production [261, 262]
				↓ Sperm counts and motility [259, 261, 263-265]
				Erectile dysfunction [242]
				Ejaculation dysfunction [261, 263]
				↓ Mating behaviour [261, 263]
				↓ Fertility [263, 265]
		ALX Rat	T2DM	↓ LH, FSH and testosterone serum levels [266]
				Disruption of seminiferous tubular morphology [266]
				↓ Number of Leydig and Sertoli cells [266]
				↓ Number of spermatogonia [266]
Clinical Studies			T1DM	Disruption of seminiferous tubular morphology [250]
				Germ-cell depletion and Sertoli-cell vacuolization [250]
				Disruption of Blood-Testis Barrier [250]
				Erectile dysfunction [242, 248]
				Ejaculation dysfunction [245, 246]
				↓ Semen volume [248, 249]
				↓ Sperm counts, motility and morphology [247-249]
			T2DM	Erectile dysfunction [241]
				↓ Semen volume [251, 254]
				↓ Sperm motility [251]
				↑ Sperm DNA fragmentation [251]

Role of insulin dysfunction in male infertility

Euglycemia is very difficult to achieve in diabetic individuals although glycemic control is crucial to reduce DM-related complications [221]. In fact, hypoglycemia and the correlated hyperinsulinemia are common events in diabetic individuals. Glucose and insulin fluctuations lead to important molecular alterations that may result in detrimental effects to the reproductive health of the diabetic men. The undesirable effects of DM can be controlled depending on the DM type and the stage of the disease progression but hyperinsulinemia and hypoglycemia are common in both T1DM and T2DM [267, 268]. Moreover, throughout the diabetic individual's life, especially T2DM subjects, become progressively more insulin deficient and, consequently, more vulnerable to the undesirable effects of a poor glycemic control. Furthermore, contrarily to non-diabetic individuals that are very sensitive to small glucose or insulin variations in plasma, diabetic individuals gradually lose their sensitiveness to glucose and insulin [269, 270]. This is very important because, as discussed earlier, the number of diabetic adolescents is increasing and their reproductive health may be compromised at a very early age. Noteworthy, although the use of insulin analogues and/or insulin is essential they do not guarantee total effectiveness. In addition, insulin therapy can be a double-edged sword and its safety has been questioned [222-224]. Thus, it is imperative to discuss the molecular mechanisms altered in testicular cells when insulin deregulation occurs.

Few studies have been focused on the molecular mechanisms that are altered in testicular cells by insulin deregulation. In the early 80's the first studies concerning the effect of insulin in SCs glucose metabolism were published. Initially, it was reported that insulin acted synergistically with other hormones but its importance to male reproduction remained unknown [271]. Others reported that insulin stimulated the uptake of total nucleotide pool and in ATP, GTP, and UTP pools [272] as well as transferrin secretion by SCs [273]. Then, it was reported that insulin at micromolar concentrations induced stimulatory effects not only in DNA and protein synthesis but also in SCs lactate production [274]. Later, it was reported that after 3 hours of insulin addition to SCs, they showed a marked stimulation of lactate production [275]. This insulin effect on cultured immature rat SCs was described as being mediated by insulin receptors [275], however it was only a few years later that the authors reported not only the presence of insulin receptors but also suggested that insulin and IGF-I had precise functions responsible for the regulation of specific SCs activities during spermatogenesis initiation and maintenance [276, 277]. Interestingly, LCs cultured in a Sertoli cell-conditioned medium had also their functions controlled by the presence or absence of insulin [278]. In the 90's it was reported that insulin could not only regulate glycine metabolism but also lipid metabolism in cultured SCs [114]. Later, a study in SCs and LCs of STZ-treated rats, evidenced that the biosynthesis of arachidonic acid, a polyunsaturated fatty acid essential for several cellular functions, was under insulin regulation [279]. Importantly, it was reported that insulin could promote the differentiation of spermatogonia into primary spermatocytes by binding to the IGF-I receptor [280]. More recently, it was suggested that

insulin restores the reproductive health in diabetic males by the normalization of the HPT axis, and thus via the LH and T levels, rather than having a direct interaction in the testis [281].

Insulin role on the male reproductive tract goes far beyond testicular cells. In the 70's the first studies pointed to a stimulation of hexoses metabolism by sperm under insulin action [282]. However, the discussion continued whether insulin could modulate sperm metabolism or not with a study reporting that spermatozoa glucose oxidation could be independent from extracellular glucose concentration and insulin was not able to alter neither glucose metabolism nor spermatozoa motility [283]. A few years later, it was reported that both plasma membrane and the acrosome of spermatozoa are targets for insulin action and thus are under insulin hormonal control [284]. Also, intratesticular injection of insulin resulted in a decrease in spermatozoa motility in vas deferens and an increase in motile spermatozoa percentage on incubation medium after removal [285]. Moreover, defects in insulin secretion may change testicular and sexual glands function [286]. Interestingly, a study about the incidence of insulin resistance in men with ED, concluded that these men have a high incidence of insulin resistance thus evidencing a possible role for insulin in the reproductive health of men [287]. Others also reported that in men with increased insulin resistance there is a decrease in Leydig cell T secretion and, thus, severe male reproductive dysfunction [288]. Importantly, a recent study from Aquila and collaborators [289] proved that not only human ejaculated spermatozoa secrete insulin, as there is a physiological role for this insulin in the autocrine glucose metabolism regulation. This finding opened a new possible role for insulin in spermatozoa capacitation by controlling their glucose metabolic pathways. Others have also reported that insulin treatment in washed human spermatozoa from normozoospermic donors significantly increased total and progressive motility and acrosome reaction, as well as nitric oxide production, thus evidencing that insulin can enhance human spermatozoa fertilization capacity [178]. Moreover, a mitochondrial citrate carrier that contributes to the acquisition of sperm fertilizing ability through insulin secretion control has been identified [290], showing that insulin can act in sperm metabolism in a more complex way than the first proposed mechanisms. In sum, from the literature overview we conclude that insulin dysfunction has a crucial role in male infertility and/or subfertility related to DM and it is vital to study how insulin and insulin analogues therapy is administered and used to maintain the glycemic control. Moreover, insulin-induced hypoglycemia, a phenomenon that is frequent and in some rare cases fatal, has unpredictable effects in the overall metabolism of testicular cells. In fact, neither the magnitude of insulin dysfunction nor the mechanisms by which insulin controls testicular cells and sperm metabolism are fully disclosed. This issue should deserve more attention in the future.

Molecular mechanisms of testicular glucose metabolism in diabetic conditions

There is no doubt that spermatogenesis is a metabolically active process that depends upon strict metabolic cooperation between the several testicular cell types. During spermatogenesis, spermatozoa are produced within the seminiferous tubule in a process that takes several days and is under endocrine and paracrine control through the SCs [291]. In addition, SCs are responsible for the conversion of glucose, a non-metabolized substrate by developing germ cells, in lactate, which is the preferential substrate for those cells. The molecular mechanisms of testicular glucose metabolism in diabetic conditions are far from being disclosed. Nevertheless, *in vitro* and morphological studies of human biopsies from diabetic men allowed the collection of small but vital information concerning those molecular mechanisms. Human biopsies from diabetic men showed that there are morphological changes in testicular cells, namely in SCs, which presented extensive vacuolization and had a high degree of degeneration [250]. Moreover, germ cells exhibited a normal morphology, but the seminiferous tubules were depleted, and the number of LCs was also very variable, with these cells presenting lipid droplets and variable number of vacuoles [250]. All these changes certainly have dramatic consequences to testicular cells glucose metabolism and to the overall metabolic cooperation between SCs and developing germ cells. In fact, impairment of glucose metabolism is often related with increased fatty acid metabolism. Early studies reported that DM caused an increased endogenous oxygen uptake and reduced lactate production by testicular cells [292]. The same study also reported that DM increased cholesterol, non-esterified fatty acids, triglycerides and phospholipids in rat testis tissues [292]. In the 80's, Hutson [293] studied the biochemical responses of rat SCs and peritubular cells cultured under simulated diabetic conditions and reported that the *in vitro* metabolic functioning of these testicular cells was very sensitive to glucose concentrations. In fact, SCs cultured with high glucose concentration increased lactate secretion. At that time, it had already been reported that lactate enhanced respiration rates and protein and RNA synthesis in isolated pachytene spermatocytes and round spermatids [78], by interacting in other metabolic pathways and producing ATP [294]. Besides, lactate was also described as a modulator of nicotinamide adenine dinucleotide phosphate-oxidase (NADH) oxidation and the pentose phosphate pathway in those cells [295]. In the 90's, new functions for the lactate produced in the testis were described. Intratesticular lactate infusion was reported to improve the spermatogenesis in adult cryptorchid rat testis [77] and later, it was described that germ cell death is inhibited in a dose-dependent way by lactate [79]. Therefore, any condition that promotes an alteration in testicular lactate levels or lactate production by SCs may compromise germ cell development and this can occur through several distinct mechanisms. Recently, *in vitro* works focused on the molecular mechanisms of glucose or insulin deprivation in SCs. It was reported that decreased glucose levels in SCs culture medium increases glucose uptake to maintain lactate production, which was only slight

decreased [97]. This was achieved by modulating GLUT1 and GLUT3 expression, through activation of AMPK, phosphatidylinositol 3-kinase (PI3K)/PKB, and p38 MAPK-dependent pathways [97]. The authors do not infer about possible alterations in the key steps of glycolysis. Although the exact mechanisms by which DM alters the glucose metabolism in testicular cells are not easy to follow *in vivo, in vitro* evidence cannot be disregarded even those dispersed and indirect. As discussed above, it is well known that DM is a metabolic disease that induces crucial alterations in sex hormones levels [296]. Recent work in both, rat SCs, showed that their energy metabolism is influenced by sex steroid hormones. Cells treated with 5α-DHT consumed less glucose and thus, produced less lactate. Both, E_2 and 5α-DHT were able to alter lactate metabolism-associated gene transcript levels such as MCT4, LDH [96]. Moreover, sex steroids are also known to regulate apoptotic signaling pathways in SCs thus deregulated levels of these hormones may lead to apoptosis and necrosis [297, 298]. Nonetheless, when analyzing all these molecular mechanisms that are certainly related to DM pathological conditions, one cannot neglect the possible contribution of glycogen. It has been reported that SCs possess glycogen and glycogen phosphorylase activity [24, 103] but these reports from the 80's were not consolidated and therefore should deserve special attention in a near future to understand the adaptive mechanisms of SCs in pathological conditions. The presence of glycogen and glycogen metabolism related machinery can be a valuable mechanism to explain some of the assumptions concerning DM-related effects in glucose testicular cells.

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Chapter 2

Aims and outline of the thesis

Aims and outline of the thesis

The metabolic cooperation established between testicular cells is a complex event and depends on the correct functioning of several metabolic pathways. All these events are controlled by a myriad of factors that include: hormones, proteins, metabolic products, growth factors, cytokines and many other factors, which through a complex network of signals ultimately control spermatogenesis. Testicular metabolism, in particular the metabolism of Sertoli cells (SCs), plays a crucial role on the normal occurrence of spermatogenesis. Recent advances have highlighted that the metabolism of testicular cells is a target of several diseases, particularly those associated with changes in whole body metabolism, explaining the subfertility/infertility problems that individuals suffering from those diseases. Spermatogenesis is highly sensible to external factors and the exposure to contaminants, food intake and factors related to lifestyle may also explain the decrease of fertility in males in reproductive age years. The general aim of the research described in this thesis was to evaluate the effects of metabolic pathologies (and its co-morbidities) associated with the overconsumption of high-energy diets (HED), such as pre-diabetes and type 2 diabetes mellitus (T2DM), on the metabolism of testicular tissue and SCs (particularly glucose metabolism), and the subsequent consequences for male reproductive health.

A key player in metabolic diseases such as pre-diabetes and T2DM is insulin, which also has an important role in glucose homeostasis. Thus, our first objective was to deepen the knowledge on the how insulin modulates the metabolism of human SCs (hSCs) (Chapter 3). So, we evaluated metabolite consumption or secretion in primary cultures of hSCs under insulin deprivation. We also evaluated how the absence of this hormone affected the expression of glucose transporters and enzymes, to further disclose the insulin-deprivation effect on lactate production and export.

Another important feature of diabetes mellitus (DM) is a deregulation of sex steroid synthesis. As in the seminiferous tubules, SCS are the key targets for these hormones and there is a growing awareness that androgens and estrogens have general metabolic roles that reach far beyond the reproductive processes, we aimed to disclose the metabolic modulation of SCs by sex steroid hormones. So, our second objective was to determine how hSCs metabolically respond when exposed *in vitro* to physiologic concentrations of 5α dihydrotestosterone (5α-DHT) and 17ß-estradiol (E_2) (Chapter 4). We aimed to evaluate the consumption of specific substrates (glucose and pyruvate) and the production of metabolites (lactate and alanine). We also evaluated how these hormones affected the expression of key proteins associated with glucose metabolism in SCs, to disclose their effect on lactate production and export.

In fact, progressive stages of DM (pre-diabetes and T2DM) are associated with inceasing alterations of testosterone (T) levels. So, our third objective was to determine the *in vitro* effects of T deficiency induced by those stages of DM on the glycolytic metabolism of rat SCs (Chapter 5). For that we exposed hSCs to different levels of T observed either in pre-diabetic state, as also in T2DM states. Key steps of the glycolytic pathway, namely glucose consumption, the protein expression of glycolysis-related enzymes and the production of

lactate were assessed in the respective conditions. We also evaluated the possible use of alternative substrates by SCs that ensure an endogenous source of glucose in those conditions. The fourth objective of our work was to determine predidabetes affected testicular glucose metabolism and the consequenses for male reproductive health. Since the consumption of HED is increasing in the modern societies and such behavior is strongly associated to high risk of DM development, we took advantage of an animal model that could mimic the initial stages of DM induced by HED consumption. We evaluated the impact of this pathological state on overall testicular glucose metabolism and the male reproductive health, evaluating the epididymal sperm parameters (Chapter 6). We also aimed to determine on how pre-diabetes affected testicular bioenergetic capacity (Chapter 7), evaluating the expression of key proteins involved in the mitochondrial function, such as peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) and its downstream target sirtuin 3 (SIRT3). We also evaluated the testicular mtDNA integrity and the functioning of testicular mitochondrial electron transport chain (ETC). As PGC-1α and SIRT3 are also involved in the activation of the antioxidant defense system, the oxidative stress parameters, such as antioxidant capacity, lipid peroxidation and protein oxidation were assessed.

The final objective of our work was to determine the impact of an established T2DM state (Chapter 8) on testicular glucose metabolism. We evaluated also impact of T2DM on the testicular expression and activity of key glycolytic-associated enzymes and transporters. As it has been proposed that T2DM induces crucial changes in testicular glycogen metabolism, we assessed the expression of key enzymes involved in the glycogen metabolism (glycogen synthase and phosphorylase). The epididymal sperm parameters were assessed to determine the impact of this pathological state on male reproductive health.

Chapter 3

Effect of Insulin Deprivation on Metabolism and Metabolism-Associated Gene Transcript Levels of *In Vitro* Cultured Human Sertoli Cells

This chapter was adapted from the published work:

*P. F. Oliveira, M. G. Alves, **L. Rato**, S. Laurentino, J. Silva, R. Sá, A. Barros, M. Sousa, R. A. Carvalho, J. E. Cavaco and S. Socorro. Effect of Insulin Deprivation on Metabolism and Metabolism-Associated Gene Transcript Levels of In Vitro Cultured Human Sertoli Cells. Biochimica et Biophysica Acta – General Subjects. (2012) 1820 (2): p. 84-89.*

Effect of Insulin Deprivation on Metabolism and Metabolism-Associated Gene Transcript Levels of *In Vitro* Cultured Human Sertoli Cells

Abstract

BACKGROUND: Sertoli cells metabolize glucose producing lactate for developing germ cells. As insulin regulates glucose uptake and its disturbance/insensitivity is associated with diabetes mellitus, we aimed to determine the effect of insulin deprivation in human Sertoli cells (hSCs) metabolism and metabolism-associated gene expression.

METHODS: hSCs-enriched primary cultures were maintained in the absence/presence of insulin and metabolite variations were determined by ^{1}H-NMR. mRNA expression levels of glucose transporters (GLUT1, GLUT3), lactate dehydrogenase (LDHA) and monocarboxylate transporter (MCT4) were determined by reverse transcriptase–polymerase chain reaction.

RESULTS: Insulin deprivation resulted in decreased lactate production and in a decrease of glucose consumption that was completely reverted after 6h. Cells of both groups consumed similar amounts of glucose. In insulin-deprived cells, transcript levels of genes associated to lactate metabolism (LDHA and MCT4) were decreased. Transcript levels of genes involved in glucose uptake exhibited a divergent variation: GLUT3 levels were decreased while GLUT1 levels increased.

CONCLUSIONS: Insulin-deprived hSCs presented: 1) altered glucose consumption and lactate secretion; 2) altered expression of metabolism-associated genes involved in lactate production and export; 3) an adaptation of glucose uptake by modulating the expression of GLUT1 and GLUT3.

GENERAL SIGNIFICANCE: This is the first report regarding the effect of insulin-deprivation on hSCs metabolism.

Keywords: Human Sertoli cells; energy metabolism; insulin; lactate; glucose

Introduction

Sertoli cells (SCs) form the blood-testis-barrier are often called as the “nurse cells”, supply the physical and nutritional support for germ cells [1, 2] and are crucial to establish an adequate luminal environment in the seminiferous tubules [3]. SCs have a high capacity to produce lactate [4] which is a key element for germ cells due to its anti-apoptotic effect and its role as energy source [5]. In fact, spermatocytes and spermatids suffer a rapid decline in their ATP content and require lactate for ATP maintenance [4]. They are unable to use glucose for their energy metabolism and preferentially use lactate [1]. Therefore, regulation of glucose metabolism is critical for normal spermatogenesis and fertility. Insulin is the leading hormone influencing this regulatory system and its dysfunction (deficiency or

resistance) has attracted much attention since it spreads the outcome results to diabetes mellitus (DM), hypertension, dyslipidaemia, among others [6]. DM is characterized by an absolute or relative insulin deficiency and by poor glucose control in the blood. In type I diabetes there is an absolute lack of insulin due to the progressive loss of pancreatic β-cells. Numerous studies in male individuals and animal models of DM suggest that it impairs male fertility with reduction in fecundity, lower lactate content in testes and impairment of sperm quality [7, 8]. Testicular biopsies of diabetic patients revealed numerous abnormalities, including altered SCs connections, degenerating SCs and vacuolization of both Sertoli and Leydig cells [9]. It has also been described that testicular cells of individuals subjected to DM present not only adaptive responses to oxidative stress [10] but also metabolic adaptations that allow them to avoid the deleterious effects promoted by this disease ensuring an adequate conditions for germ cell development [7, 11].

It remains to be determined if insulin deficiency causes a modulation of lactate secretion by SCs, which may be due to alterations in carbohydrate metabolism and/or in the expression of metabolism-associated genes, such as: (i) Glucose transporters (GLUT), that mediate the transport of glucose through the plasma membrane [12]; (ii) Lactate dehydrogenase (LDH), that catalyses the interconversion of pyruvate to lactate [13]; and (iii) Monocarboxylate transporters (MCT), that transport lactate across the plasma membrane towards the germ cells [14].

With our work, we aimed to examine the effect of insulin deprivation on metabolite secretion or consumption in primary cultures of human SCs (hSCs) using ^{1}H-NMR analysis. We also explored the hypothesis that the absence of this hormone affected the expression of GLUT1, GLUT3, LDHA and MCT4 by evaluating mRNA expression to further disclose the insulin-deprivation effect on lactate production and export.

Methods

Chemicals

Polyclonal antibodies, M-MLV RT and random primers were purchased from Invitrogen (Carlsbad, CA, USA). dNTPs were purchased from GE Healthcare (Buckinghamshire, UK). Taq DNA Polymerase was purchased from Fermentas Life Sciences (Ontario, Canada). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Patient selection, ethical issues and testicle tissue preparations

The clinical study of the patients and testicle tissue processing was performed at the Centre for Reproductive Genetics Alberto Barros (Porto, Portugal) in accordance with the Guidelines of the Local, National and European Ethical Committees. In all cases, testicular biopsies were obtained from infertile patients under treatment for recovery of male gametes and used after informed written consent. Studies have been performed according to the Declaration of Helsinki.

In the present study only cells left in the tissue culture plates after treatment of the patients were used. hSCs were isolated from five testicular biopsies with normal spermatogenesis, selected from patients with an ejaculation (psychological, vascular, neurologic), vasectomy or traumatic section of the vas deferens.

Each testicle biopsy of the selected cases was collected in sperm preparation medium (SPM-Hepes buffer; Medicult) and kept at 32ºC with 5% CO_2 in air until use.

Sertoli cell culture

Testicle biopsies were washed twice in $HBSS_f$ (Hanks Balanced Salt Solution without Ca^{2+} or Mg^{2+}; Sigma) by centrifuging at 500.*g* at room temperature, as described by Oliveira and collaborators [15]. Sertoli cells were obtained by a method previously described [16]. Briefly, the resulting pellet was suspended in SCs culture medium (DMEM:Ham's F-12 1:1, containing 15 mM HEPES, 50 U/ml penicillin and 50 mg/ml streptomycin sulfate, 0.5 mg/ml fungizone, 50 µg/ml gentamicin and 10% heat inactivated FBS) and forced through a 20G needle, in order to disaggregate large cell clusters. The concentration of cells on the cellular suspension obtained from the procedure described above was adjusted to 5000 cells/ml with SCs culture medium, plated on Cell+ culture flasks (Sarsted, Nümbrecht, Germany), and incubated at 30–32°C, 5% CO_2 in air until used.

Cultures with cell contaminants below 5% after 96 h, as examined by phase contrast microscopy, were used. Sertoli cell culture purity was revealed by immunoperoxidase detection of Anti-Mullerian hormone and Vimentin specific markers [17].

In order to discard the possibility of dedifferentiation of the Sertoli cells in culture we evaluated the mRNA expression of Cytokeratin-18 (Ck-18), an intermediate filament that has been demonstrated to be only present in immature Sertoli cells [18]. No expression of Ck-18 was observed before or after the treatment.

Experimental groups

Sertoli cells were allowed to grow until reach 90-95% of confluence, and then washed thoroughly and the medium was replaced by serum-free medium (supplement DMEM:F12 1:1, pH 7.4). In order to evaluate the effects of insulin in metabolite consumption and production, GLUT1, GLUT3, LDHA and MCT4 expression, SC medium was supplemented either with Insulin-Transferrin-Sodium Selenite (5 mg/mL - 5 mg/mL - 5 μg/mL, respectively) [Control group] or with Transferrin-Sodium Selenite (5 mg/mL - 5 μg/mL, respectively) [InsD group]. Treatments were performed for 48 hours. During treatment, 250 µL of the culture medium were collected at 6, 24, and 48 hours, for ^{1}H-NMR analysis. At the end, cells were detached from the flask using a Trypsin-EDTA solution and collected for RNA extraction. The total number of cells per flask was determined with a Neubauer chamber and a viability test was performed on the cells of the different experimental groups using the Trypan Blue Exclusion Test. Viability averaged 85-90%, always with values higher than 85%.

NMR spectroscopy

^{1}H-NMR spectroscopy was performed to determine lactate production, glucose consumption and variations in other substrates, such as pyruvate and alanine, during the 48 hours of cell incubation in the various conditions as describe previously by Alves and collaborators [19]. Sodium fumarate (final concentration of 2 mM) was used as an internal reference (6.50 ppm) to quantify metabolites in solution (multiplet, δ, ppm): lactate (doublet, 1.33); alanine (doublet, 1.45); pyruvate (singlet, 2.36); H1-α glucose (doublet, 5.22).

Reverse transcriptase-polymerase chain reaction

At the end of the 48 hours treatment, total ribonucleic acid (RNAt) was extracted from isolated SCs using TRI reagent according to the manufacturer's instructions. RNAt concentration and absorbance ratios (A260/A280) were determined by spectrophotometry (Nanophotometer^{TM}, Implen, Germany). RNAt was reversely transcribed as described previously [20]. The resulting cDNA was used to amplify GLTU1, GLUT3, LDHA and MCT4 cDNA fragments with exon-exon spanning primer sets. Primer sequences, optimal annealing temperature and the number of cycles required for exponential amplification phase of fragments are indicated in Table 3.1. For each sample the PCR reactions were carried out in triplicate and GLUT1, GLUT3, LDHA and MCT4 mRNA levels were normalized with 18S gene expression as internal control. Liver mRNA was used as positive control and cDNA-free sample was used as negative control. The PCR products were separated electrophoretically on a 1% agarose gel stained with ethidium bromide and visualized under ultraviolet light, using a CN-TFX imaging system (Vilber Lourmat, Marne-la-Vallée, France) equipped with a monochrome CCD camera. The intensities of the band of each fragment was measured by using BIO-PROFIL Bio-1D Software (Vilber Lourmat, Marne-la-Vallée, France) according to standard methods [21, 22]. For each sample, the obtained band intensity (for GLUT1, GLUT3, LDH A and MCT4) was divided by the respective 18S band intensity, to obtain the relative abundance in each experimental condition (Control and InsD group). The relative abundances of the InsD group were then divided by relative abundances of the Control group, in order to express these values as fold induction/reduction of the InsD group versus the Control group.

Statistical analysis

The statistical significance of metabolite variation and mRNA expression among the experimental groups was assessed by one-way ANOVA, followed by Dunn post-test. All experimental data are shown as mean ± SEM (n=5 for each condition). Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). $P<0.05$ was considered significant.

Table 3.1. Oligonucleotides and cycling conditions for PCR amplification of GLUT1, LDHA, GLUT3, MCT4 and 18S. Abbreviations: AT — annealing temperature; C — number of cycles during exponential phase of amplification.

Gene	Sequence (5'-3')	AT (°C)	Amplicon Size (bp)	C
GLUT1	Sense: AGCAGCAAGAAGCTGACGGGTC Antisense:CGCCGGCCAAAGCGGTTAAC	60	269	35
LDHA	Sense: GCACTCACACGTGGGTTCCCG Antisense: GCAAGTTCATCTGCCAAGTCCTTCA	60	200	35
GLUT3	Sense: TCAGGCTCCACCCTTTGCGGA Antisense: TGGGGTGACCTTCTGTGTCCCC	56	228	35
MCT4	Sense: ACCAACCCTCCTGGCCATGGGA Antisense: GCCAAACCCAACCCCGTGATG	58	396	35
18S	Sense: AAGACGAACCAGAGCGAAAG Antisense: GGCGGGTCATGGGAATAA	56	149	25

Results

Insulin deprivation decreases initial glucose consumption

In the first 6 hours of treatment, glucose consumption was practically nil for insulin-deprived hSCs, while cells in control conditions consumed 28 ± 3 pmol of glucose.cell^{-1} (Figure 3.1A). Between the 6th and the 24th hour, insulin-deprived hSCs presented a glucose consumption rate notably higher when compared with cells in the control condition. At the end of the 24th hour, hSCs cultured without insulin consumed 50±6 pmol.cell^{-1} at a rate of 2.7 ± 0.2 pmol.h^{-1}.cell^{-1} while hSCs in normal conditions consumed 30±1 pmol.cell^{-1} at a rate of 0.1 ± 0.1 pmol.h^{-1}.cell^{-1} (Figure 3.1A, B). The overall consumption of glucose after 48 hours of treatment was not significantly different in cells from both groups (Figure 3.1A). hSCs cultured in control and insulin-deprived conditions consumed similar amounts of glucose (47 ± 4 and 59 ± 5 pmol.cell^{-1}, respectively).

Pyruvate consumption is reduced in insulin deprivation conditions

Pyruvate was promptly consumed by hSCs on control conditions and almost all the pyruvate initially present on the control medium was used by the cells. After 48 hours, the pyruvate consumption was 1.8 ± 0.4 pmol.cell^{-1} for those cells (Figure 3.1C).

Insulin-deprived cells during 48 hours altered significantly the consumption of pyruvate profile. At the end of 6 hours, they had only consumed 0.6±0.2 pmol.cell^{-1} while cells in control conditions consumed 1.4 ± 0.1 pmol.cell^{-1} (Figure 3.1C). Remarkably, beyond the 24th of insulin deprivation, cells started to produce and export pyruvate to the extracellular medium, as can be seen by the shift on the pyruvate consumption rate (-0.04 ± 0.1 pmol.h^{-1}.cell^{-1}) in Figure 3.1D.

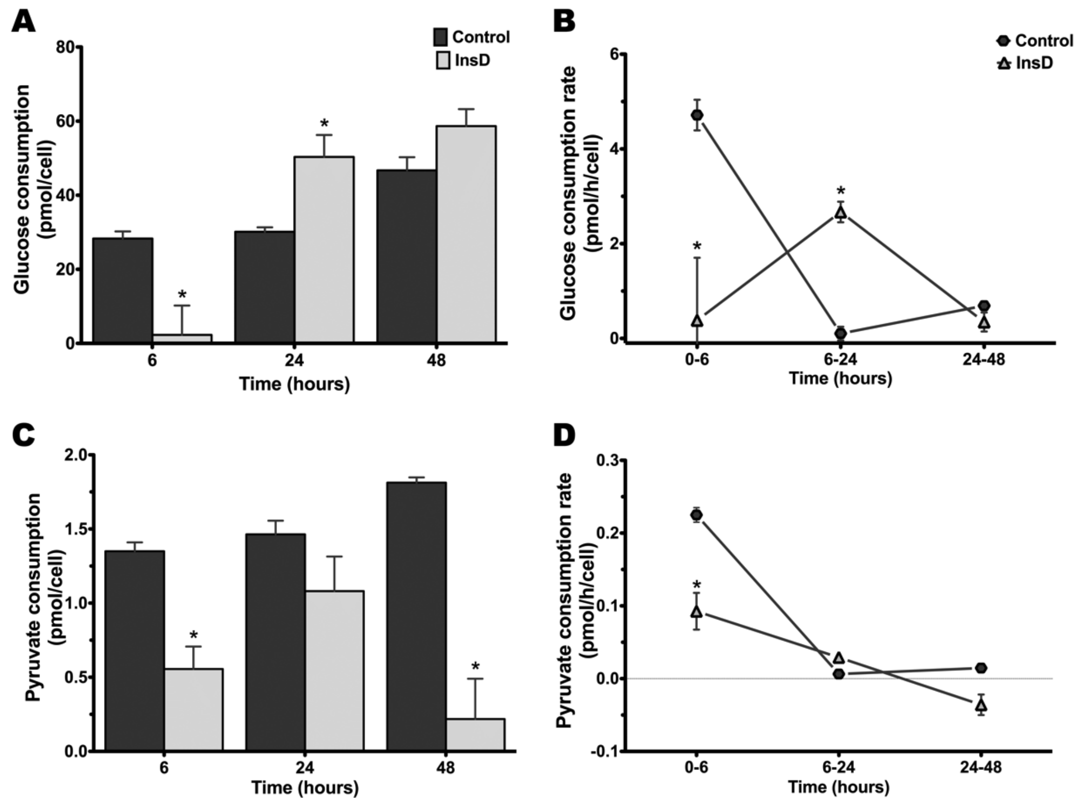

Figure 3.1. Effect of Insulin-deprivation on Glucose and Pyruvate consumption in human Sertoli cells cultured *in vitro*. (A) Glucose consumption; (B) Glucose consumption rate; (C) Pyruvate consumption; (D) Pyruvate consumption rate. Control - control group; InsD- Insulin deprived group. Results are expressed as means ± SEM (n=5). * Significantly different when compared to control (P<0.05).

Insulin deprivation decreases lactate production

The amount of lactate produced during the first 6 hours of treatment was similar for control and insulin-deprived cells, 2.3 ± 0.1 and 2.1 ± 0.1 pmol.cell^{-1}, respectively. During the following 42 hours, hSCs in control conditions continued to produce lactate at similar rates, however insulin-deprived cells secreted significantly lower amounts of lactate (Figure 3.2A,B). At the end of the treatment, hSCs from control and insulin-deprived group produced 12.1 ± 0.3 and 4.1 ± 0.2 pmol.cell^{-1}, respectively. The lactate production rate was similar for the first 6 hours for hSCs cultured in all experimental conditions but at the end of the 48th hour of culture, the lactate production rate reaches values near zero in insulin-deprived cells which was much lower than that observed in control conditions (0.23 ± 0.01 pmol.h^{-1}.cell^{-1}) (Figure 3.2B).

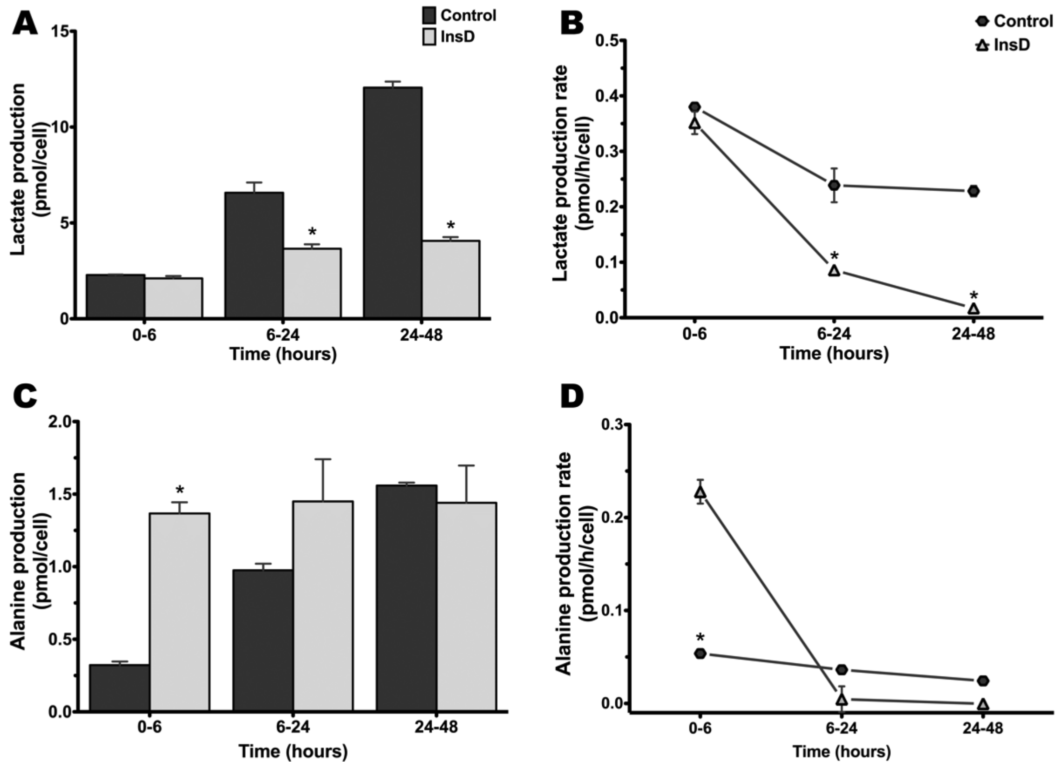

Figure 3.2. Effect of Insulin-deprivation on Lactate and Alanine production in human Sertoli cells cultured *in vitro*. (A) Lactate production; (B) Lactate production rate; (C) Alanine production; (D) Alanine production rate. Control - control group; InsD- Insulin deprived group. Results are expressed as means ± SEM (n=5). * Significantly different when compared to control ($P<0.05$).

Alanine production is highly increased in the first hours of insulin deprivation

During the initial 6 hours of treatment, alanine production was highly increased in insulin-deprived hSCs. These cells produced 1.37 ± 0.08 pmol of alanine.cell^{-1} while cells in control conditions produced 0.32 ± 0.02 pmol of alanine.cell^{-1}. At the end the 48 hours treatment, alanine production was very similar in all conditions (Figure 3.2C). Accordingly, alanine production rate was significantly higher in the first 6 hours in insulin-deprived hSCs (0.23 ± 0.01 pmol.h^{-1}.cell^{-1} versus 0.05 ± 0.01 pmol.h^{-1}.cell^{-1} in control condition) but after that period it dropped to values near zero, as opposed to the control situation where it remained constant (Figure 3.2D).

Insulin deprivation decreases GLUT3 but increases GLUT1 mRNA levels

GLUT1 and GLUT3 are high affinity glucose transporters known to be present on the plasma membrane of SCs and usually are associated with the transport of glucose in cells with high-energy demands [12]. To analyse the possible effect of insulin-deprivation on the expression levels of GLUT1 and GLUT3 we performed a semi-quantitative RT-PCR to quantify the mRNA levels of these GLUTs after 48 hours of culture. In insulin-deprived SCs, GLUT3 mRNA levels decreased significantly to 0.67 fold (Figure 3.3) and GLUT1 mRNA levels significant increased to 1.25 fold (Figure 3.3) after the 48 hours treatment.

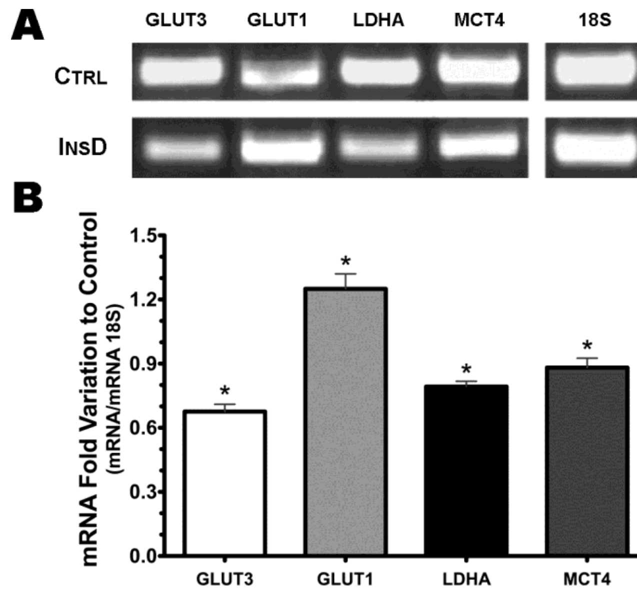

Figure 3.3. Effect of 48 hours of Insulin-deprivation on the expression of metabolism-associated genes in human Sertoli cells cultured *in vitro*. Panel A shows a representative agarose gel electrophoresis showing expression differences in insulin-deprived cells (INSD) and Controls (CTRL). Panel B shows pooled data of independent experiments (n=5), indicating the fold variation of GLUT1, GLUT3, LDHA and MCT4 mRNA levels. For each sample, the obtained band intensity (for GLUT1, GLUT3, LDH A and MCT4) was divided by the respective 18S band intensity, to obtain the relative abundance in each experimental condition (Control and InsD group). The relative abundances of the Insulin Deprived group (InsD) were then divided by relative abundances of the Control group, in order to express these values as fold induction/reduction of the InsD group versus the Control group. Results are expressed as mean ± SEM (n=5). * Significantly different when compared to Control ($P<0.05$).

LDHA mRNA levels are decreased in insulin-deprived cells

After observing a decrease on lactate levels produced by the insulin-deprived SCs, we investigated the possibility of an effect of this hormone on mRNA levels of LDHA. We observed a significant 0.79 fold decrease in LDHA mRNA levels when compared to control conditions, after 48 hours of treatment (Figure 3.3).

Insulin deprivation decreases MCT4 mRNA levels

Following the noted decrease on the amount of lactate exported to extracellular medium by the SCs of the insulin-deprived group, we also investigated the possibility of an alteration on the levels of MCT4, a monocarboxylate transporter that serves primarily as a lactate exporter [23]. These cells showed a significant 0.88 fold decrease on MCT4 mRNA levels when compared with SCs cultured in control conditions (Figure 3.3).

Discussion

In hSCs, the study of carbohydrate metabolism has been somewhat overlooked, although for cultured rat SCs it has been widely debated since the early 80s. Rat testes show an unusual dependence on glucose as a source of energy [24, 25] to produce lactate, which is the preferred energy substrate for spermatocytes and spermatids [4, 26]. Thus, the mechanisms

that regulate SCs glucose metabolism are particularly relevant in maintaining spermatogenesis and male fertility. We have recently described that 17β-estradiol and 5α-dihydrotestosterone are metabolic modulators of rat [20] and hSCs [15], demonstrating that SCs metabolism is under strict hormonal control. DM is a hormonal condition classically associated with insulin deprivation or insulin resistance in the cells, which is often described as a disorder of carbohydrate, lipid and protein metabolism. It has been described that DM impairs spermatogenesis, although none of these studies were carried using hSCs.

In the present work, insulin-deprived hSCs consumed significantly less glucose and pyruvate during the first 6 hours, although the lactate production was not severely affected. We hypothesize that during this period, the SCs consumed other energetic substrates such as ketone bodies and fatty acids, since they have also been described as possible substrates in cultured SCs [27, 28]. The glycogenolysis pathway may also be participant in this process as glycogen and glycogen phosphorylase activity have been detected in SCs, making possible that glycogen hydrolysis fuels the glycolytic pathway in these cells [29, 30]. These substrate storages, known to exist in these cells, could be consumed during the first hours and after being depleted, SCs adapt their glucose uptake mechanisms, as indicated by GLUT1 and GLUT3 mRNA modulation, in order to maintain the lactate production within a physiological range. After 48 hours the glucose consumption was not significantly different between hSCs from both groups, although lactate production was significantly lower in insulin-deprived cells. SCs can take up glucose from the external medium [31] through the action of hexose transporters (GLUTs) present on their plasma membranes and usually this is the rate-limiting step for glucose metabolism in the cells [32]. Three glucose transporter isoforms (GLUT1, GLUT3 and GLUT8) have been so far identified in SCs [12, 33]. However, GLUT8 is not expected to be involved in glucose transport from the extracellular *milieu* since it has not been identified in the plasma membrane of tissues [34] and thus we investigated the alteration of the mRNA levels of GLUT1 and GLUT3. After the 48 hours insulin-deprivation treatment, there was a significant decrease in GLUT3 and a concurrent increase in GLUT1 mRNA levels. Although these changes on mRNA levels are not a direct measure of the protein functioning, they clearly indicate an effect on the regulatory mechanisms of action of these proteins by insulin. Thus, insulin-deprived hSCs cultured *in vitro* adjust their glucose uptake by modulating GLUT1 and GLUT3 transcript levels. This phenomenon was also observed in rat SCs cultured *in vitro* under glucose deprivation [11] and described as an adaptation to ensure an adequate lactate concentration in the microenvironment where germ cells develop. In our experimental conditions, after 48 hours culture the insulin-deprived hSCs produced significantly less lactate than control cells. This may be explained by a reduction of lactate export through the MCT or by a reduced lactate conversion from pyruvate by LDHA. After 48 hours treatment, there was a significant decrease in mRNA levels of MCT4 and LDHA, which indicates that lactate interconversion from pyruvate and the export of lactate are modulated by insulin. The decreased expression of both MCT4 and LDHA mRNA is consistent with a lower lactate concentration in extracellular media of insulin-deprived cells.

As discussed above, in insulin-deprived cells the pyruvate consumption is significantly reduced in the first 6 hours of treatment, following closely the decrease on glucose consumption. However, after the 24th hour of insulin deprivation the pyruvate consumption profile changed dramatically. During the 24th and the 48th hour of treatment, the insulin-deprived SCs produced and exported pyruvate to the extracellular medium in contrast to what happened in control conditions. Pyruvate is a key branch point in central carbon metabolism and its metabolic fate is regulated by the availability of other substrates and in large part by the redox state of the cell [19]. The cytosolic pyruvate is originated from glycolysis and can be: (1) interconverted into lactate, (2) interconverted into alanine via transaminase reaction or (3) enter the Krebs cycle [35]. During the 24th and the 48th hour of treatment, the pyruvate production and export is concomitant with glucose consumption. The equimolar disappearance of glucose is not stoichiometrically linked to lactate production, denoting that pyruvate production at this time interval is caused by glucose metabolism (glycolysis) rather than an inversion of the metabolic profile of lactate or alanine production. The produced cytosolic pyruvate is not converted either in alanine or lactate but is exported, as was detected. Interestingly, alanine production was significantly increased during the first 6 hours. In other somatic cell types, an increase of alanine production is associated with the rapid oxidation of aminoacids [36]. It has also been described that SCs can metabolize aminoacids and that low amounts of acetyl-CoA arising from glucose metabolism can modulate alanine oxidation to CO_2. Furthermore the conversion alanine to lipids is suggested as a result of pentose cycle [24] and glucose metabolism [37] stimulation. Therefore a low glucose uptake during the first 6 hours by insulin-deprived cells results in low alanine conversion in lipids and thus and therefore in an alanine accumulation. As said, the ratio lactate-alanine is an index of the redox state of the cell. Since the reduction of pyruvate into lactate or its conversion into alanine is related with the $NADH/NAD^+$ ratio [19] the appearance of higher levels of alanine in insulin-deprived cells can be associated with a reduced redox cytosolic state (low ratio $NADH/NAD^+$) of the cells in these condition. After the 48 hours treatment, the alanine production was similar in both conditions.

In SCs, carbohydrate metabolism has been shown to present some unique characteristics. Others have described that when subjected to conditions of glucose deprivation, rat SCs cultured *in vitro* have the ability to adapt their metabolism. We demonstrated that hSCs cultured in insulin deprivation conditions adapted their glucose metabolism by modulating the expression of GLUT1 and GLUT3. There was a significant decrease on GLUT1 mRNA levels, which was accompanied by a contrasting increase on GLUT3 mRNA levels. We concluded that the first 24 hours of insulin-deprivation are critical on this adaptive mechanism in hSCs cultured *in vitro*. After the 48 hours, insulin-deprived hSCs consumed the same amount of glucose but lactate production is severely altered, most likely due to an alteration of the two main mechanisms of lactate metabolic control: the export by MCT and the conversion from pyruvate by LDH. Thus, insulin controls not only glucose uptake, as expected, but also the mRNA expression of important metabolism-associated genes in *in vitro* cultured hSCs.

In type I diabetes there is an absolute lack of insulin and several studies using animal models of DM suggested that it impairs male fertility with a marked reduction in sperm quality and as a consequence in fecundity [7]. Testicular biopsies of diabetic patients also revealed numerous abnormalities in SCs [9] but the mechanisms remained unknown. So, although observations in Sertoli cell primary cultures may not exactly represent an *in vivo* situation, the results presented here are the first report of the effect of insulin deprivation on the metabolism of hSCs and a first step to identify key mechanisms by which this hormone can regulate hSCs function, with direct influence over spermatogenesis and thus male fertility. Our work gives new insights over the regulation of glucose metabolism in insulin-deprivation conditions. Nevertheless, further knowledge on the functioning and regulation of these biochemical mechanisms is essential for the enlightenment of a process that is central to spermatogenesis and fertility. As DM grows to epidemic proportions, the key regulatory mechanisms by which spermatogenesis is affected in this disease is critical in order to highlight new therapeutic approaches.

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Chapter 4

Influence of 5α-Dihydrotestosterone and 17β-Estradiol on Human Sertoli Cells Metabolism

This chapter was adapted from the published work:

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Influence of 5α-Dihydrotestosterone and 17β-Estradiol on Human Sertoli Cells Metabolism

Summary

Sertoli cells metabolize glucose converting it to lactate that is used by developing germ cells for their energy metabolism. Androgens and estrogens have metabolic roles that reach far beyond reproductive processes. So, the main purpose of this study was to examine the effect of sex steroid hormones on metabolite secretion/consumption in human Sertoli cells. Human Sertoli cell-enriched primary cultures were maintained in a defined medium for 50 hours and glucose, pyruvate, lactate and alanine variations were determined by ^{1}H-NMR spectra analysis, in the absence or presence of 100 nM 17β-estradiol (E_2) or 100 nM 5α-dihydrotestosterone (DHT). The mRNA expression levels of glucose transporters, lactate dehydrogenase and monocarboxylate transporters were also determined by semi-quantitative RT-PCR. Cells cultured in the absence (control) or presence of E_2 consumed the same amounts of glucose at similar rates during the 50h. During the first 15h of treatment with DHT, glucose consumption and glucose consumption rate were significantly higher. Nevertheless, DHT-treated cells secreted a significantly lower amount of lactate than control and E_2-treated cells. Such a decrease was concomitant with a significant decrease in lactate dehydrogenase A mRNA levels after 50h treatment, in DHT-treated groups. Finally, alanine production was significantly increased in E_2-treated cells after 25h treatment, which indicated a lower redox/higher oxidative state for the cells on those conditions. These results support the existence of a relationship between sex steroid hormones action and energy metabolism, providing the first assessment of androgens and estrogens as metabolic modulators of human Sertoli cells.

Keywords: Human Sertoli cell; energy metabolism; androgens; estrogens; lactate

Introduction

Sertoli cells (SCs) face the lumen of the seminiferous tubule providing structural support and creating an immunologically protected space for the developing germ cells. They have been classified as the “nurse cells” within the seminiferous epithelium [1, 2], playing a key role on the establishment of an adequate luminal environment in the seminiferous tubules of the male reproductive tract [3]. Developing germ cells are unable to use glucose for their energy metabolism and preferentially use lactate as an energy source [4]. There are evidences that lactate has a crucial role in spermatogenesis, having also an anti-apoptotic effect on germ cells [5]. The importance of lactate for normal spermatogenesis was highlighted in a report showing that, in adult cryptorchid rat testis, spermatogenesis is improved by intratesticular infusion of lactate [6]. The lactate used by the developing germ cells is produced by SCs [7]. SCs metabolize glucose and the majority of it is converted to lactate, and not oxidized via the

citric acid cycle [8], although the reasons why SCs preferentially export lactate and pyruvate for germ cells are not entirely understood [9]. There is a growing awareness that androgens and estrogens have general metabolic roles that reach far beyond reproductive processes. In rat Sertoli cells it has been described that lactate production is decreased in animals exposed *in utero* to flutamide, an antagonist of the androgen receptor [10]. Several cellular systems might contribute to a modulation in lactate secretion by SCs: glucose transporters, lactate dehydrogenase isozyme system, and lactate transporters. Glucose transporters (GLUTs) mediate the transport of glucose through the plasma membrane, a rate-limiting step in glucose metabolism [11-13]. Lactate is produced from pyruvate following lactate dehydrogenase (LDH) catalysis and, in testis, LDHA is the predominantly expressed isoform [14]. Lactate is then transported across the plasma membrane to the germ cells by specific proton/monocarboxylate transporters (MCTs). These biochemical systems are key targets to be regulated for achieving an adequate lactate offer to germ cells, namely by androgens and estrogens. With our work, we aimed to examine the effect of 17β-estradiol and 5α-dihydrotestosterone on metabolite secretion or consumption in primary cultures of human SCs (hSCs), using ^{1}H-NMR spectra analysis. We also explored the hypothesis that those hormones affected the expression of GLUT3, LDHA and MCT4, by evaluating its mRNA expression, to disclose their effect on lactate production and export.

Materials and Methods

Chemicals

Polyclonal antibodies, M-MLV RT and random hexamer primers were purchased from Invitrogen (CA, USA). dNTPs were purchased from GE Healthcare (Buckinghamshire, UK). Taq DNA Polymerase was purchased from Fermentas Life Sciences (Ontario, Canada). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Patient selection, ethical issues and testicle tissue preparations

The clinical study of the patients and testicle tissue processing was performed at the Centre for Reproductive Genetics Alberto Barros (Porto, Portugal) in accordance with the Guidelines of the Local, National and European Ethical Committees. In all cases, testicular biopsies were used after informed, written patient consent and studies have been performed according to the Declaration of Helsinki. In the present study only cells left in the tissue culture plates after treatment of the patients were used. hSCs were obtained from five testicular biopsies of infertile patients under treatment for recovery of male gametes. Only patients with normal spermatogenesis were selected. Cases with normal spermatogenesis included the following patients: anejaculation (psychologic, vascular, neurologic), vasectomy or traumatic section of the vas deferens. Each testicle biopsy of the selected cases was collected in sperm preparation medium (SPM-Hepes buffer; Medicult) and kept at 32ºC with 5% CO_2 in air until use.

Sertoli cell culture

Testicle biopsies were treated as described by Sousa and collaborators [15]. Briefly, each testicle biopsy (5-10 mg) was collected in sperm preparation medium (SPM; Medicult, Copenhagen, Denmark) and squeezed with surgical blades. After washing, samples were digested in a solution of SPM containing DNase and collagenase-IV (Sigma). The resulting pellet was washed and resuspended in IVF medium (Medicult) and incubated at 30–32°C, 5% CO_2 in air until use. SCs were obtained by a method previoulsy described by Oliveira and collaborators [16, 17]. Sertoli cell culture purity was revealed by the immunoperoxidase technique. Briefly, cells were incubated with primary polyclonal antibody and processed by the labeled streptavidin–biotin method using an ExtrAvidin-Peroxidase Staining Kit (Sigma Aldrich). Specific protein markers, Anti-Mullerian hormone and Vimentin, were used to assess the purity of the hSCs cultures [18]. Cultures with cell contaminants below 5% after 96 h, as examined by phase contrast microscopy, were used.

Experimental groups

SCs were allowed to grow until reach 90-95% of confluence, and then washed thoroughly and medium was replaced by serum-free medium (DMEM:F12 1:1 with ITS supplement, pH 7.4). In order to evaluate the effects of sex hormones in metabolic secretion and production, LDH A and MCT4 expression, SCs were treated with 100 nM of E_2 or 100 nM of DHT. DHT was chosen as representative of the androgen family since is not conversable to estrogens within the cells [19]. The sex steroid hormone concentrations were chosen based on published data, which reported that testicular interstitial fluid concentrations of those hormones are notably higher than those of circulating plasma, reaching values up to 200 nanomolar [20-23]. Treatments were performed during 50 h, for in previous studies of our research group the presence of DHT and E_2 in the culture media was confirmed up to 72 h (data not shown). Control groups were treated with same amount of solvent (EtOH) used in DHT and E_2 groups (<0,025% v/v). At the end of the 50 h treatment, the total number of cells per flask was determined with a neubauer chamber and cells were collected for RNA extraction.

NMR spectroscopy

During the 50 hours of hormonal treatment, 250 µL of the culture medium were collected at 5, 10, 25, 35, and 50 hours, for ^{1}H-NMR analysis. ^{1}H-NMR spectroscopy was performed to determine lactate production, glucose consumption and variations in other substrates, such as pyruvate and alanine, during the 50 hours of cell incubation in the various conditions as describe previously by Alves and collaborators [24]. Sodium fumarate (final concentration of 2 mM) was used as an internal reference (6.50 ppm) to quantify metabolites in solution. The following metabolites were determined whenever present: lactate, doublet located at 1.33 ppm; alanine, doublet at 1.45 ppm; pyruvate, singlet at 2.36 ppm; and H1-α glucose, doublet at 5.22 ppm (Figure 4.1).

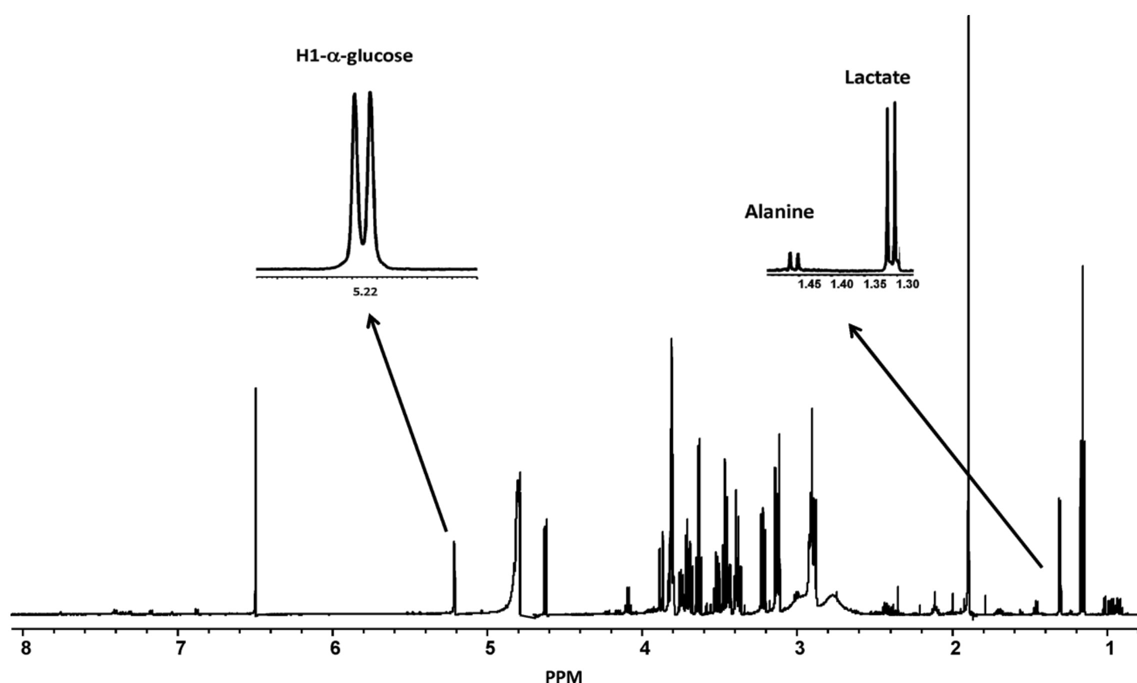

Figure 4.1. Representative ^{1}H-NMR spectrum attained for the ITS supplement DMEM:F12 showing the localization of alanine, lactate and H1-α-glucose peaks.

Reverse transcriptase-polymerase chain reaction

At the end of the 50 h treatment, total ribonucleic acid (RNAt) was extracted from isolated Sertoli cells using TRI reagent according to the manufacturer's instructions. RNA concentration and absorbance ratios (A260/A280) were determined by spectrophotometry (Nanophotometer^{TM}, Implen, Germany). RNAt was reversely transcribed as described previously by Cavaco and collaborators [25]. The resulting cDNA was used to amplify GLUT3, LDH A and MCT4 cDNA fragments with exon-exon spanning primer sets. Both optimal annealing temperature and the number of cycles required for exponential amplification phase of fragments are shown in Table 4.1. PCR reactions were carried out in triplicate. GLUT3, LDH A and MCT4 mRNA levels were normalized with 18S gene expression as internal control. Liver mRNA was used as positive control and cDNA-free sample was used as negative control. Densities from each band were obtained with BIO-PROFIL Bio-1D Software from Quantity One (Vilber Lourmat, Marne-la-Vallée, France) according to standard methods [26]. The band density obtained was then divided by the respective 18S band density and results expressed as fold induction/reduction versus the control group.

Table 4.1. Oligonucleotides and Cycling Conditions for PCR Amplification of LDHA, GLUT3, MCT4 and 18S. Legend: AT: annealing temperature; C: Number of cycles during exponencial phase of amplification.

Gene	Sequence (5'-3')	AT (°C)	Amplicon Size (bp)	C
LDHA	Sense: GCACTCACACGTGGGTTCCCG Antisense: GCAAGTTCATCTGCCAAGTCCTTCA	60	200	35
GLUT3	Sense: TCAGGCTCCACCCTTTGCGGA Antisense: TGGGGTGACCTTCTGTGTCCCC	56	228	35
MCT4	Sense: ACCAACCCTCCTGGCCATGGGA Antisense: GCCAAACCCAACCCCGTGATG	58	396	35
18S	Sense: AAGACGAACCAGAGCGAAAG Antisense: GGCGGGTCATGGGAATAA	56	149	25

Statistical analysis

The statistical significance of differences in glucose and pyruvate consumption, lactate and alanine production, and in GLUT3, LDHA and MCT4 expression among the experimental groups was assessed by two-way ANOVA, followed by Bonferroni post-test. All experimental data are shown as mean ± S.E.M (n=5 for each condition). Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). $P<0.05$ was considered significant and those results are signalized.

Results

DHT treatment increases glucose consumption

From the first hours of treatment, glucose consumption was higher in DHT-treated SCs. After 50 hours, SCs cultured in control conditions presented an overall glucose consumption of 34 ± 2 pmol/cell, while those cells treated with E_2 consumed 36 ± 2 pmol/cell and those treated with DHT consumed 58 ± 3 pmol/cell. The treatment of cultured SCs with DHT resulted in significantly higher glucose consumption after 50 hours being that the majority of the glucose consumed during the first 15 hours of treatment (Figure 4.2A).

The glucose consumption rate is notably higher in the first 5 hours in DHT-treated SCs (4.0 ± 0.1 pmol/h/cell) when compared with E_2-treated (1.8 ± 0.2 pmol/h/cell) and control condition (2.4 ± 0.3 pmol/h/cell). After the first 15 hours of incubation, glucose consumption rate highly decreases in DHT-treated cells, becoming similar to the other groups. DHT-treated cells presented a rate of 0.6 ± 0.2 pmol/h/cell, while cells in the control condition and those treated with E_2 consumed glucose at a rate of 0.5 ± 0.1 and 0.4 ± 0.3 pmol/h/cell, respectively (Figure 4.2B).

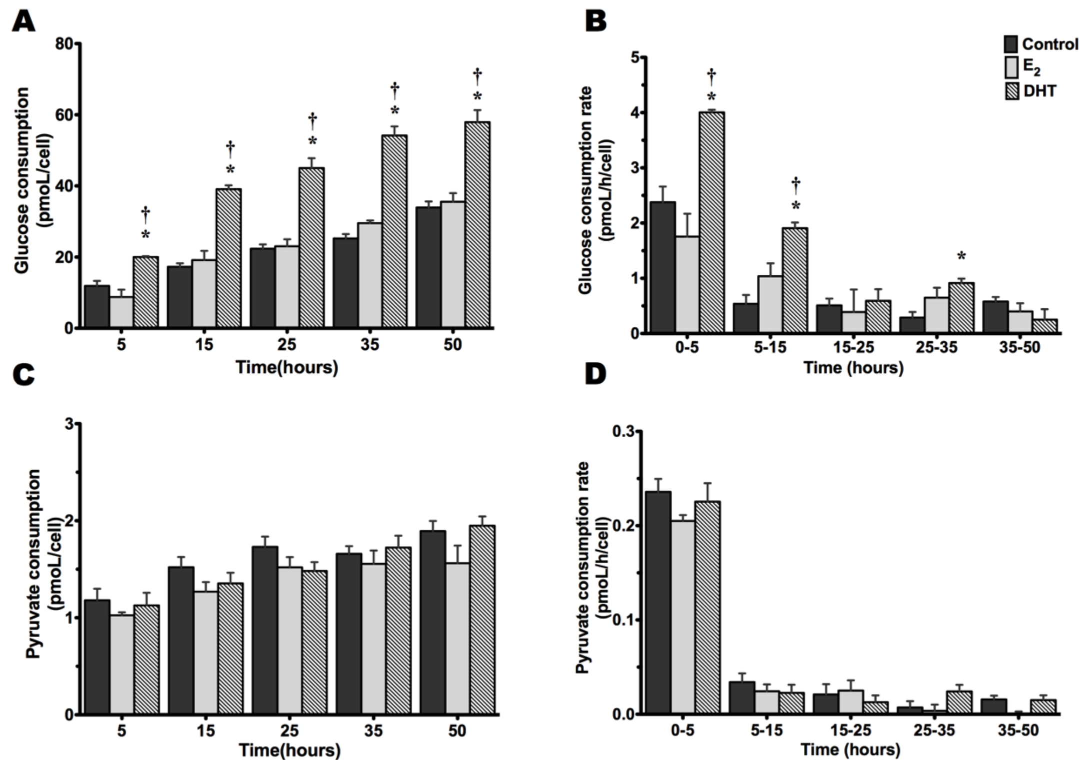

Figure 4.2. Glucose and Pyruvate consumption by human Sertoli cells. (A) Glucose consumption; (B) Glucose consumption rate; (C) Pyruvate consumption; (D) Pyruvate consumption rate. C- control; E_2- 17β estradiol; DHT- dihydrotestosterone. Results are expressed as means ± SEM (n=5). * significantly different when compared to control ($p<0.05$); † significantly different when compared to E_2 ($p<0.05$).

There were no hormonal-related effects in pyruvate consumption

The pyruvate consumption was not dependent on hormonal treatment. After 50 hours, the pyruvate consumption was 1.9 ± 0.1, 1.6 ± 0.2 and 1.9 ± 0.1 pmol/cell in control, E_2-treated and DHT-treated cells, respectively (Figure 4.2C). There were also no significant differences in pyruvate consumption rate between control, E_2-treated and DHT-treated SCs throughout the 50 hours (Figure 4.2D).

DHT treatment decreases lactate production

The amount of lactate produced during the 50 hours was similar for control and E_2-treated cells, 13.0 ± 0.4 and 13.8 ± 0.2 pmol/cell, respectively. During the first 15 hours, SCs in all the conditions produced similar amounts of lactate, however after DHT treatment for 50 hours cells secreted significantly lower amounts of lactate (10.4 ± 0.4 pmol/cell; Figure 4.3A). The lactate production rate is also similar for the first 15 hours in SCs cultured in all conditions. Between the 15th and 25th hour of culture, the lactate production rate highly increases in E_2-treated cells (0.44 ± 0.01 pmol/h/cell) when compared with control (0.30 ± 0.04 pmol/h/cell) and DHT-treated cell cultures (0.21 ± 0.01 pmol/h/cell). Between the 25th and 35th hour of culture, the lactate production rate remains higher in control and E_2-treated cells, which presented rates of 0.40 ± 0.03 pmol/h/cell and 0.27 ± 0.03 pmol/h/cell, when compared to those cells treated with DHT (0.06 ± 0.02 pmol/h/cell; Figure 4.3B).

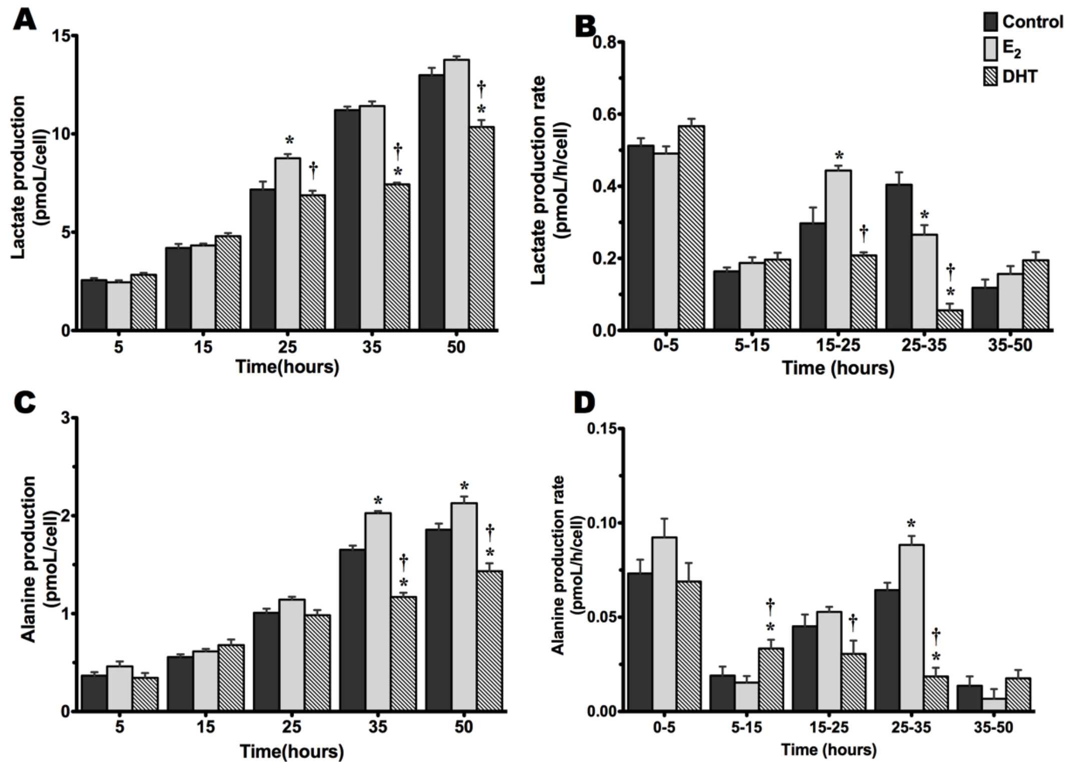

Figure 4.3. Lactate and Alanine production by human Sertoli cells. (A) Lactate production; (B) Lactate production rate; (C) Alanine production; (D) Alanine production rate. C- control; E_2- 17β estradiol; DHT- dihydrotestosterone. Results are expressed as means ± SEM (n=5). * significantly different when compared to control ($p<0.05$); † significantly different when compared to E_2 ($p<0.05$).

E_2 treatment highly increases alanine production

The alanine production was very similar in all conditions during the first 25 hours of treatment. Throughout that period, control cells, cells treated with E_2 and those treated with DHT secreted 1.01 ± 0.04, 1.14 ± 0.05 and 0.98 ± 0.05 pmol/cell of alanine, respectively. After the first 25 hours, the production of alanine significantly increased in control conditions (1.65 ± 0.4 pmol/cell) and in SCs treated with E_2 (2.02 ± 0.02 pmol/cell) when compared with those treated with DHT, which produced 1.16 ± 0.04 pmol/cell. At the end of the 50 hours, E_2-treated cells secreted significantly higher alanine amounts than cells in other experimental conditions (Figure 4.3C). Alanine production rate was not significantly different in the first 5 hours in E_2-treated SCs (0.09 ± 0.01 pmol/h/cell), DHT-treated (0.07 ± 0.01 pmol/h/cell) and in control condition (0.07 ± 0.01 pmol/h/cell). Between 25 and the 35 hours of incubation, the alanine production rate highly increased in control (0.06 ± 0.01 pmol/h/cell) and E_2-treated cells (0.09 ± 0.01 pmol/h/cell), while cells treated with DHT produced alanine at a rate of 0.02 ± 0.01 pmol/h/cell (Figure 4.3D).

DHT treatment decreases GLUT3 mRNA levels after 50 hours

To analyze the possible effect of the selected sex steroid hormones on the expression of GLUT1 and GLUT3, we performed a semi-quantitative RT-PCR to quantify the mRNA levels of these glucose transporters on cultured cells after the 50 hours of incubation. GLUT1 and

GLUT3 are high affinity glucose transporters, present on the plasma membrane of the cells, usually associated with the transport of glucose in cells with high-energy demands [27]. As described earlier for the human whole testis [11], the expression of GLUT1 mRNA was also not detected on hSCs (data not shown). Conversely, the presence of GLUT3 mRNA was confirmed in cultured hSCs (Figure 4.4). Furthermore, cells treated with E_2 showed levels of GLUT3 mRNA similar to cells in control conditions, while in cells treated with DHT, GLUT3 mRNA levels decreased significantly to 0.80 fold after the 50 h treatment (Figure 4.4).

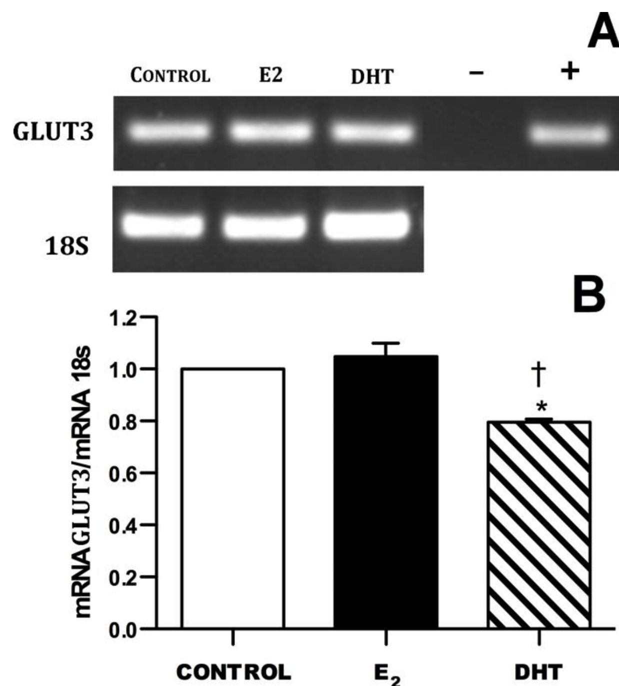

Figure 4.4. Effect of DHT and E_2 on GLUT3 mRNA levels in human Sertoli cells. Panel A shows a representative agarose gel electrophoresis. (-) Negative control, (+) Positive control. Panel B shows pooled data of independent experiments, indicating the fold variation LDHA mRNA levels found in cultures with DHT 100 nM or E_2 100 nM when compared with cultures on control conditions. Results are expressed as means ± SEM (n=5). * Significantly different when compared to control ($p<0.05$); † significantly different when compared to E_2 ($p<0.05$).

DHT treatment decreases LDHA mRNA levels after 50 hours

After observing a decrease on lactate levels produced by the SCs in DHT treated groups, we investigated the possibility of an effect of this hormone on mRNA levels of LDHA in hSCs. In DHT-treated cells a significant decrease in LDHA mRNA levels was observed, after 50 hours of treatment (Figure 4.5). The levels of LDHA in those cells were reduced to 0.88 fold when compared to control conditions. As regards E_2-treated cells, no significant variation of LDHA mRNA levels was observed after 50 hours of treatment, when compared to the control group (Figure 4.5).

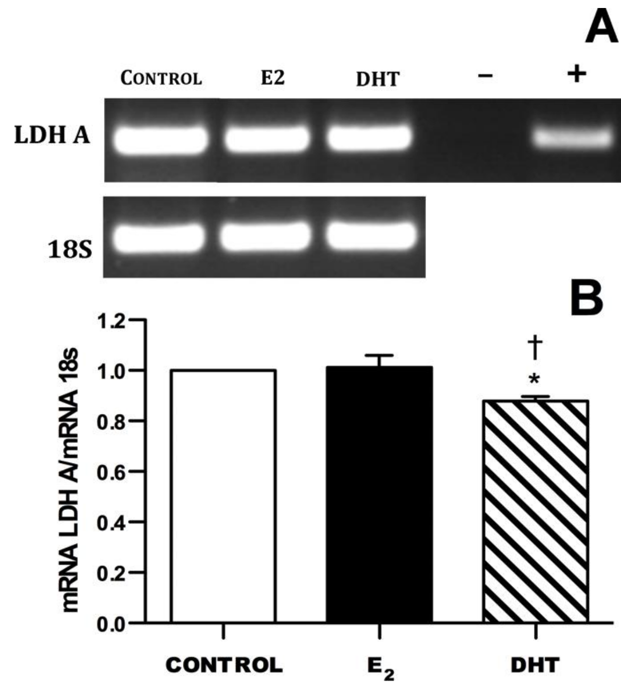

Figure 4.5. Effect of DHT and E_2 on LDHA mRNA levels in human Sertoli cells. Panel A shows a representative agarose gel electrophoresis. (-) Negative control, (+) Positive control. Panel B shows pooled data of independent experiments, indicating the fold variation LDHA mRNA levels found in cultures with DHT 100 nM or E_2 100 nM when compared with cultures on control conditions. Results are expressed as means ± SEM (n=5). * significantly different when compared to control ($p<0.05$); † significantly different when compared to E_2 ($p<0.05$).

E_2 treatment increases MCT4 mRNA levels after 50 hours

Following the noted decrease on the amount of lactate produced and exported to extracellular medium by the hSCs of the DHT-treated group, we also investigated the possibility of an alteration on the levels MCT4, a monocarboxylate transporter that serves primarily as a lactate exporter [28, 29]. DHT-treated cells showed levels of MCT4 mRNA similar to those present in cells cultured in control conditions. In opposition, E_2-treated cells presented an increase in MCT4 mRNA levels to 1.16 fold when compared to the control group, after 50 hours of treatment (Figure 4.6).

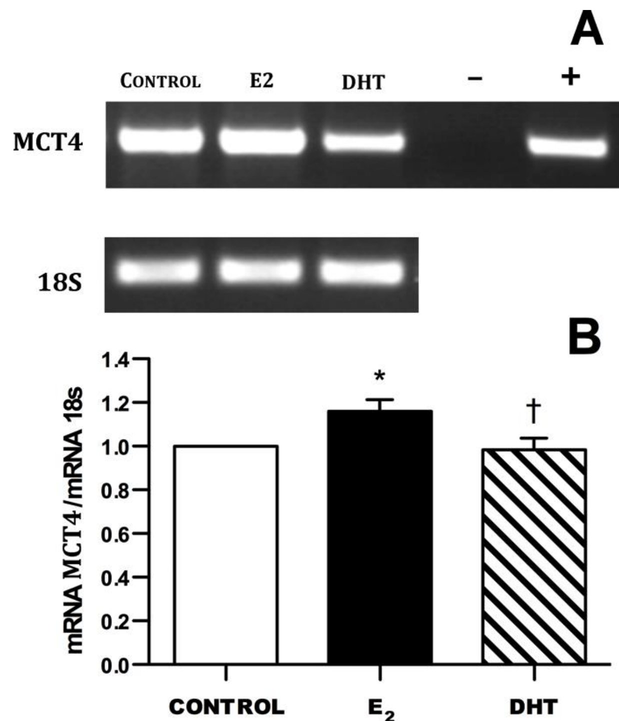

Figure 4.6. Effect of DHT and E_2 on MCT4 mRNA levels in human Sertoli cells. Panel A shows a representative agarose gel electrophoresis. (-) Negative control, (+) Positive control. Panel B shows pooled data of independent experiments, indicating the fold variation MCT4 mRNA levels found in cultures with DHT 100 nM or E_2 100 nM when compared with cultures on control conditions. Results are expressed as means ± SEM (n=5). * significantly different when compared to control ($p<0.05$); † significantly different when compared to E2 ($p<0.05$).

Discussion

Although little is known on glucose metabolism in hSCs, this subject has been widely discussed for cultured rat SCs, particularly in the 80's [8, 30, 31]. Carbohydrate metabolism in SCs has been shown to present some unique characteristics [1]. These cells have the ability to adapt their metabolism, to ensure an adequate lactate concentration in the microenvironment where germ cells develop [32]. In the present study, DHT-treated hSCs produced less lactate than those treated with E_2 or in control conditions, which in turn consumed higher amounts of glucose. This may be due to several factors such as a decrease in lactate synthesis, by lactate dehydrogenase isozyme, or a delay in lactate transport to extracellular medium, via MCTs. In fact, mRNA levels of LDHA were significantly decreased in DHT-treated cells, which may contribute to the lower lactate production levels seen in cells from these groups. Furthermore, in E_2-treated cells the mRNA expression of MCT4, a membrane protein involved mainly in the export of lactate to the extracellular medium in cells with high glycolytic capacity [28, 33] was increased. Although these changes on mRNA levels are not a direct measure of the variations on the protein functioning, they can be a clear indication on the effectiveness of the regulation exerted on the studied protein. An increase in the MCT4 levels would contribute to an increase in the accumulation of lactate on the extracellular medium. On the other hand, GLUT3 mRNA levels significantly decreased in DHT-treated cells, which seems not consistent with higher glucose consumption observed,

because higher glucose demands are usually associated with higher levels of GLUTs [34]. However, in these cells the glucose consumption rate is higher in the first 15 hours, but markedly decreases in the following 35 h of incubation. This may be responsible for the decreased GLUT3 mRNA levels observed in cells of this group after 50 hours, given that Mahraoui and collaborators [34] described that GLUT3 mRNA levels can be in close correlation with the rates of glucose consumption and cells transiting from an exponential to a stationary phase showed a decrease in GLUT3 mRNA levels.

Pyruvate was promptly consumed in all conditions, which confirms that cultured SCs highly consume pyruvate when available, in agreement with previous studies [31]. The exogenous pyruvate is a substrate in intermediary metabolism that can be converted into lactate, until pyruvate/lactate reaches equilibrium. This conversion is an NADH-dependent reduction, but pyruvate may also be converted into alanine via transaminase reaction [35]. The ratio lactate/alanine is an index of redox state of the cell [36] as the reduction of pyruvate into lactate or its conversion into alanine is related with the $NADH/NAD^+$ ratio [37]. The appearance of higher levels of alanine in E_2-treated cells can be associated with a reduced redox cytosolic state (low ratio $NADH/NAD^+$).

Alanine and lactate production were significantly decreased in DHT-treated cells. This may suggest that DHT is redirecting glucose metabolism to Krebs cycle. In this condition, cells become metabolically more efficient. Results from Gupta and collaborators [38] obtained in the epididymis are in accordance with this hypothesis. They investigated the role of sex steroids in the regulation of energy metabolism of epididymis of *rhesus monkey* and measured the activity of succinate dehydrogenase and malate dehydrogenase in castrated estrogen- and DHT-treated animals [38]. Their results indicated that DHT alone stimulated the activities of those enzymes whereas E_2 failed to stimulate any of the enzymes [38]. Thus, at least in the epididymis of monkey testis, DHT has been proven to stimulate the activity and expression of enzymes involved in Krebs cycle and in intermediate pathways coupling with Krebs cycle.

Our results lead us to propose that DHT and E_2 are modulators of lactate production/export in hSCs. Few reports have been presented on the hypothesis that sex steroids might regulate GLUT3, LDH A and MCT4 expression in these cells [10]. It has been described that in mice testes, particularly in differentiated germ cells, the expression of MCT2 is under hormonal control [39] and testosterone reduced MCT2 mRNA levels in a dose-dependent manner. In the lizard (*Hemidactylus flaviviridis)*, DHT inhibited lactate production by SCs in a dose and time-dependent mode and E_2 that markedly suppressed lactate production in a dose-dependent manner [40]. In primary cultures of rat SCs, a modulatory effect of testosterone on fatty acid biosynthesis has also been described [41]. Apart from that, little is known on the influence of sex steroid hormones on the energy metabolism of SCs, particularly in the human testes.

In conclusion, glucose metabolism in SCs was shown to be regulated by sex hormones, namely by DHT and E_2. However, we cannot exclude the metabolic utilization of ketone bodies and fatty acids by SCs in these conditions, as they have also been described as a energy substrate for cultured rat Sertoli cells [42, 43]. Furthermore, the glycogenolysis pathway, known to be

present in SCs, may also be hormonally regulated. The presence of glycogen and of glycogen phosphorylase activity has been described in SCs, making possible for glycogen hydrolysis to fuel the glycolytic pathway in these cells [44, 45]. So, although observations in SCs primary cultures may not exactly represent an *in vivo* situation, the results presented here are the first report of the effect of androgens and estrogens in the metabolism of hSCs and a first step to identify key mechanisms by which these hormones can regulate SCs function with direct influence over spermatogenesis and thus male fertility.

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P. F. Oliveira and M. G. Alves participated in the study experimental design, execution and analysis. L Rato participated in the RT-PCR experimental execution. J. Silva was IVF laboratory work supervisor. R. Sá participated in the testicle tissue preparation. A Barros participated in the patient recruitment and analysis. M. Sousa participated in the selection and testicle tissue analysis. R. A. Carvalho participated in the ^{1}H-NMR experimental analysis and discussion. J. E. Cavaco participated in the RT-PCR experimental design and analysis. He also participated in manuscript discussion. S. Socorro participated in the overall study design and critical discussion.

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Chapter 5

Testosterone deficiency induced by progressive stages of diabetes mellitus differentially modulates glycolytic flux of cultures rat Sertoli cells

This chapter was adapted from the submitted work:

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Testosterone deficiency induced by progressive stages of diabetes mellitus differentially modulates glycolytic flux of cultured rat Sertoli cells

Abstract

The mechanisms that govern Sertoli cells' (SCs) glucose metabolism are essential for spermatogenesis. These metabolic processes that result in an elevated production of lactate are regulated by several hormones and factors. Testosterone (T) deficiency has been associated with decreased insulin sensitivity and deregulation of glucose metabolism. However, little is known of how SCs respond to T alterations, especially to reduced T levels induced by diabetes mellitus (DM). Herein, we evaluate the effect of different T concentrations, detected in physiologic, pre-diabetic (PreD) and type 2 *diabetic mellitus* (T2DM) conditions, in cultured SCs obtained from rodent models of PreD and T2DM. We evaluated the expression and activities of glycolysis and glycogen metabolism-related proteins, as well as metabolite secretion/consumption by cultured SCs.

Both glucose and pyruvate consumption were significantly decreased in PreD conditions, whereas T2DM conditions reversed this profile. Lactate production was not significantly altered at the end of the treatment, although the expression and activities of the lactate production-associated proteins were increasingly affected by progressive T-deficiency conditions. Alanine production was significantly increased in SCs of both groups suggesting an alternative metabolic fuel. Notably, intracellular glycogen content was only increased in SCs of the T2DM group. Taken together these results illustrate that gradually reduced T levels, induced by progressive stages of DM, impair glycolysis favoring glycogen metabolism. This report highlights the physiologic significance of T in the regulation of the glycolytic profile of SCs metabolism, in particular when associated with the progression of T2DM.

Keywords: Pre-diabetes; Type 2 diabetes mellitus; Testosterone deficiency; Sertoli cell; Glucose metabolism; Glycogen.

Introduction

Diabetes mellitus (DM) is a metabolic disease that directly compromises male fertility through the induction of whole metabolic and hormonal deregulation, particularly in the reproductive axis. A direct consequence of DM on testicular secretory function is the inhibition of testosterone (T) synthesis [1-3]. Epidemiological studies have shown that 40% of men with type 2 diabetes mellitus (T2DM) present T deficiency [3]. Whole body metabolic alterations are "sensed" by testicular tissue and the significant T reduction induced by diabetic states [1, 2, 4-6] seems to be in part closely associated to the several abnormalities that occur throughout the reproductive tract [7] and particularly in the Leydig cells [1, 8].

Within the seminiferous epithelium, androgen receptors are expressed exclusively in SCs [9], making them major targets for those hormones [10-12]. In fact, the physiology of SCs is modulated by T and its metabolites, namely 5α-dihydrotestosterone (5α-DHT), which has a biological activity two or three times higher than T [13, 14]. Previous works have shown that 5α-DHT modulates the metabolism of SCs, increasing glucose consumption without enhancing the production of lactate [15, 16]. Those works suggested that androgens may induce a metabolic reprogramming of SCs, promoting the shift from a Warburg-like [17] into an oxidative Krebs cycle metabolism [16, 18].

The metabolism of SC presents particular features, as this cell metabolizes glucose to lactate, which is the main energy substrate [19], and also an anti-apoptotic factor [20] for developing germ cells. Often referred as "nurse cell", SCs confers physical and nutritional support to germ cells, creating an adequate environment for the occurrence of spermatogenesis [16, 21]. SCs uptake glucose, via specific glucose transporters (GLUTs), that once inside the cell undergoes the glycolytic pathway producing pyruvate. The majority of this glycolysis-derived pyruvate is converted to lactate by lactate dehydrogenase (LDH) [22], subsequently exported to the developing germ cells via specific monocarboxylate transporters.

Interestingly, SCs of under diabetic conditions use alternative substrates as a compensatory mechanism to ensure the adequate conditions for germ cell development and to counteract the deleterious effects of DM [23-26]. In fact, SCs are the major source of glycogen within seminiferous tubules [27] and present glycogen metabolism-related enzymes [28, 29]. However little is known of how SCs metabolically respond the reduced T levels induced by progressive stages of diabetes, pre-diabetes (PreD) and established T2DM. We hypothesize that T deficiency promoted by PreD and T2DM induces crucial alterations in SCs glycolytic profile, evidencing mechanisms by which hormonal deregulation promoted by DM may affect spermatogenesis. To test our hypothesis, we evaluated the effect of different T levels on metabolite secretion/consumption in primary cultures of rat SCs obtained from animal models of PreD and T2DM. We also determined the protein levels of key glycolysis-related interveners. Finally, we also evaluated the effect of T levels on SC glycogen content and glycogen metabolism-related enzymes expression.

Material and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A) unless specifically stated.

Animals

Eighteen male Wistar rats (Charles River Laboratories, Barcelona, Spain) were used. Animals were housed in our accredited animal facilities and maintained with food and water *ad libitum* in a constant room temperature (20 ± 2ºC) on a 12-hour cycle of artificial lighting. All

experiments were performed according to the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996) and the European directives (Directive 86/609/EEC).

Experimental animal procedure

Animal models of progressive stages of T2DM establishment (PreD and T2DM) were obtained by methods previously described [4, 30]. In brief, eighteen male Wistar rats (*Rattus norvegicus*) were randomly divided in control (n=6), T2DM (n=6) and pre-diabetic (n=6) groups.

For the induction of T2DM, six 2-day-old male Wistar rats were intraperitoneally injected with a low-dose (40 mg/kg) of streptozotocin (STZ) freshly diluted in citrate buffer (0.1 M sodium citrate, pH 4.5), as described by Weir and collaborators [30]. Animals from the other groups received the vehicle solution in equivalent volumes. PreD was induced according to the method reported by Rato and collaborators [4]. In brief, six 2-month-old Wistar rats from the PreD group were daily fed with an additional high-energy emulsion during one month. Animals were given progressively 1 to 5 mL of emulsion in the first 5 days of administration. Thereafter, they were administrated daily with 5 mL of the emulsion.

All animals were fed with standard chow diet (4RF21 certificate, Mucedola, Italy) and water. After treatment, animals were killed by decapitation and testicles were aseptically removed for evaluation of T and 17ß-estradiol (E_2) testicular levels and for primary cultures of SCs.

Glucose tolerance and Insulin resistance tests

At 3 months of age, animals were submitted to a glucose tolerance test, as described by Rato and collaborators [4]. The animals were also subjected to an insulin tolerance test as described by Holmes and collaborators [31]. The areas under the curve for the glucose tolerance and insulin resistance tests were calculated using the trapezoidal rule, as described [5].

Testosterone and 17ß-estradiol determination

Testicular hormonal levels were measured as described by Rato and collaborators [4], using commercial rat EIA kits, according to manufacturer instructions (Cayman Chemical Company, Ann Arbor, MI, USA). EIA kits had detection limits of approximately of 6 pg/mL (for T) and 20 pg/ml (for E_2).

Primary Sertoli cell cultures

Sertoli cells were isolated as described [32]. In brief, testicles were decapsulated and dispersed seminiferous tubules were incubated in 0.5 mg/ml collagenase and in a 0.5 mg/mL trypsin solution at 37ºC. Cell suspension suspended in an adequate volume of culture medium (Dulbecco’s Modified Eagle Medium:Ham's F-12 (DMEM:F12) 1:1, containing 10% heat inactivated fetal bovine serum) and incubated at 33°C (5% CO_2 in air) until use.

Experimental groups

SCs were allowed to grow until reaching 85-90% of confluence and then washed with phosphate buffered saline solution. The medium was replaced by serum-free medium (DMEM:F12) (1:1) with insulin (10 mg/L)-transferrin (5.5 mg/L)-sodium selenite (5 μg/L) (ITS) supplement, pH 7.4. In the first group, SCs obtained from animals of the control group were exposed to sex steroid concentrations within the physiologic range as reported in the testicular interstitial fluid (TIF) by Rato and collaborators [4] and these were considered the control conditions (T-CTR group). SCs from T-CTR group were cultured in ITS medium supplemented with 2500 nM of T and 3 nM of E_2. In a second group, SCs obtained from the animals of the pre-diabetic group were exposed to sex steroid concentrations reported in the TIF from the pre-diabetic animal model. In this group, the ITS medium was supplemented with 600 nM of T and 3 nM of E_2 (T-PreD group). In a third group, SCs obtained from the animals of T2DM group were exposed to sex steroid concentrations determined in the TIF from the stage of T2DM. In this group, the ITS medium was supplemented with 7 nM of T and 3 nM of E_2 (T-T2DM group).

As it has been described that sex steroids are able to modulate SCs metabolism for periods longer than 50 h [18], treatments were performed for a period of 96 h. At 96 h of treatment, culture medium was collected for proton nuclear magnetic resonance (^{1}H-NMR) analysis and cells were detached and collected. The total number of cells per flask was determined with a Neubauer chamber. A viability test was performed on the cells of the different experimental groups using the Blue Exclusion Test. Viability averaged 90-95%, always with values higher than 90%.

NMR spectroscopy

^{1}H-NMR spectra of the culture media were acquired at 14.1 T, 25°C, using a Bruker Avance 600 MHz spectrometer equipped with a 5-mm QXI probe with a z-gradient (Bruker Biospin, Karlsruhe, Germany) using standard methods [4]. Sodium fumarate (1 mM) was used as reference (singlet, 6.50 ppm) to quantify the metabolites in solution (multiplet, ppm): H1-α glucose (doublet, 5.22 ppm) pyruvate (singlet, 2.36 ppm); lactate (doublet, 1.33 ppm) and alanine (doublet 1.45 ppm). Relative areas of ^{1}H-NMR resonances were quantified using the curve-fitting routine supplied with NUTSpro NMR spectral analysis program (Acorn NMR, Inc, Fremont, CA).

Glycogen content

Glycogen content of SCs was determined by using a commercial kit (Abnova KA0861, CA, USA) following the manufacturer's instructions. Glycogen content was expressed as picomole of glycogen per microgram of protein. Protein concentration was determined by the Bradford micro-assay, using bovine serum albumin as standard.

Western Blot

Western Blot procedure was performed as described [33]. Membranes were incubated with rabbit anti-glucose transporter 1 (GLUT1) (1:500, Sc-7903, SantaCruz Biotechnology, Heidelberg, Germany), rabbit anti-glucose transporter 3 (GLUT3) (1:500, ab41525, Abcam, Cambridge, MA, USA), rabbit anti-phosphofructokinase 1 (PFK1) (1:500, Sc-67028, SantaCruz Biotechnology), rabbit anti-MCT4 (1:1000, Sc-50329, SantaCruz Biotechnology), rabbit anti-LDH (1:10000, ab52488, Abcam, Cambridge, MA, USA), rabbit anti-glycogen synthase 1 (GYS1) (1:100, Sc-81173, SantaCruz Biotechnology) and rabbit anti-glycogen phosphorylase (PYG) (1:500, Sc-66913, SantaCruz Biotechnology). Mouse anti-tubulin (1:5000, T9026, Sigma-Aldrich) was used as protein loading. The immuno-reactive proteins were detected separately with goat anti-rabbit IgG-AP (1:5000, Sc-2007, SantaCruz Biotechnology) or goat anti-mouse IgG-AP (1:5000, Sc-2008, SantaCruz Biotechnology). Membranes were reacted with enhanced chemifluorescence detection system (GE Healthcare, Weßling, Germany). The densities from each band were obtained using the Quantity One Software (Bio-Rad, Hemel Hempstead, UK), divided by the respective tubulin band density and then normalized against the respective control.

Enzymatic activities

LDH activity was determined using a commercial assay kit (Promega, Madison, USA) following the manufacturer's instructions. Alanine aminotransferase (ALT) was determined by a colorimetric assay as described [34]. The attained activities were expressed as fold variation *versus* the control group.

Analysis of adenine nucleotides content

Adenine nucleotides levels were measured according to Rego and collaborators [35]. In brief, cellular pellet was homogenized in lysis buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, pH 7.4) supplemented with 1% protease inhibitor cocktail and centrifuged at 14000.*g*, for 2 min at 4°C. After neutralization (3 M KOH, 1.5 M Tris) samples were centrifuged at 14000.*g* for 2 min (4°C). Resulting supernatants were used to determine protein concentration by the Bradford micro-assay, using bovine serum albumin as standard. Supernatants were assayed for adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) by separation in a reverse-phase high-performance liquid chromatography, as described [36]. The chromatographic apparatus used was a Beckman-System Gold (Beckman Instruments, Fullerton, USA). The detection wavelength was 254 nm, and the column used was a Lichrospher 100 RP-18 (5 μm) from Merck (Darmstadt, Germany). An isocratic elution with 100 mM phosphate buffer (KH_2PO_4; pH 6.5) and 1.0% methanol was performed with a flow rate of 1 mL/min. The required time for each analysis was 5 min. Peak identity was determined by the retention time compared with standards. The amounts of nucleotides were determined by a concentration standard curve.

Testicular adenylate energy charge (AEC) was determined according the formula: ATP+0.5xADP/(ATP+ADP+AMP), as described previously [37].

Statistical Analysis

Statistical differences between the experimental groups were assessed by two-way ANOVA, followed by Bonferroni post-test (Graph Pad Software 6.0, San Diego, CA, USA). All experimental data are shown as mean ± standard error of the mean (SEM); $P<0.05$ was considered significant.

Results

High-energy fed animals developed PreD, while those administered with streptozotocin developed T2DM, exhibiting progressively lower testicular testosterone levels

The HED fed rodent model developed PreD, as previously described by our team [4]. At the end of treatment, the animals presented mild hyperglycaemia and glucose intolerance (while not insulin resistance). On the other hand, as previously described [30], STZ-treated rats developed T2DM, exhibiting hyperglycaemia, glucose intolerance and insulin resistance (see Annex, Figure 1).

Furthermore, PreD rats exhibited a significant decrease in T levels in both serum and TIF, comparatively to the control animals. However, E_2 levels were not altered. STZ-treated rats also showed a pronounced T deficiency either in serum as in TIF, but no differences were observed regarding to E_2 levels (see Annex, Figure 2).

Testosterone deficiency is critical for the glycolytic flux on Sertoli cells

After 96 h of culture the overall glucose consumption was significantly higher in the SCs of the T-T2DM group (60.11 ± 2.31 $pmol.cell^{-1}$) than in cells of the T-PreD and T-CTR groups, which consumed 36.42 ± 2.95 $pmol.cell^{-1}$ and 54.84 ± 1.32 $pmol.cell^{-1}$, respectively (Figure 5.1A). In SCs, extracellular glucose uptake is mainly achieved through GLUT1 and GLUT3 and at the end of treatment no differences were observed in GLUT1 and GLUT3 protein levels in the SCs of the T-PreD group, when compared to the control (Figure 5.1B). However, SCs of the T-T2DM group showed a decrease by 43% (0.57 ± 0.09 fold variation) in GLUT1 protein levels comparatively to control (Figure 5.1B).

In these cells the majority of glucose taken up from extracellular media is oxidized via glycolysis and phosphofructokinase (PFK) is known as a key rate-limiting enzyme of this process. We observed a decrease of 21% (0.79 ± 0.09 fold variation) of PFK1 protein levels in SCs of the T-PreD group comparatively to the cells of the T-CTR group (Figure 5.1B). In SCs of the T-T2DM group PFK1 protein levels were also decreased by 59% (0.41 ± 0.05 fold variation) as compared with the T-CTR group (Figure 5.1B).

Regarding pyruvate consumption, after the 96 h of treatment, SCs of the T-PreD group only consumed 2.70 ± 1.27 pmol.cell^{-1}, while SCs of the T-T2DM and T-CTR groups consumed 3.39 ± 1.27 pmol.cell^{-1} and 3.26 ± 0.11 pmol.cell^{-1}, respectively (Figure 5.1C).

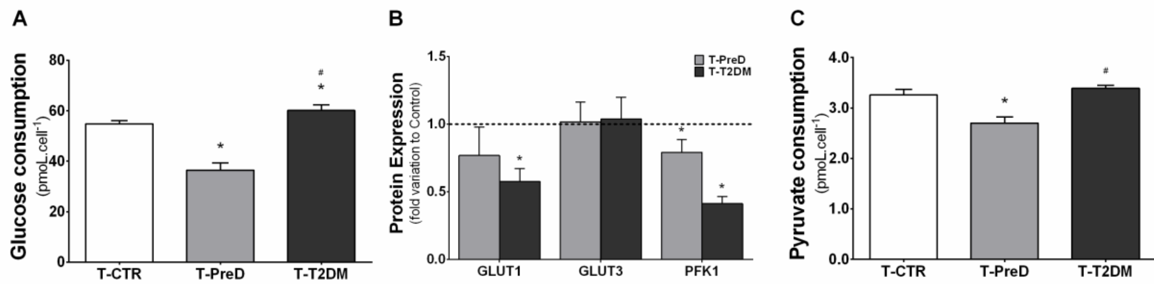

Figure 5.1. Effect of the different testosterone (T) concentrations on metabolite consumption and expression of glycolysis-related proteins of Sertoli cells from the T-CTR, T-PreD and T2DM groups. (A) Glucose consumption; (B) GLUT1, GLUT3 and PFK1 protein levels; (C) Pyruvate consumption. Results are expressed as mean ± SEM (n=6 for each condition). *Significantly different when compared to T-CTR group ($p < 0.05$). #Significantly different when compared to T-PreD group ($p < 0.05$). CTR- control, PreD- pre-diabetes; T2DM - type 2 diabetes mellitus.

T deficiency progressively affects LDH expression and activity in Sertoli cells

The lactate produced by SCs is a critical “fuel” for developing gem cells. At the end of treatment SCs of all groups produced similar amounts of lactate. Cells from the T-CTR group produced 75.62 ± 4.20 pmol.cell^{-1}, while cells from the T-PreD and T-T2DM produced 67.55 ± 5.38 pmol.cell^{-1} and 70.67 ± 5.79 pmol.cell^{-1}, respectively (Figure 5.2A).

Contrastingly, T deficiency induced a decrease in LDH protein levels. In SCs of the T-PreD group we observed a decrease of 33% (0.67 ± 0.18 fold variation) when compared to cells of the T-CTR group (Figure 5.2B). The decrease was more pronounced in SCs of the T-T2DM group, where LDH protein levels were decreased by 48% (0.52 ± 0.07 fold variation) comparatively to SCs of the T-CTR group (Figure 5.2B). The LDH activity of the SCs the control group was 188±7 nmol.min^{-1}.mg protein^{-1}, while in the SCs from T-T2DM group the activity of the enzyme was decreased by 81% (0.19 ± 0.02 fold variation) where T deficiency was more pronounced, and in SCs from the T-PreD group LDH activity was decreased by 8% (0.92 ± 0.12 fold variation) (Figure 5.2C).

The lactate produced by SCs is exported through MCT4 to be delivered to developing germ cells. In SCs from the T-PreD group, MCT4 protein levels remained unaltered. However, in SCs from T-T2DM group a decrease of 28% (0.72 ± 0.11 fold variation) was observed, as compared with cells of the T-CTR group (Figure 5.2B).

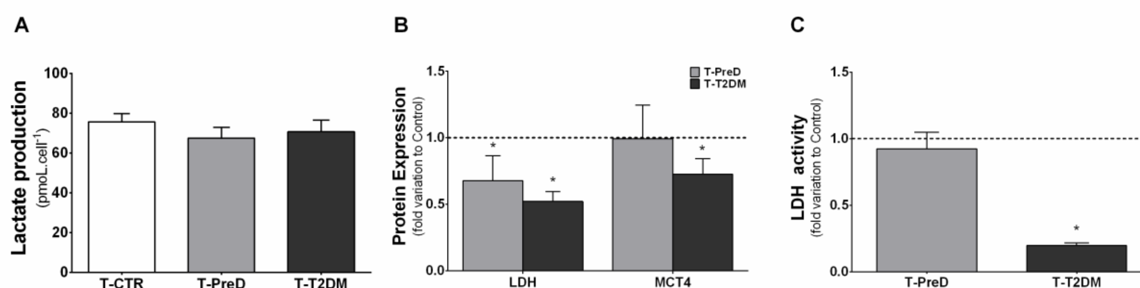

Figure 5.2. Effect of the different testosterone (T) concentrations on lactate production, and on LDH and MCT4 protein levels and LDH activity of Sertoli cells from the T-CTR, T-PreD and T2DM groups. (A) Lactate production; (B) LDH and MCT4 protein levels; (C) LDH enzyme activity. Results are expressed as mean ± SEM (n=6 for each condition). *Significantly different when compared to T-CTR group ($p < 0.05$). #Significantly different when compared to T-PreD group ($p < 0.05$). CTR- control, PreD- pre-diabetes; T2DM - type 2 diabetes mellitus.

Alanine metabolism in cultured SCs is severely altered by T deficiency

Pyruvate derived from glycolysis can be reversibly converted either to lactate (by LDH) or to alanine (by ALT). The overall alanine production was significantly increased in the SCs from both T-PreD and T-T2DM groups after 96 h of culture which produced 7.4 ± 1.4 $pmol.cell^{-1}$ and 7.1 ± 0.7 $pmol.cell^{-1}$, respectively, while SCs from the T-CTR group only produced 5.0 ± 0.2 $pmol.cell^{-1}$ (Figure 5.3A). The ALT activity of the SCs from control group was 23.0 ± 2.8 U/L and SCs from T-PreD group exhibited a significant increase of ALT activity (by 153%) (2.53 ± 0.48 fold variation), whereas SCs of the T-T2DM group presented a decrease of 22% (0.78 ± 0.10 fold variation) (Figure 5.3B).

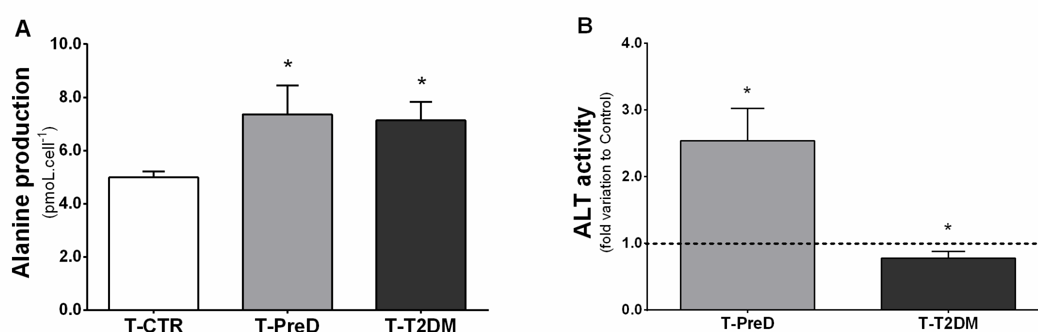

Figure 5.3. Effect of the different testosterone (T) concentrations on alanine production and ALT activity of Sertoli cells from the T-CTR, T-PreD and T2DM groups. (A) Alanine production; (B) ALT enzyme activity. Results are expressed as mean ± SEM (n=6 for each condition). *Significantly different when compared to T-CTR group ($p < 0.05$). #Significantly different when compared to T-PreD group ($p < 0.05$). CTR- control, PreD- pre-diabetes; T2DM - type 2 diabetes mellitus.

Reduced T levels induced by T2DM favor glycogen synthesis in Sertoli cells

Cells of the T-PreD group exhibited similar amount of intracellular glycogen (17.4 ± 3.7 pmol/µg protein) when compared to cells of the T-CTR group (23.7 ± 4.3 pmol/µg protein) (Fig.5.4A). However, SCs from the T-T2DM group exhibited a significant increase of 69.4% in intracellular glycogen content (40.1 ± 1.9 pmol/µg protein) when compared to cells of the T-CTR group (23.7 ± 5.3 pmol/µg protein) (Figure 5.4A). When we evaluated the expression

levels of glycogen metabolism-associated enzymes, protein levels of GYS1 (the main GYS isoform in testicles and in SCs) from T-PreD group were decreased by 25% (0.75 ± 0.06 fold variation) when compared to control cells (Figure 5.4B), while in SCs from T-T2DM group the GYS1 protein levels remained unaltered. On the other hand, the expression levels of PYG, a key enzyme on glycogen degradation, were unaltered in SCs from T-PreD group (Figure 5.4B), while in cells from T-T2DM group, its protein levels were decreased by 19% (0.81 ± 0.09 fold variation) comparatively to cells of T-CTR group (Figure 5.4B).

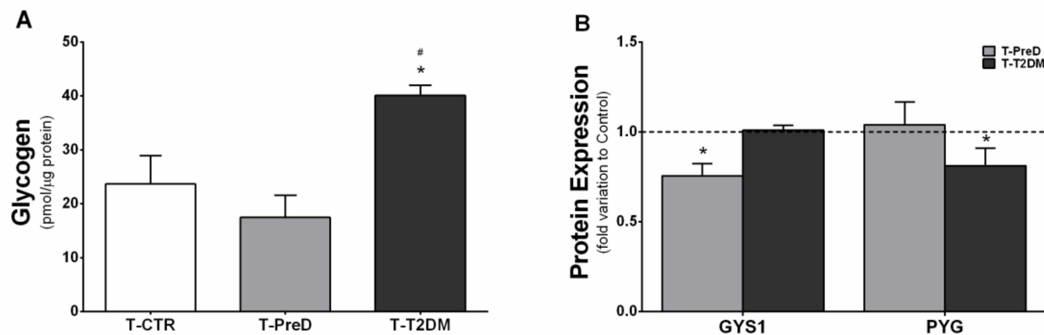

Figure 5.4. Effect of the different testosterone (T) concentrations on intracellular glycogen and on the expression of glycogen metabolism-related enzymes of Sertoli cells from the T-CTR, T-PreD and T2DM groups. (A) Glycogen content; (B) GYS1 and PYG protein levels. Pooled data of independent experiments, indicating the fold variation levels found in T-PreD and T-T2DM group cells when compared with the T-CTR group cells (dashed line). Results are expressed as mean ± SEM (n=6 for each condition). *Significantly different when compared to T-CTR group ($p < 0.05$). #Significantly different when compared to T-PreD group ($p < 0.05$). CTR- control, PreD- pre-diabetes; T2DM - type 2 diabetes mellitus.

Energy charge is progressively increased in Sertoli cells cultured in T deficiency conditions

AMP levels were decreased by 65% in SCs from T-PreD group (15.9 ± 4.9 pmol/mg protein) and by 75% in SCs from the T-T2DM group (11.2 ± 2.6 pmol/mg protein) when compared to SCs of the T-CTR group (45.2 ± 6.7 pmol/mg protein) (Figure 5.5A). Similarly to AMP, ADP content was also decreased by 64% (0.28 ± 0.09 pmol/mg protein) in SCs from T-PreD group and by 93% (0.05 ± 0.01 pmol/mg protein) in cells from the T-T2DM group, comparatively to T-CTR group (0.82 ± 0.17 pmol/mg protein) (Figure 5.5B). No differences were observed concerning to ATP levels (data not shown). Taking into account the unaltered levels of ATP and the significant decrease observed in AMP, the ATP/AMP ratio in SCs of the T-T2DM group was increased by 79% (0.43 ± 0.08) when compared to cells of the T-CTR group (0.09 ± 0.01) (Figure 5.5C). So, on face these results the overall AEC of the SCs from T-T2DM group was significantly increased by 53% (0.15 ± 0.02) comparatively to control (0.07 ± 0.01), while no significant alteration was observed for SCs of the T-PreD group (0.10 ± 0.02) (Figure 5.5D).

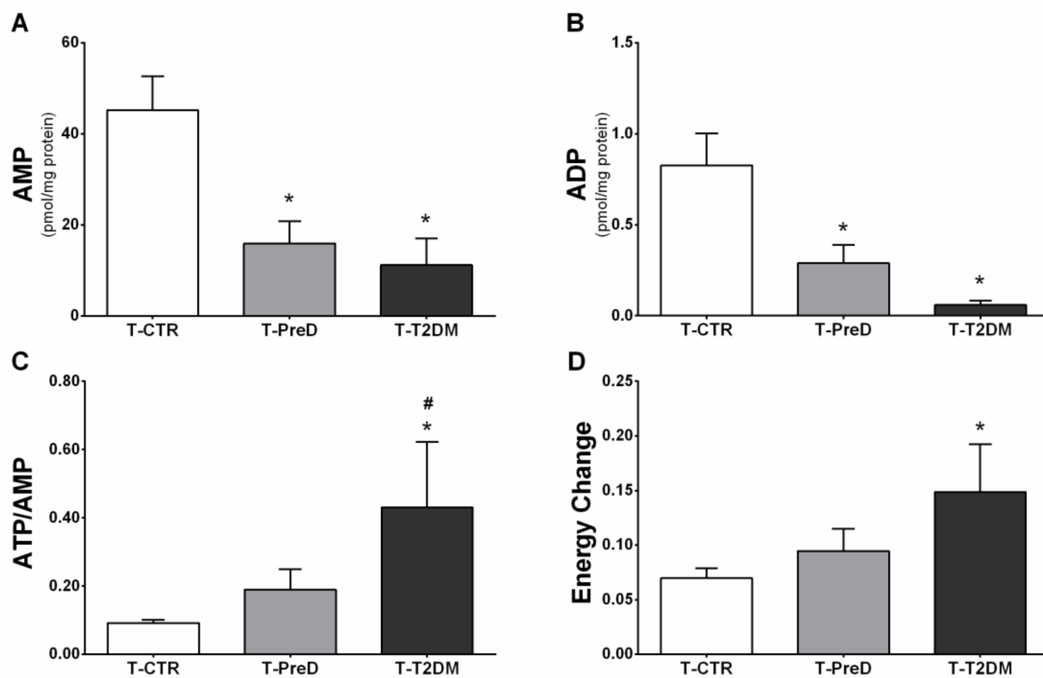

Figure 5.5. Effect of the different testosterone (T) concentrations on adenine nucleotides levels of Sertoli cells from the T-CTR, T-PreD and T2DM groups. (A) AMP content; B) ADP content; (C) ATP/AMP ratio; (D) Testicular adenylate energy charge. Results are expressed as mean ± SEM (n=6 for each condition). *Significantly different when compared to T-CTR group ($p < 0.05$). #Significantly different when compared to T-PreD group ($p < 0.05$). CTR- control, PreD- pre-diabetes; T2DM - type 2 diabetes mellitus.

Discussion

Type 2 diabetes mellitus is one of the most prevalent and serious metabolic diseases of the modern societies [38]. Recently, a prodromal stage of this metabolic disease named PreD has been described, which includes some (but not all) diagnostic criteria for established T2DM [39]. It has been reported that these two pathological conditions induce detrimental alterations in male reproductive function [40, 41], with special emphasis on testicular metabolism [4, 5, 25]. Testicular cells, and particularly the somatic SCs, are highly dependent on glucose as main carbon source to produce lactate, which is the preferred energy substrate for spermatocytes and spermatids [19, 42, 43]. Within the seminiferous tubules, SCs are the key targets for several hormones and growth factors and their functions are highly regulated by hormonal action, particularly by T [15, 16, 18, 24]. Recent works from our group highlighted that 5α-DHT modulates the metabolism of glucose in both human and rat SCs [16, 24, 25]. Moreover, the establishment of both PreD and T2DM have been consistently associated with severe hormonal deregulation, particularly concerning androgen levels [4, 40, 44]. Hence, we evaluated how T deficiency, promoted by PreD and T2DM, induced crucial alterations in the glycolytic profile of SCs obtained from rat models of those development stages of DM, evidencing mechanisms by which T deregulation may affect spermatogenesis. In our work, we found distinct metabolite consumptions and enzyme expression patterns of specific glycolytic intervenients among the experimental groups, with the most pronounced

effects being observed in higher T deficiency conditions, which correspond to the ones observed in the more severe stages of DM.

SCs obtained from pre-diabetic rats (T-PreD group) were exposed to intermediate levels of T and presented a decrease on glucose consumption, although no alterations were observed on GLUT1 and GLUT3 protein levels, when compared with SCs from the T-CTR group (exposed to the highest levels of T). T has been implicated in the regulation of glucose uptake and metabolism, including in cells of the blood-brain barrier [45], which exhibits remarkable metabolic similarities with the blood-testis barrier (for review see [46]). Furthermore, previous studies from our team have shown that 5α-DHT alone is able to upregulate the *in vitro* consumption of glucose by human and rat SCs [15, 16]. This effect has been associated with a modulation of GLUT1 expression and a decrease on lactate production [16, 18]. Yet, it has been reported that PreD rats show increased testicular content of lactate due to a favoring of testicular glycolytic metabolism and GLUT1 and GLUT3 expression [4]. In contrast, T-PreD SCs exhibited a lower expression of PFK, which was concurrent with the decrease observed on glucose consumption and suggests a lower glycolytic activity. Nonetheless, no differences were observed on lactate production, which illustrate the metabolic flexibility suggested for these cells [17] that even in under unfavorable *in vitro* conditions are able to modulate their metabolism [25, 26] or use alternative fuels in order to sustain the production of lactate [47]. Still, neither pyruvate or glycogen (which have been reported as alternative fuels for SCs under detrimental conditions [32] seem to be the sustainers of lactate production in the T-PreD SCs. In fact, pyruvate consumption was decreased in SCs from the T-PreD group, following the concomitant decrease in the glycolytic activity of these cells. Pyruvate is primarily derived from glycolysis and may be converted into lactate, alanine or it may enter the Krebs cycle [48]. The amino acid alanine is an important end-product of glucose utilization, which is required to cytosolic amino acid transformations and protein synthesis. In SCs from the PreD-T group, not only alanine production but also ALT activity were increased. ALT plays an important role in amino acid metabolism and gluconeogenesis and increased serum ALT levels have been associated with the development of PreD, with high risk of developing T2D and impaired fasting glucose [49, 50], which illustrates a common metabolic featured associated to this pathology. Hence, alanine production may be enhanced to sustain pyruvate metabolism (and hence, lactate delivery to germ cells). Furthermore, glycogen content was not altered, although the levels of GYS were decreased. Notably, insulin resistance and T2DM have been associated with a partially reversible decrease on GYS action in biopsies of human skeletal muscle, which involved altered GYS activity and reduced GYS expression [51].

Contrarily, SCs from the T-T2DM group consumed higher amounts of glucose at the end of the treatment, when compared to SCs from control and T-PreD groups. These results are not in line with the decreased expression of GLUT1 observed in SCs from these T-T2DM group, suggesting the existence of compensatory mechanisms, such as increased GLUTs activity. In fact, as discussed, SCs present a metabolic flexibility which allow them, even in insulin- or

glucose-deprivation conditions, to maintain or enhance glucose consumption in order to sustain the production of lactate [25, 26]. While glucose deficit and insulin deprivation caused an increase in GLUT1 and a decrease in GLUT3 expression in SCs [26], 5α-DHT deprivation caused no apparent alteration on GLUTs levels still maintaining glucose consumption [16]. It has been proposed that, under detrimental conditions, both GLUT1 and GLUT3 activities and/or expression may be modulated to provide the cells with sufficient amounts of glucose loading into the cells [46, 47, 52]. However, decreased levels of T in SCs of T-T2DM groups compromised the expression of PFK. Notably, PFK was shown to be tightly regulated by T in accessory reproductive glands, such as prostate and seminal vesicles. Indeed, PFK was downregulated in castrated animals, and those effects were reverted upon T administration, illustrating that this enzyme is responsive to the hormonal action of T [53]. Also, PFK is dependent on the cellular energy state and allosterically responds to other regulatory factors, such as ATP and AMP levels. The unaltered levels of ATP observed among the experimental groups may be explained due to the creatine and phosphocreatine system, which is widely present throughout male reproductive tract and have important roles as components of an energy buffer mechanism in cells [54]. In those conditions phosphocreatine may be used by creatine kinase in the interconversion between ADP to ATP to maintain adequate ATP levels during energy demands. SCs of the T-T2DM group showed an increase in ATP/AMP ratio, which is concurrent with an increased energy charge. The increased ATP/AMP ratio may exacerbate the inhibitory effects of T deficiency on PFK [55] and point towards an down-modulation of the glycolytic activity on these cells. In fact, although higher amounts of glucose were consumed, SCs from the T-T2DM group were not able to produce higher amounts of lactate than control cells. This may be due also to the decreased expression and activity of LDH. Interestingly, as T levels diminish, the effects in this biochemical process are more pronounced, since the protein expression and activity of LDH, as well as the protein expression of MCT4, in the SCs of the T-T2DM group were highly decreased.

As referred, one of the key functions of SCs is to ensure an adequate nurture of developing germ cells. Although our results show that T deficiency promoted by different stages of DM alters glucose metabolism in SCs, these cells can use alternative substrates to produce lactate. This is particularly relevant under pathological conditions to sustain a correct nutrition of developing germ cells [56]. In testicles, glycogen accumulation has a major physiological significance since it ensures an endogenous "glucose disposal". Testicular glucose metabolism is of the extreme importance, since key events of spermatogenesis depend on it. For instance, the absence of this hexose impairs steroidogenesis by Leydig cells [57]. Moreover, one of the major functions of SCs is to ensure the transport and metabolism of glucose to produce the metabolic precursors for developing germ cells [16, 18, 19]. Under pathological conditions, as is the case of DM, glucose metabolism is compromised, however SCs are able to adapt and use glycogen to ensure an endogenous source of glucose. The presence of glycogen and enzymes-related to glycogen metabolism in SCs [28, 29] has suggested that glycogen is a mobilizable fuel storage that can be readily metabolized by SCs

under abnormal physiologic conditions, thus explaining why glycogenesis may be enhanced in testicles of diabetic individuals [58]. In fact, glycogen is known to play a preponderant role in T2DM, contributing to glucose disposal [59]. Within seminiferous epithelium, SCs are the main producers of glycogen [27]. We observed an increase on glycogen accumulation in SCs from the T-T2DM group at the end of the treatment, illustrating that decreased T levels promoted by T2DM alter glycogen synthesis and its accumulation in these cells. These results are consistent with previous reports showing that testicles of diabetic rats presented an increased accumulation of glycogen precursors, such as uridine diphosphate glucose [58]. The increased levels of glycogen may be due to the significant decrease of PYG protein levels and/or to the increased glucose availability. Interestingly, in other tissues, T deficiency has been associated with decreased glycogen synthesis and storage, however the mechanisms that govern glycogen metabolism may differ between in testicular tissue and other tissues [60].

Altogether the present data illustrate that T deficiency promoted by different development stages of DM do not favor glycolytic flux in SCs. Glucose taken up by SCs is not as efficiently converted into lactate as in control conditions, being partly redirected to other metabolic pathways. The more pronounced effects were concurrent with the lower T levels, in which SCs adapted their glucose metabolism and synthesized more glycogen. In fact, deregulation of T levels is reported to contribute to the impairment of glucose metabolism mediated by insulin [61]. Moreover, it also affected the expression of lactate metabolism-related enzymes and transporters [25]. However, it was also evident that even in the most detrimental experimental conditions (lower T levels, as in T2DM individuals), SCs were still able to adapt their metabolism in order to sustain lactate metabolism. The results obtained herein highlight the physiologic significance of T in the regulation of the glycolytic profile of SCs metabolism, in particular when associated with the progression of T2DM. Further knowledge on the function and regulation of the molecular mechanisms that govern SCs metabolism will be essential to provide new insights on effects of the different stages of DM on SCs' metabolic (dys)function that may hamper spermatogenesis and contribute to male infertility.

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Chapter 6

High-energy diets may induce a pre-diabetic state altering testicular glycolytic metabolic profile and male reproductive parameters

This chapter was adapted from the published work:

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High-energy diets may induce a pre-diabetic state altering testicular glycolytic metabolic profile and male reproductive parameters

Abstract

Diabetes mellitus is a metabolic disorder that may arise from diet habits and is growing to epidemic proportions. Young male diabetic patients present high infertility/subfertility prevalence resulting from impaired reproductive function and poor semen quality. We aimed to evaluate the effects of a high-energy diet (HED) on glucose tolerance/insulin levels and correlate the observed effects on male reproductive function with overall testicular metabolism. After one month, HED fed rats showed increased glycemic levels, impaired glucose tolerance and hypoinsulinemia. Moreover, an imbalance of intratesticular and serum testosterone levels was observed, whereas those of 17β-estradiol were not altered. High-energy diet also affected the reproductive parameters, with HED rats exhibiting a significant increase in abnormal sperm morphology. Glucose glycolytic metabolism was favored in testicles of HED rats with an increased expression of both glucose transporters 1 (GLUT1) and 3 (GLUT3) and the enzyme phosphofrutokinase 1. Moreover, lactate production and the expression of metabolism-associated genes and proteins involved in lactate production and transport were also enhanced by HED. Alanine testicular content was decreased and thus intratesticular lactate/alanine ratio in HED rats was increased suggesting increased oxidative stress. Other energetic substrates such as acetate and creatine were not altered in testis from HED rats but intratesticular glycine content was increased in those animals.

Taken together these results suggest that HED induce a pre-diabetic state that may impair reproductive function by modulating overall testicular metabolism. This is the first report on testicular metabolic features and mechanisms related with the onset of a pre-diabetic state.

Keywords: High-energy diet; Testicular metabolism; Male reproductive function; Pre-diabetes; Glucose metabolism

Introduction

Diabetes mellitus (DM) includes a group of metabolic diseases that can be caused by a physical or functional loss of β-cell mass, due to an autoimmune process (type-1 diabetes) and/or increased insulin need (type-2 diabetes) [1]. DM is considered one of the greatest threats to the modern global health and its incidence is rising rapidly, in part due to the current eating habits, particularly among young people [1-3]. Infertility and subfertility prevalence is high in male patients with type-1 and type-2 DM [4, 5]. Moreover, data from animal models strongly suggest that DM impairs male fertility at multiple levels, such as endocrine control of spermatogenesis, spermatogenesis itself, or by impairing penile erection

and ejaculation [6-8]. Nevertheless, although the problems arising from DM have been widely investigated, the mechanisms responsible for the reported male reproductive dysfunction are still poorly understood [9, 10]. Recent reports have highlighted changes in whole body metabolic profile in diabetic conditions [11, 12], though only one has reported the testicular metabolic profile under diabetic state [13]. The detrimental influence of DM on testicular metabolism is receiving increased attention and recently it was suggested that testicular cells of diabetic individuals may present metabolic adaptations that allow them to minimize the negative effects promoted by the disease [14, 15]. In fact, glucose metabolism is crucial for normal development of spermatogenesis [16]. Amongst testicular cells, the Sertoli cells (SCs), the key somatic component of the seminiferous epithelium [17], are essential in the metabolic control of spermatogenesis because their carbohydrate metabolism presents some unique characteristics [18, 19]. After entering into these cells through glucose transporters 1 (GLUT1) and 3 (GLUT3), glucose undergoes the glycolytic pathway producing pyruvate, being the majority of it converted into lactate that functions as “fuel” for developing germ cells [16, 20, 21]. Even in non-physiological conditions, SCs adapt their metabolism to maintain lactate production. Recently, we reported the effect of sex hormones, which are known to be deregulated in DM [22], and of insulin deprivation in SCs concluding that these cells adapt their metabolism by modulating the expression of key metabolic enzymes and transporters of glucose metabolism [23]. Pre-diabetes is characterized by mild-hyperglycemia and arises as a high-risk factor for diabetes [24]. The most common alterations evidenced in pre-diabetic state are impaired fasting glucose levels and/or glucose intolerance due to the glucose control deregulation, as well as β-cell dysfunction [1, 25]. Increasing levels of circulating glucose cause overall metabolic changes, which may be reflected at testicular level. Testicular metabolism present unique characteristics being highly dependent on glucose metabolism and may adapt to peripheral glucose fluctuations that occurs under diabetic conditions [23, 26]. These mild increased glycemic levels may lead to the production of free oxygen radicals, which could themselves cause tissue damage and impair reproductive function [14]. Nevertheless, the knowledge concerning the impairment of the overall testicular metabolism caused by the pre-diabetic state is virtually null and scarce in concerning DM, being that in most cases the available information is associated with more advanced or severe stages of the disease. Thus, we aimed to use an animal model that could mimic the initial stages of DM induced by food intake. We used a high-energy diet (HED) fed male rat model, in order to evaluate diet effect on the glucose tolerance/insulin levels and correlate with the male reproductive function and overall testicular metabolism. To our knowledge, the present study is the first to apply a metabolomics technology approach to give new insights regarding the effect of a diet-induced pre-diabetic state on the testicular metabolism to disclose some of the molecular mechanisms related to poor quality diets intake and subfertility/infertility associated with DM.

Material and Methods

Animals

Twelve2-months old male Wistar rats (Charles River Laboratories, Barcelona, Spain) were used. The animals were housed in our accredited animal colony and maintained with food and water ad libitum in a constant room temperature (20 ± 2ºC) on a 12-hour cycle of artificial lighting. All experiments were performed according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the European directives for the care and handling of laboratory animals (Directive 86/609/EEC).

Experimental design

Rats were randomly divided (6 per group) in control and high-energy diet (HED) groups. The control group animals were fed with a standard chow diet (4RF21 certificate, Mucedola, Italy) and the HED group received an additional high-energy emulsion as described elsewhere [27-29]. Briefly, in the first 5 treatment days, animals were given progressively 1 to 5 mL of emulsion consisting of 20 g lard oil, 1 g thyreostat, 5 g cholesterol, 1 g sodium glutamate, 10 g sucrose, 20 mL Tween 80, 30 mL propylene glycol prepared in a final volume of 100 mL by adding distilled water. Thereafter, they were administrated daily with 5 mL of the emulsion until they reach one month of treatment. Water, food consumption and animal's weight were monitored every 2 days. After the treatment, animals were killed by cervical dislocation. Blood was collected by cardiac puncture to non-heparinized tubes. Testes were removed, weighed and processed for testicular interstitial fluid (TIF) collection, according to Porter and collaborators [30] or stored at -80º C.

Blood glycaemia

Non-fasting glycaemia was determined every 6 days. Blood samples were collected from the tail vein and blood glucose levels were measured through glucose oxidase reaction by using a glucometer (One Touch Ultra Lifescan-Johnson).

Glucose tolerance test

At 3 months of age the animals were subjected to a glucose tolerance test as described by Nunes and collaborators [31]. Briefly, 16-18 hours before the test, access to food was removed from the animals. An intraperitoneal injection with 6 mL glucose 30% (w/v) per kg of body weight was given to each animal. Blood glucose levels were measured every 30 minutes during 2 hours.

Insulin, testosterone and 17β-estradiol determination

Insulin, Testosterone (T) and 17β-estradiol (E_2) levels were determined using commercial rat EIA kits according to manufacturer instructions. T and E_2 EIA Kits were purchased from

Cayman Chemical Company (Ann Arbor, MI, USA). Insulin ELISA measurement kit was purchased from Mercodia (Uppsala, Sweden). The EIA kits used had detection limits of approximately of 40 µU/mL (for insulin), 6 pg/mL (for T) and 20 pg/ml (for E_2).

Epididymal sperm parameters

Epididymis were isolated and placed in pre-warmed (37ºC) HBSS (pH 7.4), minced with a scalpel blade and the suspension was incubated for 5 minutes (37ºC). Sperm motility was evaluated by placing a drop of sperm suspension (2-3 x 10^6 sperm cells) in a warmed slide (37ºC)and motile sperm percentage was assessed in 10 random fields, using an optical microscope (x100 magnification), and the average value was used as the total sperm motility. Sperm viability was assessed examining eosin-nigrosin stained epididymal sperm smears. A total of 333 spermatozoa were counted in random fields under a light microscope. Dead sperm stained pink, because its membrane integrity is compromised, causing the dye (eosin) uptake. Epididymal sperm concentration was determined using a dilution of 1:50 in HBSS solution to fill the two grids of a Neubauer counting chamber. The number of sperm cells was then counted under an optical microscope (x400 magnification). For the assessment of sperm morphology we used standard methods [32]. Sperm morphology was evaluated using Diff-Quick™ (Baxter Dale Diagnostics AG, Dubinger, Switzerland) stained smears according to manufacturers' instructions. A total of 333 sperm were evaluated, in random fields. To be classified as normal a sperm cell must have a hook-shaped head and no defects of head, neck or tail. Otherwise, sperm were considered abnormal.

NMR spectroscopy

A combined extraction of polar and apolar metabolites was performed as previously described [33]. The aqueous phase containing the water-soluble metabolites was lyophilized. NMR spectra was acquired as described previously [33]. Sodium fumarate was used as an internal reference (6.50 ppm) to quantify the following metabolites (multiplet, δ, ppm): lactate (doublet, 1.33 ppm); alanine (doublet, 1.45 ppm); acetate (singlet, 1.90 ppm); creatine (singlet, 3.0 ppm); glycine (singlet, 3.54 ppm). The relative areas of ^{1}H-NMR resonances were quantified using the curve-fitting routine supplied with the NUTSpro™ NMR spectral analysis program (Acorn, CA, U.S.A.).

Reverse transcriptase-polymerase chain reaction

At the end of the treatment, total ribonucleic acid (RNAt) was extracted from testicular tissue using TRI reagent according to the manufacturer's instructions. RNA concentration and absorbance ratios (A260/A280) were determined by spectrophotometry (NanophotometerTM, Implen, Germany). RNAt was reversely transcribed as described previously [20]. Semi-quantitative PCR was performed to analyze lactate dehydrogenase A (LDHA), monocarboxylate transporter 4 (MCT4), glucose transporter 1 (GLUT1), glucose transporter 3 (GLUT3), phosphofrutokinase 1 (PFK1) and lactate dehydrogenase C (LDHC) mRNA expression

as described by Oliveira and collaborators [34]. Both optimal annealing temperature and the amplification size of fragments are shown in Table 6.1. mRNA expression was normalized with 18S gene expression and expressed as fold variation (induction/reduction) versus the control group.

Table 6.1. Oligonucleotides and cycling conditions for PCR amplification of LDHA, MCT4, GLUT1, GLUT3, PFK1, LDHC and 18S. AT — annealing temperature; C —number of cycles during exponential phase of amplification.

Gene	Sequence (5'-3')	AT (C°)	Amplicon size (bp)
LDHA	Sense:CGTCGTCCCCCATCGTGCAC Antisense:GGGCCCCCGCGGTGATAATG	60	345
MCT4	Sense:CGTCGTCCCCCATCGTGCAC Antisense:GGGCCCCCGCGGTGATAATG	60	629
GLUT1	Sense:TCCGGCGGGAGACGCATAGT Antisense:CCCGCATCATCTGCCGACCC	61	842
GLUT3	Sense:GCGCAGCCCTTCCGTTTTGC Antisense:CCCCTCGAAGGCCCGGGTAA	63	806
PFK1	Sense:GAGTGCTGACAAGCGGCGGT Antisense:GTGGCCCAGCACGGTCACTC	61	839
LDHC	Sense:ATGTGGGCATGGCGTGTGCC Antisense:CCCAGCCATGGCAGCTCGAA	66	477
18S	Sense:AAGACGAACCAGAGCGAAAG Antisense:GGCGGGTCATGGGAATAA	56	149

Western blot

Western Blot procedure was performed as previously described by Alves and collaborators [35]. The resulting membranes were incubated with rabbit anti-GLUT1 (1:300, CBL242), rabbit anti-GLUT3 (1:1000, ab41525), rabbit anti-PFK1 (1:500, Sc67028), rabbit anti-MCT4 (1:1000, Sc50329), rabbit anti-LDH (1:10000, ab52488), rabbit anti-ALT (1:500, Sc99088). Mouse anti-tubulin (1:5000, A5441) was used as protein loading control for testicular tissue. The immuno-reactive proteins were detected separately with goat anti-rabbit IgG-AP (1:5000, Sc2007) or goat anti-mouse IgG-AP (1:5000, Sc2008). Membranes were reacted with ECF detection system. The densities from each band were obtained using the Quantity One Software (Bio-Rad, UK), divided by the respective tubulin band density and then normalized against the respective control.

Enzymatic activity assays

Lactate dehydrogenase (LDH) activity was determined using a commercial assay kit (Promega, Madison, USA) and following the manufacturers' instructions. Phosphofructokinase 1 (PFK1) activity was determined as previously described [36]. The enzyme activity was expressed as units per milligram of protein. The attained activities were expressed as fold variation versus the control group.

Statistical analysis

The statistical significances of differences of all experimental data were assessed by Student-t test (GraphPad Software). All experimental data are shown as mean ± S.E.M; P<0.05 was considered significant. Further analysis of the statistical power (SP) of differences of experimental data was evaluated with a one-tail test assuming an alpha of 0.05 that corresponds to a 0.95 confidence interval, as described by Levin [37], using the software provided by:

http://www.dssresearch.com/KnowledgeCenter/toolkitcalculators/statisticalpowercalculators.aspx.(Accessed in December 2012).

Results

High-energy diet fed rats developed mild hyperglycemia, glucose intolerance and hypoinsulinemia

Glycemic values of HED fed animals were not significantly different, when compared with the control group, until the12th treatment day. Afterwards, the glycaemia in rats from the HED group rose slightly but significantly (SP = 95%) to moderate hyperglycemic values that were maintained up until 30th day (Figure 6.1A).

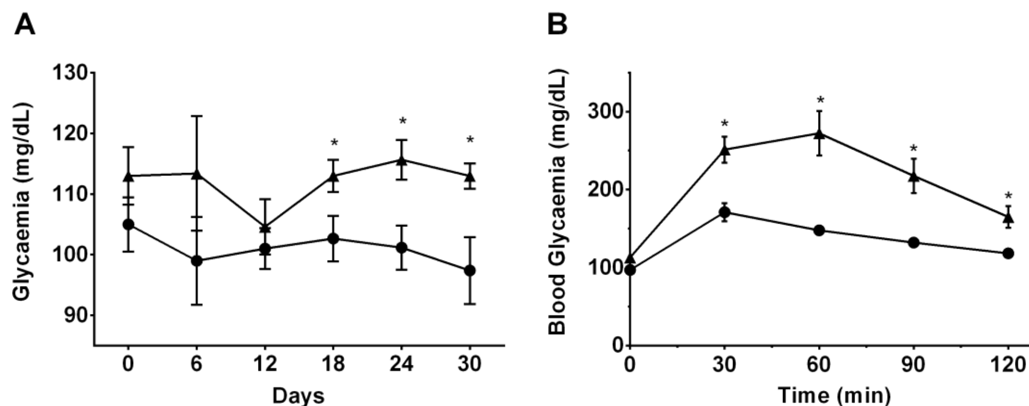

Figure 6.1. Blood glucose profile of the Control group (●) and the High-energy diet(HED) group animals (▲) during the 30 days of HED treatment. (Panel A) and blood glucose levels of the Control group (●) and the High-energy diet (HED) group animals (▲) measured during the intraperitoneal glucose tolerance test (Panel B). Figure shows pooled data of independent experiments, indicating the blood glucose levels variation. Results are expressed as mean ± SEM (n=6 for each condition). * Significantly different relatively to control (p< 0.05).

Since mild hyperglycemia could lead to a glucose intolerance status, we performed an intraperitoneal glucose tolerance test that shown a significant difference in glucose profile between the animals of both groups (Figure 6.1B). The HED rats showed a significant increase in blood glycaemia during the 120 minutes of the glucose tolerance test indicating that HED rats developed glucose intolerance. These results pointed towards to an insulin dysfunction status thus, we evaluated fasting insulin levels. HED rats showed a decrease of blood insulin levels of 2.64-fold (SP = 100%) when compared to control animals (Table 6.2).

Table 6.2. Average values of the animals weight, insulin and gonads measured in Control group and HED group animals after 30 days treatment. HED – high-energy diet. Results are expressed as means ± SEM (n=6for each condition). * Significantly different relatively to control (P<0.05).

Parameters	Control Group	HED Group
Insulin (µU/mL)	12.54 ± 1.46	4.75 ± 1.51*
Body weight (g)	335.67 ± 8.69	274.50 ± 8.91*
Gonad weight (g)	3.61 ± 0.07	3.30 ± 0.04

Testosterone serum and intratesticular levels are highly reduced in HED rats

When we evaluated total T serum concentration, we observed a significant decrease of 8.5-fold in HED group when compared to the control (SP = 81%) (Figure 6.2A). However, serum E_2 levels were not altered in both groups (Figure 6.2B).

Testicular cells are bathed by the TIF and the establishment of an appropriate fluid is crucial for an adequate hormonal control of spermatogenesis. As expected, our results showed that the sex hormones levels in TIF are 100-fold higher than in serum, since the sex hormones levels in the TIF are known to be significantly higher than those found in serum [38-40]. Additionally, the HED rats showed a significant decrease in T levels present in TIF when compared to control animals (SP = 100%) (Figure 6.2A). On the other hand HED did not significantly alter E_2 concentration in TIF (Figure 6.2B).

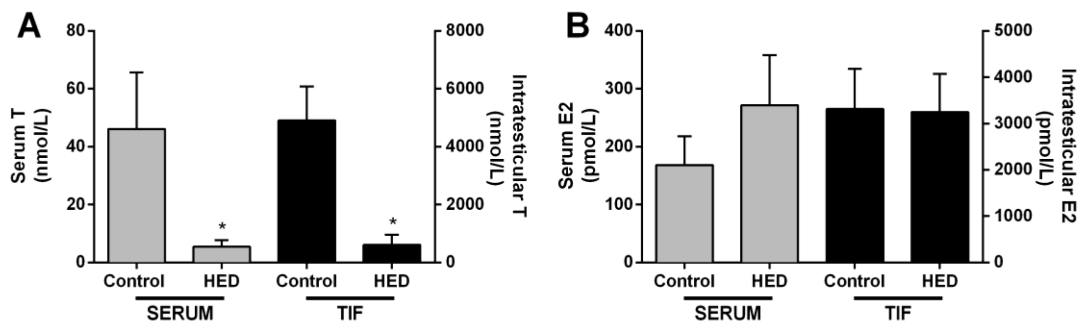

Figure 6.2. Testosterone levels in testicular interstitial fluid and serum of Control group and High-energy diet (HED) group animals. (Panel A).17β-estradiol levels in testicular interstitial fluid and serum of Control group and high-energy diet (HED) group animals (Panel B). Results are expressed as mean ± SEM (n=6 for each condition). * Significantly different relatively to control (p< 0.05).

Epididymal sperm motility and morphology are affected by the HED

No differences were observed for sperm viability and concentration between the HED group and the control group (Table 6.3). However, sperm motility was significantly higher (SP = 74%) in HED animals (68 ± 2 %) than in control animals (58 ± 5 %) (Table 6.3). Another important parameter to evaluate male fertility potential is sperm morphology [41]. The average percentage of normal epididymal spermatozoa (Table 6.3) was significantly lower in the HED group animals (45.9 ± 2.3 %) than in the control group (61.0 ± 0.6 %) (SP = 100%).

Table 6.3. Epididymal sperm motility, viability, concentration and morphology of Control group and HED group animals. HED-high-energy diet. Results are expressed as means ± SEM (n=6for each condition). * Significantly different relatively to control ($P<0.05$).

SpermParameters	Control Group	HED Group
Motility (%)	58.0 ± 5.0	68.0 ± 2.0*
Viability (%)	67.0 ± 2.0	76.0 ± 3.0
Concentration (x10^7 cell/mL)	2.80 ± 0.3	2.80 ± 0.7
Morphology (% normal spermatozoa)	61.0 ± 0.6	45.90 ± 2.3*

High-energy diet causes a significant increase on intratesticular GLUT1 and GLUT3 levels and PFK enzymatic activity

Testicles are high-energy demand tissues, presenting a high glycolytic flux. A rate-limiting step for glucose metabolism is its import through the cytoplasmic membrane hexose transporters [42] thus, we evaluated the possible effect of HED on GLUT1 and GLUT3 transporters transcript and protein levels. Concerning GLUT1, the mRNA levels showed a slight non-significant increase (SP = 46 %) in testicles of the HED animals (Figure 6.3A) that was followed by a 1.58-fold increase in protein levels compared to the control group (SP =100%) (Figure 6.3B). GLUT3 mRNA expression in testicles from HED rats was 1.39-fold increased (SP = 93%) relatively to control and was followed by a 1.17-fold increase in protein levels (SP = 66%) (Figure 6.3B). After glucose transport, a key regulatory step of glycolytic pathway is mediated by PFK1 that irreversibly converts fructose 6-phosphate to fructose 1,6-bisphosphate [43]. Thus, we evaluated the HED effect in PFK1. The mRNA levels of PFK1 were 1.31-fold increased (SP = 68%) in HED rats compared to the control (Figure 6.3A), however protein levels showed a 0.65-fold reduction when compared to control (SP = 100%) (Figure 6.3 B). These apparent contradictory results in mRNA and protein levels, led us to evaluate PFK1 activity in testicular tissue homogenates. Although HED animals presented lower testicular PFK1 protein levels, the correspondent enzyme activity was significantly higher (0.039 ± 0.013 U/mg protein) than in control animals (0.013 ± 0.002 U/mg protein), exhibiting a 3-fold increase (SP = 88%) (Figure 6.4).

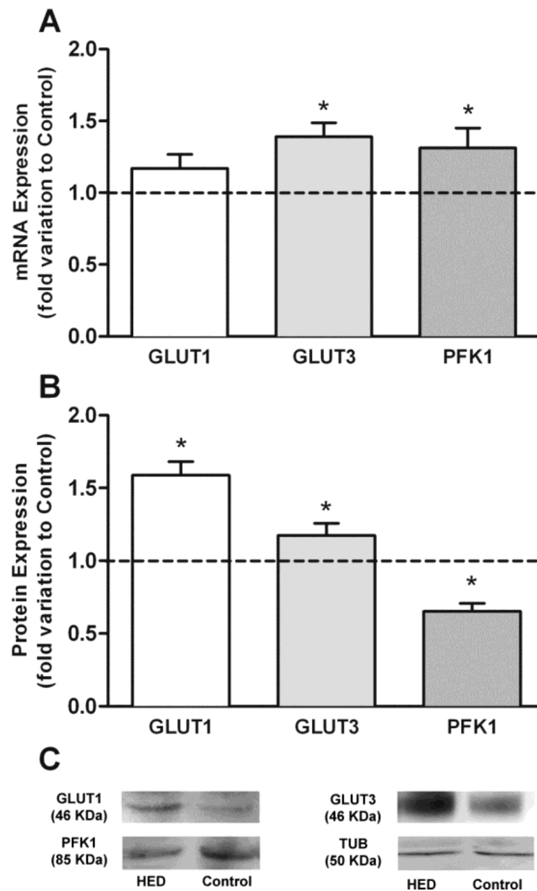

Figure 6.3. Effect of high-energy diet (HED) on testicular GLUT1, GLUT3 and PFK1 mRNA (Panel A) and protein (Panel B) levels. Pooled data of independent experiments, indicating the fold variation levels found in testis of HED group rats when compared with the Control group rats(dashed line). Panel C represents an illustrative Western Blot experiment. Results are expressed as mean ± SEM (n=5 for each condition). * Significantly different relatively to control ($p< 0.05$).

HED increased intratesticular lactate content, LDH and MCT4 levels

For a successful spermatogenic event, lactate plays a crucial role, acting as "fuel" for germ cells [16]. Thus, we aimed to analyze the HED effect in the intratesticular lactate content and on the expression and activity of lactate-related enzymes and transporters.

Lactate content was significantly increased by 33% (SP = 74%) in the testes of HED rats to 8.34 ± 0.65 µmol/mg tissue (Table 6.4), thus it would be expectable that overall LDH levels would be increased in these animals. In testes there are two main LDH isoforms: a typical LDHA and the testis-specific LDHC [44].

Table 6.4. Variation in the relative intratesticular metabolite content in Control group and HED group animals. HED – high-energy diet. Results are expressed as means ± SEM (n=5 for each condition). * Significantly different relatively to control (P<0.05).

Metabolites (µmol/mg tissue)	Control Group	HED Group
Lactate	6.29 ± 0.81	8.34 ± 0.65*
Alanine	3.31 ± 0.35	2.91 ± 0.13*
Acetate	0.37 ± 0.06	0.51 ± 0.09
Creatine	54.9 ± 4.60	61.3 ± 2.50
Glycine	5.15 ± 0.54	6.44 ± 0.36*

We analyzed the mRNA levels of both, LDHA and LDHC, and the overall LDH protein levels. Although testicular LDHA mRNA levels were not significantly different, the LDHC levels were significantly increased by 1.37-fold (SP = 87%) in HED rats when compared to control (Figure 6.5A). In agreement, whole-testis LDH protein content was also higher (2.10-fold) in HED than in control rats (SP = 100%) (Figure 6.5B). However LDH activity was not significantly different between both control and HED groups (Figure 6.4). After being produced, in the blood-testis barrier, lactate is exported through MCT4 [45]. Therefore we evaluated the effect of HED on MCT4 expression levels. MCT4 mRNA levels presented a significant 1.44-fold increase (Figure 6.5A) that was followed by a significant 2.19-fold increase of MCT4 protein levels (Figure 6.5B) in testis from HED rats when compared with the control group (SP = 100%).

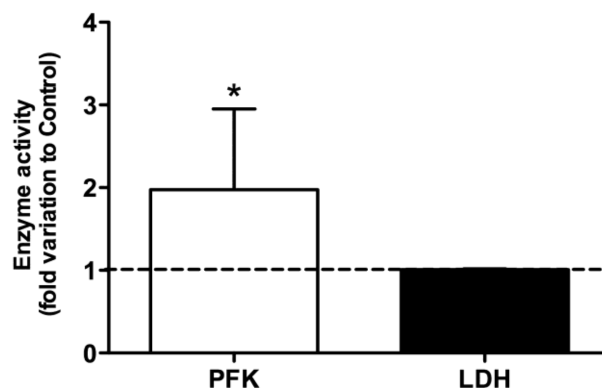

Figure 6.4. Effect of high-energy diet (HED) on testicular Phosphofructokinase-1 and lactate dehydrogenase activities. Panel shows pooled data of independent experiments, indicating the fold variation of enzymes activitiesin testis of high-energy diet HED group rats when compared with the Control group rats (dashed line). Results are expressed as mean ± SEM (n=5 for each condition). * Significantly different relatively to control (p< 0.05).

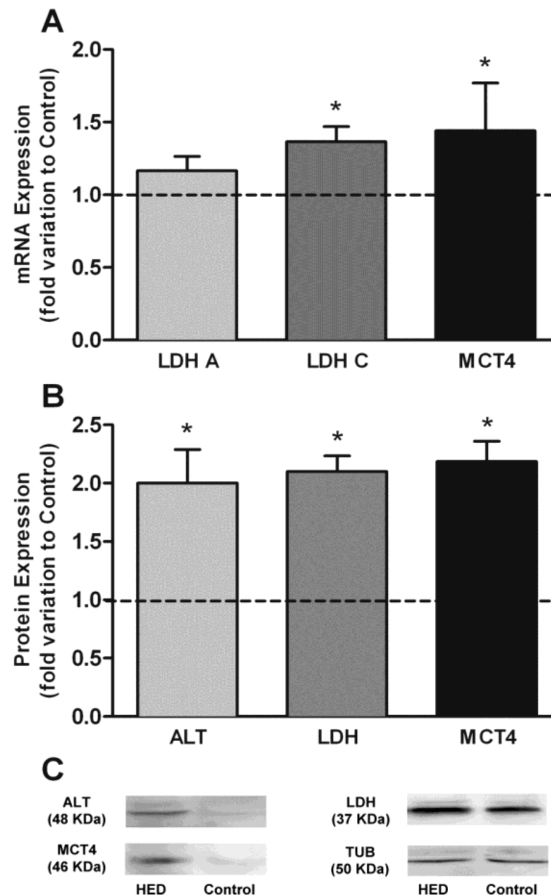

Figure 6.5. Effect of high-energy diet (HED) on testicular LDHA, LDHC, MCT4 mRNA (Panel A) or LDH, MCT4, ALT protein (Panel B) levels. Pooled data of independent experiments, indicating the fold variation levels found in testis of HED group rats when compared with the Control group rats (dashed line). Panel C represents an illustrative Western Blot experiment. Results are expressed as mean ± SEM (n=5 for each condition). * Significantly different relatively to control ($p < 0.05$).

HED modulates intratesticular alanine and glycine concentrations

Alanine metabolism is closely related to lactate production and metabolism, which can be converted to pyruvate by alanine aminotransferase (ALT) prior to the eventual conversion of pyruvate to lactate by LDH [20]. So we measured alanine intratesticular content and ALT protein levels in the testes of animals from both groups. Alanine content was decreased by 12% in HED rats (SP = 31%), which presented an intratesticular concentration of 2.91 ± 0.13 µmol/mg tissue. This was concomitant with the significant 2.90-fold increase (SP = 100%) of ALT protein expression in HED rat testes (Figure 6.5B). We also measured the intratesticular concentration of acetate since we have previously reported that acetate metabolism, which is important to membrane remodeling in germ cells and thus to spermatogenesis, is under strict insulin control [21]. Intratesticular acetate concentration in HED rats was slightly increased (not significantly). We also detected and measured the intratesticular concentration of two aminoacids, glycine and creatine, known to be used as energetic sources for testicular cells [46]. Intratesticular glycine concentration significantly increased (SP = 75%) from 5.1 ± 0.5 in

control group to 6.4 ± 0.3 in HED group (Table 6.4), while intratesticular creatine concentration was also increased by the HED (but not significantly) (Table 6.4).

Discussion

Diabetes mellitus is a heterogeneous metabolic disorder affecting whole body system and can result as a consequence of diet habits. There are evidences that DM negatively affects male reproductive function at multiple levels [6-8]. In the present study, we used a high-energy fed rodent model, to evaluate the effects of the initial stages of development of DM on the male reproductive function. Pre-diabetes is the state in which some but not all of the diagnostic criteria for diabetes are met. Pre-diabetes is often connected with the metabolic syndrome, which in turn is closely associated with obesity [47, 48]. The pre-diabetic state is characterized by impaired fasting glucose or glucose intolerance and by mild-hyperglycemia [24]. In our study, the administration of a HED to adult rats led to the development of those two characteristics, glucose intolerance and mild-hyperglycemia, suggesting that HED animals developed a pre-diabetic state. HED animals also presented a significant lower weight gain when compared to control animals, a feature consistent with what is reported in several other studies using high-energy/high-fat diets that did not induce weight gain [49, 50]. Indeed, those authors described that rats fed with high-fat diets displayed a lower body weight gain probably due to low protein content in high-fat diets, which favors an increase of brown adipose tissue and not the heavier white adipose and muscle tissues. This may explain why the HED animals exhibited a lower weight gain after high-energy diet treatment. Nonetheless, control and HED animals presented similar gonadal weights, which indicate that the testicular architecture is maintained. However, those animals presented a slight but significant increase in glycaemia levels. The mild glycaemia observed in the HED animals does not meet the criteria for established DM[51], but these animals shown an impaired glucose tolerance (2-h post load glucose, levels of glycaemia remained >140 mg/dL and <200 mg/dL) indicating a “status” of increased risk for DM, often designated as pre-diabetic state [52, 53]. The pre-diabetic state has also been associated with impairment of insulin secretion and decreased insulin serum levels [52]. Indeed, our results showed a significant decrease in serum insulin concentration in HED rats. Taken together these markers exhibited by HED rats suggest that a pre-diabetic state was developed in these animals [54, 55] increasing the relevance of this study. DM has also been associated with sex steroid levels imbalance [22, 56]. In fact, HED rats showed a significant decrease in intratesticular and serum T levels that could be explained by the lower serum insulin levels, since it has been reported that insulin may act directly in brain through its receptors located in hypothalamus and pituitary [57] and that low insulin levels negatively affect T secretion. Additionally, it was demonstrated that insulin administration is able to restore the hypothalamus-pituitary-gonad axis functioning and normalize T levels in diabetic mice [58]. Moreover, studies with neuron-specific insulin receptor knockout mouse have evidenced the role of insulin in male fertility maintenance [59]. Concerning estradiol levels we did not observe significant differences at serum and

intratesticular levels between animals from both groups. These results are in accordance with those obtained by Burul-Bozkurt and collaborators [60] that reported no alterations on the plasma estradiol levels in streptozotocin-treated rats. The results obtained in our study can be explained by the fact that the animal model developed a pre-diabetic state and not an advanced stage of disease where a marked sex steroid hormone deregulation occurs [56, 61]. It is well documented that sperm parameters are compromised in diabetic individuals [10, 62, 63]. In the present study, evaluation of reproductive parameters showed altered motility and morphology, although no differences in sperm concentration and viability were observed between animals of both groups. As referred above, glycaemia is slightly increased in pre-diabetes. Additionally, there are evidence that glucose metabolism may be enhanced by the pre-diabetic state, thus favoring oxidative stress. Indeed, higher glucose availability may lead to an increased glycolytic activity and therefore an ROS overproduction. In the seminiferous tubules, ROS are continuously produced by the Sertoli cells and the germ cells as a result of their continuous metabolic activity [64]. Different testicular cells show [64, 65]. Thus, an increased oxidative environment leads to cellular damages such as lipid peroxidation and DNA fragmentation with consequent sperm abnormalities [66]. Nevertheless, a higher availability of glucose will increase sperm ATP production. There are evidences regarding a direct relation between glucose availability and consumption by spermatozoa [67]. Others authors have also reported that epididymal cells uptake glucose by facilitated transport [68] and gradient concentration [69]. These facts suggest that a high serum glucose concentration, as happens in a mild hyperglycemic state, may increase the epididymal glucose content, that after being internalized by sperm is readily metabolized to produce ATP by oxidative phosphorylation and may lead to an increase in sperm motility [70]. Crucial for sperm formation and normal spermatogenesis is the overall testicular metabolism. Recently, we have reported the influence of sex steroid hormones on testicular cells metabolism [20, 21, 34] concluding that hormonal deregulation can modulate SCs metabolism, and thus spermatogenesis. Glucose is a key substrate in testicular energy metabolism, since testes present high glycolytic flux, but these organs are not able to accumulate this metabolite [13] because glucose is rapidly oxidized in testes [71]. It was also reported that SCs cultured in the absence of glucose [15] and under insulin deprivation presented an altered glucose metabolism and modulation of GLUT1 and GLUT3 transcript levels [23]. As expected, due to lower insulin and higher glucose levels in HED rats, GLUT1 and GLUT3 protein levels increased to favor the glucose uptake by the cells. Once inside the cells, glucose enters in the glycolytic process and PFK1 irreversibly catalyzes fructose-6-phosphate to fructose-1-6-bis-phosphate [43] in a rate-limiting step for glucose metabolism. Following the GLUT1 and GLUT3 increase, testicular PFK1 activity was also increased in HED rats supporting that the glycolytic process was stimulated in HED rats. Interestingly, at translational level, HED reduced the protein levels but increased the mRNA levels of PFK1. In fact the observed increase in PFK1 mRNA levels may not be immediately reflected in protein levels. This may be consequence of several factors associated with gene expression regulatory mechanisms complexity, such as,

mRNA retention in the nucleus, processing, stability and half-life time of mRNA molecule, control of protein translation efficiency, post-translational modifications, and protein degradation [36]. Taking into account these facts, the authors assessed PFK1 activity and observed that HED PFK1 activity was 2-fold higher than control group, suggesting that glycolytic process was stimulated in HED animals. Also, it has been described that lactate is a modulator of glycolytic enzymes expression and activity [72]. In fact, intratesticular lactate concentration was found to be increased in HED rats and therefore one cannot exclude the possible regulatory role of this metabolite in PFK1 protein levels. Overall, the HED stimulated glucose uptake by GLUTs and PFK1 activity. In testis, lactate is no longer considered a dead-end waste product of glycolysis, but an active metabolite that is known to be produced in high quantities by SCs and acts as a fuel for developing germ cells [16]. Therefore, as expected, intratesticular lactate concentration in HED rats was significantly increased. This is also concomitant with the significant increase in mRNA and protein levels of LDH, which is responsible for the interconversion of pyruvate derived from glucose metabolization into lactate [20]. Once produced by testicular cells, namely by SCs, lactate is exported through MCT4 [20]. Accordingly, MCT4 protein and mRNA levels were also increased suggesting that HED stimulates lactate production and export by testicular cells. The lactate produced by cells is derived from pyruvate that is at a crossroad between lactate and alanine. These interconversion reactions are crucial in testicular cells for the occurrence of a normal spermatogenesis [18]. Therefore, we evaluated the possible role for alanine in lactate intratesticular concentration. In fact, intratesticular alanine concentration was significantly decreased in HED group suggesting that this metabolite is also highly consumed for lactate production. This aminoacid is converted from pyruvate via transaminase reaction, catalyzed by ALT [73] and as expected HED rats presented an increase in ALT protein levels. Importantly, the lactate/alanine ratio is of great importance, and often used as an index of the redox state of tissues and cells[74], because it reflects the NAD^+/NADH ratio. The decreased levels of alanine together with the increased lactate concentration leads to an oxidized redox state, thus, an increased testicular lactate/alanine ratio in HED rats reflects a higher degree of overall testicular oxidative stress. Testicular cells often consume several other substrates than lactate and glucose. For instance SCs can consume a variety of substrates to maintain lactate production for the developing germ cells. Thus, we detected and quantified relevant metabolites that act as energy sources for testicular cells [20, 21]. Glycine was found to be significantly increased in HED rats. This aminoacid is an important precursor for the Krebs cycle and protein synthesis [46] and, as shown above, glycolysis was highly stimulated by HED and is the metabolic preferential pathway under these conditions thus explaining the increased levels of glycine. Besides, concomitant with this hypothesis, creatine levels increased in HED rats, although creatine can also be used as an energy source [75]. Recently we reported the hormonal control of acetate metabolism by sex steroids and insulin in cultured human SCs [21] and showed that under insulin deprivation acetate production is completely suppressed while E_2 favored acetate production and

dihydrotestosterone decrease its production. Thus, it would be expected that intratesticular acetate concentration could be decreased since insulin concentration was significantly decreased, but one cannot forget that SCs present a unique hormonal control of their metabolic profile [19] that may not be followed by other testicular cells. Also, this intermediate of fatty acids synthesis and cholesterol is important for spermatogenesis since its metabolism produces essential sub-products to maintain lipid synthesis and remodeling in developing germ cells. These are clear evidences that testes develop adaptive mechanisms to ensure an adequate microenvironment for germ cells development. Intratesticular metabolic flux has arisen as the one of the new challenges that reproductive biology faces in next century. In this study we describe important metabolic features and possible mechanisms within testes concerning the onset of a pre-diabetic state. Pre-diabetes is often associated an initial stage of development of DM and, moreover, it is well known that pre-diabetes can be reversed to normoglycaemia [76, 77]. To the best of our knowledge, this is the first report giving new insights over testicular metabolic mechanisms at this very earlier stage of DM development. Our results show that pre-diabetes may lead to subtle testicular metabolic changes, with altered sperm parameters. These results are of great significance and this topic should deserve special attention in future, since there is an increasing incidence of DM among young diabetic individuals and the reasons for subfertility/infertility associated to the early stages of this pathology remain to be clarified. Further knowledge on the functioning and regulation of these mechanisms, especially in early onset of the disease, will be essential in order to counteract the undesirable effects of DM on male reproductive function.

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Chapter 7

Pre-diabetes alters testicular PGC-1α/SIRT3 axis modulating mitochondrial bioenergetics and oxidative stress

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Pre-diabetes alters testicular PGC-1α/SIRT3 axis modulating mitochondrial bioenergetics and oxidative stress

Abstract

Pre-diabetes, a risk factor for type 2 diabetes development, leads to metabolic changes at testicular level. Peroxisome proliferators-activated receptor γ coactivator 1 α (PGC-1α) and Sirtuin 3 (SIRT3) are pivotal in mitochondrial function. We hypothesized that pre-diabetes disrupt testicular PGC-1α/SIRT3 axis, compromising testicular mitochondrial function. Using a high-energy-diet induced pre-diabetic rat model, we evaluated testicular levels of PGC-1α and its downstream targets, nuclear respiratory factor 1 (NRF-1) and 2 (NRF-2), mitochondrial transcription factor A (TFAM) and SIRT3. We also assessed mitochondrial DNA (mtDNA) content, mitochondrial function, energy levels and oxidative stress parameters. Protein levels were quantified by Western Blot, mtDNA content was determined by qPCR. Mitochondrial complexes activity and oxidative stress parameters were spectrophotometrically evaluated. Adenine nucleotides levels, adenosine and its metabolites (inosine and hypoxanthine), were determined by reverse-phase HPLC. Pre-diabetic rats showed increased blood glucose levels and impaired glucose tolerance. Both testicular PGC-1α and SIRT3 levels were decreased. NRF-1, NRF-2 and TFAM were not altered. Testicular mtDNA content was decreased. Mitochondrial complex I activity was increased, whereas mitochondrial complex III activity was decreased. Adenylate energy charge was decreased in pre-diabetic rats, as were ATP and ADP levels. Conversely, AMP levels were increased, evidencing a decreased ATP/AMP ratio. Concerning to oxidative stress pre-diabetes decreased testicular antioxidant capacity and increased lipid and protein oxidation. In sum, pre-diabetes compromise testicular mitochondrial function by repressing PGC-1α/SIRT3 axis and mtDNA copy number, declining respiratory capacity and increasing oxidative stress. This study gives new insights into overall testicular bioenergetics at this prodromal stage of disease.

Keywords: High-energy diet; Mitochondria; PGC-1α/SIRT3 axis; Pre-diabetes; Testicular bioenergetics

Introduction

Pre-diabetes is a major risk factor for the development of type 2 diabetes mellitus (T2DM) and is accompanied by elevated blood glucose levels, although not sufficient to meet the criteria for established diabetes [1]. It is characterized by impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT), and its prevalence is increasing among young people [1]. Every year, about 5–10% of the individuals with pre-diabetes become diabetic [2], and population habits may increase these rates. Moreover, in developed societies, the decrease of fertility rates has been associated with the increased incidence of diabetes mellitus (DM) [3]. Indeed, the effect of DM on male fertility has emerged as an alarming issue, further

exacerbated by the increasing number of children and adolescents with T2DM [4], which present a transition time between pre-diabetes and T2DM even shorter than adult individuals [5, 6]. “Western” lifestyle habits, such as overeating and sedentary life, have contributed to the increased infertility and subfertility prevalence associated to DM [7, 8]. Emerging evidence supports that dietary lifestyle affects male fertility. For instance, Attaman and collaborators [9] showed a moderate association between dietary fats and semen quality. Similarly, Jensen and collaborators [10] also reported that high fat intake affects sperm count and sperm concentration of Danish men. These results are also in line with data from animal studies [11, 12], supporting that saturated fatty acids intake is deleterious for male reproductive performance. The hyperglycemic state resultant from diabetic conditions contributes for an impaired reproductive function [13-15]. Recently our team reported that diet-induced pre-diabetes alters testicular metabolism, compromising sperm quality parameters, with a marked increase in abnormal sperm morphology [14]. However, little is known on the molecular mechanisms underlying male reproductive dysfunction in the diet-induced pre-diabetic state. Mitochondria are the best-known cellular powerhouses and form an interconnected network that is integrated with other cellular compartments. This organelle is of the great importance, since is essential for functional sperm, and thus, to ensure a normal spermatogenic event. Peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) is member of a small family of transcriptional regulators which controls the expression of genes involved in energy homeostasis, mitochondrial biogenesis, fatty acid oxidation and glucose metabolism [16, 17]. PGC-1α stimulates the expression of transcriptional regulators, the nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) that act on the nuclear genes coding for subunits of the oxidative phosphorylation (OXPHOS) system. Mitochondrial transcription factor A (TFAM) is a downstream target of both NRF-1 and NRF-2 that activates the transcription via sequence specific binding in the mitochondrial promoters [18]. TFAM gene presents consensus-binding sites for both NRF-1 and NRF-2 providing a unique mechanism for the cell integrates the expression of nuclear DNA-encoded proteins with the transcription of genes encoded by mtDNA [18]. Undoubtedly, PGC-1α is pivotal for mitochondrial function, as well as for the expression of key mitochondrial proteins, such as Sirtuin 3 (SIRT3) [16]. SIRT3 is a member of sirtuins family of NAD^+-dependent class III histone deacetylases and/or protein ADP-ribosyltransferases that mediate adaptive responses to a variety of stresses [19]. Among the seven members of the sirtuins family, SIRT3 is of particular interest concerning the mitochondrial function, as this protein is preferentially allocated in this organelle [19]. Similarly to PGC-1α, SIRT3 promotes metabolic reprogramming by activating enzymes involved in mitochondrial fuel catabolism [20]. SIRT3 modulates mitochondrial energy homeostasis by regulating ATP generation from OXPHOS [21] and interacts with the enzymatic complexes of the electron transport chain (ETC), resulting in increased activity of complexes and contributing to an efficient electron flow through ETC [22]. Interestingly, PGC-1α and SIRT3 act synergistically to maintain mitochondrial biogenesis, functional OXPHOS and an active ROS defense system [16]. It has been reported that PGC-1α

and SIRT3 are downregulated in the skeletal muscle of high-fat fed rats [23], and the lack of PGC-1α and/or SIRT3 favor oxidative stress, due to an imbalance between ROS and antioxidant defenses [24]. ROS overproduction damages mtDNA, which compromises the expression of OXPHOS genes resulting in a mitochondrial respiratory dysfunction. Herein we hypothesize that PGC-1α/SIRT3 axis could play a major role in testicular bioenergetic metabolism and ultimately in spermatogenesis of pre-diabetic individuals. Available information concerning mitochondrial testicular bioenergetics in DM is often associated with more advanced or severe stages of the disease [25] and there are no studies at the initial stages of this pathology. Thus, using a high-energy diet (HED) male rat model that develops a pre-diabetic state, we aimed to evaluate the effects of pre-diabetes on testicular expression of PGC-1α and its downstream targets SIRT3, NRF-1, NRF-2, TFAM and on testicular ETC function. We also evaluated the effects on testicular mtDNA integrity, testicular adenylate energy charge (AEC) and on particular oxidative stress parameters, such as antioxidant capacity, lipid peroxidation and carbonyl content.

Material and methods

Chemicals

DNeasy® Blood & Tissue kit (Cat. Nº 69504, Qiagen, Hilden, Germany), Maxima SYBR Green qPCR Master Mix (Fermentas, Vilnius, Lithuania), DCPIP (D1878, Sigma-Aldrich, St Louis, MO, USA), NADH (N6005, Sigma-Aldrich, St Louis, MO, USA); Coenzyme Q1 (C7956, Sigma-Aldrich, St Louis, MO, USA), Decylubiquinone (D7911, Sigma-Aldrich, St Louis, MO, USA), cytochrome c (C30398, Sigma-Aldrich, St Louis, MO, USA), n-Dodecyl ß-D-maltoside (D4641, Sigma-Aldrich, St Louis, MO, USA), protease cocktail inhibitor (P8340, Sigma-Aldrich, St Louis, MO, USA), all other chemicals were purchased at Sigma-Aldrich (St Louis, MO, USA), anti-SIRT3 polyclonal antibody was purchased to Cell Signaling (Beverly, USA), anti-PGC-1α, anti-TFAM and anti-NRF-1 were purchased to Santa Cruz Biotechnology, Inc. (USA), anti-NRF-2 was purchased to Abcam (Cambridge, UK). ECF™ substrate was purchased to GE, Healthcare (Orsay, France).

Animals

In the present study, we used 12 two-month-old male Wistar rats (Charles River Laboratories, Barcelona, Spain). The animals were housed in our accredited animal colony (Health Sciences Research Centre, University of Beira Interior) and maintained on ad libitum food and water, at constant temperature (20 ± 2ºC) and with a 12-hour cycle of artificial lighting. All animal experiments were performed according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the European rules for the care and handling of laboratory animals (Directive 86/609/EEC).

Animal model and experimental design

Rats were randomly divided (6 per group) in control and high-energy diet (HED) groups. In control group, animals were fed with a standard chow diet (4RF21 certificate, Mucedola, Italy), while HED group received an additional high-energy emulsion, as described elsewhere [14, 26-28]. Briefly, in the first 5 days of treatment, animals were given progressively 1 to 5 mL of emulsion by gavage consisting of 20 g lard oil, 1 g thyreostat, 5 g cholesterol, 1 g sodium glutamate, 10 g sucrose, 20 mL Tween 80, 30 mL propylene glycol, prepared in a final volume of 100 mL by adding distilled water. Then, they were administrated daily with 5 mL of the emulsion until reaching one month of treatment. Water was administered to the respective control group. Animal's blood glucose levels were monitored every 6 days. After treatment, animals were killed by cervical dislocation. Blood was collected by cardiac puncture to non-heparinized tubes. Testicles were removed, weighed and stored at -80º C. Non-fasting glycaemia was determined using a glucometer (One Touch Ultra Lifescan-Johnson, Milpitas, CA, USA) and insulin levels were determined using commercial rat EIA kits (Mercodia, Uppsala, Sweden), according to manufacturer instructions. At 3 months of age, animals were submitted to a glucose tolerance test, as described by Rato and collaborators [14]. Briefly, 14-18 hours before the test, food was removed and animals were kept in fast. An intraperitoneal injection with 6 mL glucose 30% (w/v) per kg of body weight was given to each animal. Blood glucose levels were measured at 30, 60, 90 and 120 min after glucose loading. The area under the curve for glucose tolerance (AUC_g) was calculated using trapezoidal rule.

Western Blot

Western Blot procedure was performed as previously described by Simões and collaborators [29]. The resulting membranes were incubated with rabbit anti-PGC-1α (1:1000, sc-13067, Santa Cruz Biotechnology), rabbit anti-NRF-1 (1:1000, sc-33771, Santa Cruz Biotechnology), goat anti-TFAM (1:500, sc-23588, Santa Cruz Biotechnology), rabbit anti-NRF-2 (1:1000, ab31163, Abcam), rabbit anti-SIRT3 (1:1000, C73E3, Cell Signaling Technology Inc.) or Mouse anti-tubulin (1:5000, T9026, Sigma-Aldrich) was used as protein loading control for testicular tissue. The immuno-reactive proteins were detected separately with goat anti-rabbit IgG-AP (1:5000, Sc2007, Santa Cruz Biotechnology) or goat anti-mouse IgG-AP (1:5000, Sc2008, Santa Cruz Biotechnology) or rabbit anti-goat IgG-AP (1:20000, A4187 Sigma-Aldrich). Membranes were reacted with ECF™ detection system (GE, Healthcare). The densities from each band were obtained using the Quantity One Software (Bio-Rad, Hemel Hempstead, UK), divided by the respective tubulin band density and then normalized against the respective control.

mtDNA relative copy number

Total DNA was extracted from tissues using the DNeasy® Blood & Tissue kit, according to the manufacturer's instructions. mtDNA relative copy number of the experimental groups was determined by qPCR analysis, as described by Wai and collaborators [30] with slight

modifications. Relative quantification of mtDNA levels was determined by the ratio between the mitochondrial ND1 (mtND1) gene and the single-copy, nuclear-encoded beta-2-microglobulin (ß2MG) gene. Reactions were carried out in an iQ5 system (Bio-Rad, Richmond, CA, USA), and the efficiency of the reactions was determined for the selected primers using serial dilutions of DNA samples. The specificity of the amplicons was determined by melting curve analysis. The reactions mixture consisted of Maxima SYBR Green qPCR Master Mix, 200 nM of each sense and antisense primers (see Table 7.1 for details) and 20 ng of DNA. Each reaction was run in triplicate to calculate relative mtDNA copy number. Ct values of all samples were within the linear range. Ct value differences were used to quantify mtDNA copy number relative to the ß2MG gene with the following equation: Relative copy number = $2^{\Delta Ct}$, where ΔCt is $Ct_{ß2MG}$-Ct_{ND1}.

Table 7.1. Oligonucleotides and cycling conditions for qPCR amplification of ND1 and ß2-microglobulin. Abbreviations: AT - annealing temperature.

Gene	Sequence (5'- 3')	AT (°C)	Amplicon Size (bp)
ND1	Sense: GAG CCC TAC GAG CCG TTG CC Antisense: GCG AATG GTC CTG CGG CGTA	58	271
ß2MG	Sense: GCG TGG GAG GAG CAT CAG GG Antisense: CTCATCACCACCCCGGGGACT	58	264

Citrate synthase activity

Citrate synthase activity was by modification of a method previously described by Core and collaborators [31]. Briefly, testicular tissue was homogenized using lysis buffer (250 mM Sucrose, 5 mM HEPES, pH 7.4). Protein concentration was determined by the Bradford micro assay using BSA (bovine serum albumin) as a standard. 25 µg of tissue homogenate were incubated at 37ºC in a reaction buffer containing 100 mM Tris pH 8.0 plus 200 µM Acetyl-CoA, 200 µM 5,5'-dithiobis-2-nitrobenzoic acid. Enzymatic activity was determined in a VICTOR X3 plate reader (Perkin-Elmer Cetus, Norwalk, CO, USA), at 37ºC, by following the increase in absorbance (412 nm) upon addition of 100 µM freshly-prepared oxaloacetate. Enzyme activity was calculated through the mean of the slopes of duplicates obtained during the linear phase. Citrate synthase-specific activity was calculated by subtracting the basal activity in presence of 0.1% Triton-X100. A molar extinction coefficient of ε_{412}=13.6 $mM^{-1}.cm^{-1}$ and normalization to protein amount were applied. Enzyme activity was expressed as nmol of oxaloacetate min^{-1}.mg $protein^{-1}$.

NADH-ubiquinone oxidoreductase activity

Complex I activity was determined by modification of a method previously described by Long and collaborators [32]. Briefly, 30 µg of tissue homogenate (obtained as previously described) were diluted in reaction buffer containing 25 mM KH_2PO_4 pH 7.5, 5 mM $MgCl_2$, 300 µM KCN, 4 µM antimycin A, 3 $mg.mL^{-1}$ BSA, 60 µM coenzyme Q1, 160 µM 2,6-dichlorophenolindophenol

(DCPIP). Complex I activity was measured at 37ºC, in a VICTOR X3 plate reader, by following the decrease in absorbance (600 nm) of DCPIP upon addition of 100 µM freshly-prepared NADH. Enzyme activity was calculated through the mean of slopes of duplicates, obtained during the linear phase. Mitochondrial complex I specific activity was determined as the difference between basal activity in the absence or presence of 10 µM rotenone (specific inhibitor of complex I). A molar extinction coefficient of ε_{600}=19.1mM^{-1}.cm^{-1} and normalization to protein amount were applied. Complex I activity was expressed as nmol DCPIP min^{-1}.mg protein^{-1}.

Succinate-cytochrome c reductase activity

Complex II/III activity was determined by modification of a method previously described by Tisdale [33]. Briefly, 100 µg of tissue homogenate (obtained as described above) were preincubated for 5 min, at 37ºC, in 200 µl of phosphate buffer (166 mM KH_2PO_4, 166 mM K_2HPO_4, pH 7.4) supplemented with 100 mM KCN and 500 mM sodium succinate. The reaction was initiated by the addition of 120 µl of phosphate buffer supplemented with 2 mM oxidized cytochrome c (cyt c_{ox}) plus 15 mM EDTA-dipotassium. Enzyme activity was calculated through the mean of slopes of duplicates, obtained during the linear phase. Complex II/III activity was measured by following the reduction of cyt c_{ox} (increased absorbance at 550 nm), using a VICTOR X3 plate reader. Mitochondrial complex II/III specific activity was determined as the difference between basal activity in the absence or presence of 4 mM antimycin A (specific inhibitor of complex III). A molar extinction coefficient of ε_{550}=19.1 mM^{-1}.cm^{-1} and normalization to protein amount were applied. Results were express as nmol cyt c_{ox} min^{-1}.mg protein^{-1}.

Cytochrome c reductase activity

Complex III activity was determined by modification of a method previously described Luo and collaborators [34]. Briefly, 100 µg of tissue homogenate (obtained as previously referred) were incubated in reaction buffer containing 25 mM KH_2PO_4 pH 7.5, 4 uM rotenone, 0.025% Tween-20, 100µM fresh decylubiquinone solution at 37ºC. Enzymatic activity was followed by an increase in absorbance of cyt c_{ox} at 550 nm, upon addition of 75 µM cyt c_{ox} in a VICTOR X3 plate reader. Enzyme activity was calculated through the mean of slopes of duplicates, obtained during the linear phase. Mitochondrial complex III specific activity was determined as the difference between basal activity in the absence or presence of 2.5 mM antimycin A (complex III specific inhibitor). A molar extinction coefficient of ε_{550}=19.1mM^{-1}.cm^{-1} and normalization to protein amount were applied to express the activity as nmol cyt c_{ox} min^{-1}.mg protein^{-1}.

Cytochrome c oxidase activity

Complex IV activity was determined by modification of a method previously described by Brautigan and collaborators [35]. Briefly, 25 µg of tissue homogenate (obtained as previously

described) were incubated at 37ºC, in reaction buffer containing 50 mM KH_2PO_4 pH 7.0, 4 µM antimycin A, 0.05% n-dodecyl-ß-D-maltoside. Enzymatic activity was followed by a decrease in absorbance of reduced cytochrome c (cyt c_{red}) at 550 nm, upon addition of 57 µM freshly-prepared cyt c_{red} in a VICTOR X3 plate reader. Enzyme activity was calculated through the mean of slopes of duplicates, obtained during the linear phase. Mitochondrial complex IV specific activity was determined as the difference between basal activity in the absence or presence of 10 mM of KCN (complex IV specific inhibitor). A molar extinction coefficient of ε_{550}=19.1mM^{-1}.cm^{-1} and normalization to protein amount were applied. Activity was expressed as nmol cyt c_{red} min^{-1}.mg protein^{-1}.

Analysis of adenine nucleotides and adenosine metabolites

Adenine nucleotides and adenosine metabolites levels were measured according to previously described methods Rego and collaborators [36]. Briefly, testicular tissue was homogenized in lysis buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, pH 7.4) supplemented with 1% protease inhibitor cocktail, 1 mM DTT and 1 mM PMSF and centrifuged at 14000 xg, for 2 min at (0-4°C). After neutralization with 3 M KOH in 1.5 M Tris, samples were centrifuged at 14000 x g for 2 min (0–4°C). The resulting supernatants were used to determine protein concentration by the Bradford micro assay using BSA as a standard. The supernatant were assayed for ATP, ADP, AMP, adenosine and its metabolites (inosine and hypoxanthine), by separation in a reverse-phase high-performance liquid chromatography (HPLC), as described by Stocchi and collaborators [37]. The chromatographic apparatus used was a Beckman-System Gold (Beckman Instruments, Fullerton, USA), consisting of a 126 Binary Pump Model and 166 Variable UV detector, controlled by a computer. The detection wavelength was 254 nm, and the column used was a Lichrospher 100 RP-18 (5 µm) from Merck (Darmstadt, Germany). An isocratic elution with 100 mmol/l phosphate buffer (KH_2PO_4; pH 6.5) and 1.0% methanol was performed with a flow rate of 1 ml/min. The required time for each analysis was 6 min. Peak identity was determined by the retention time compared with standards. The amounts of nucleotides and metabolites were determined by a concentration standard curve. Testicular adenylate energy charge (AEC) was determined according the formula: ATP+0.5x ADP/(ATP+ADP+AMP).

Ferric reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) of the media samples was performed according to the colorimetric method described by Benzie and Strain [38]. Briefly, testicular tissue was homogenized in phosphate buffer (pH 7.4). Protein concentration was determined by the Bradford micro assay using BSA as a standard. Working FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), 2,4,6-Tripyridyl-s-Triazine (TPTZ) (10 mM in 40 mM HCl) and $FeCl_3$ (20 mM) in a 10:1:1 ratio (v:v:v). 180 µL of this reagent were mixed with 10 µg of tissue homogenate. The reduction of the Fe^{3+}-TPTZ complex to a colored Fe^{2+}-TPTZ complex by the samples was monitored immediately after adding the sample and 40 min later, by measuring

the absorbance at 595 nm using an Anthos 2010 microplate reader (Biochrom, Berlin, Germany). Antioxidant potential of the samples was determined against standards of ascorbic acid, which were processed in the same manner as the samples. Absorbance results were corrected by using a blank, with H_2O instead of sample.

Thiobarbituric acid reactive species assay

Thiobarbituric acid reactive species (TBARS) are formed as a byproduct of lipid peroxidation, which can be detected by the TBARS assay using thiobarbituric acid (TBA) as a reagent. This peroxidation reaction produces malonaldehyde (MDA) that reacts with TBA in conditions of high temperature and low pH, generating a pink colored complex, which absorbs at 532 nm [39]. TBARS assay was carried out by the method described by Iqbal and collaborators [40] with slight modifications. Testicular tissue was homogenized in phosphate buffer (pH 7.4). Protein concentration was determined by the Bradford micro assay using BSA as standard. Briefly, 20µg of tissue homogenate, 0.01 mL Tris-HCl buffer (150 mM, pH 7.1), 0.01 mL ferrous sulphate (1.0 mM), 0.01 mL ascorbic acid (1.5 mM) and 0.06 mL H_2O were mixed in a reaction tube. This mixture was incubated at 37ºC for 15 min. The reaction was stopped by addition of 0.1 mL of trichloroacetic acid (10% w/v). Subsequently, 0.2 mL of TBA (0.375% w/v) were added and all samples were incubated for 15 min at 100ºC. Finally, samples were centrifuged at 1000 x g for 10 min. The amount of MDA formed in each sample was estimated by measuring optical density at 532 nm using a UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan) against a blank. The results were expressed as nmol of TBARS/mg protein.

Analysis of carbonyl groups

Protein carbonyl content is commonly used as a marker for protein oxidation. To evaluate protein carbonyl groups a Slot Blot was performed. First, samples were derivatized using 2,4-dinitrophenylhydrazine (DNPH) according to the method developed by Levine and collaborators [41]. Briefly, a volume containing 40µg of lyophilized testicular tissue homogenized in phosphate buffer was mixed with the same volume of SDS 12% and centrifuged to minimize nucleic acid interference in the assay. The samples were then mixed with two volumes of DNPH 20mM diluted in TFA 10% and incubated for 30 min in a dark environment. The reaction was stopped using 1.5 volumes of Tris 2M diluted in ß-mercaptoethanol. Samples were then diluted to a concentration of 0.001µg/µL using phosphate buffer. A previously activated polyvinylidenedifluoride membrane was used in the Slot-Blot, which was performed in a Hybri-slot manifold system (Biometra, Germany). The membranes were then incubated for 90 min with a 5% non-fat milk solution. Afterwards, membranes were incubated overnight with rabbit anti-DNP antibody (1:5000 D9656; Sigma Aldrich). Samples were visualized using anti-rabbit IgG peroxidase. Membranes were then reacted with ECF^{TM} substrate (GE, Healthcare) and read using a BioRad FX-Pro-plus (Bio-Rad, UK). Densities from each band were quantified using the BIO-PROFIL Bio-1D Software from Quantity One (VilberLourmat, Marne-la-Vallée, France).

Statistical analysis

The statistical significances of the differences between all experimental data were assessed by unpaired Student t-test (GraphPad Software, San Diego, CA, USA). All experimental data are shown as mean ± S.E.M of the indicated number of independent experiments. P<0.05 was considered significant.

Results

High-energy diet fed rats developed mild hyperglycemia, glucose intolerance and hypoinsulinemia

HED rodent model was developed as previously described by our team [14, 42]. At the end of HED treatment, animals presented mild hyperglycemia (Figure 7.1A). Moreover, HED rats had significantly increased (by ~57%) AUC_g values compared to control group (Figure 7.1B), evidencing that HED rats developed significant glucose intolerance. These results suggested an insulin dysfunction status, so we measured fasting blood insulin levels and, as expected, insulin levels of HED rats were significantly decreased (by ~61%) when compared to control group (Figure 7.1C). These characteristics, particularly glucose intolerance and mild-hyperglycaemia, indicated that HED animals developed a pre-diabetic state.

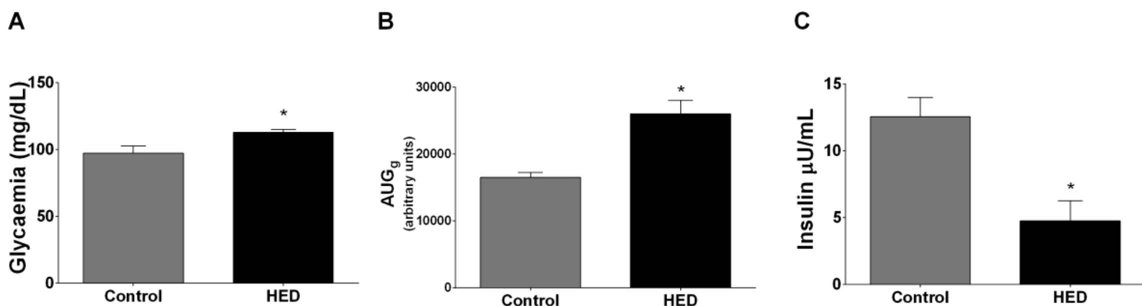


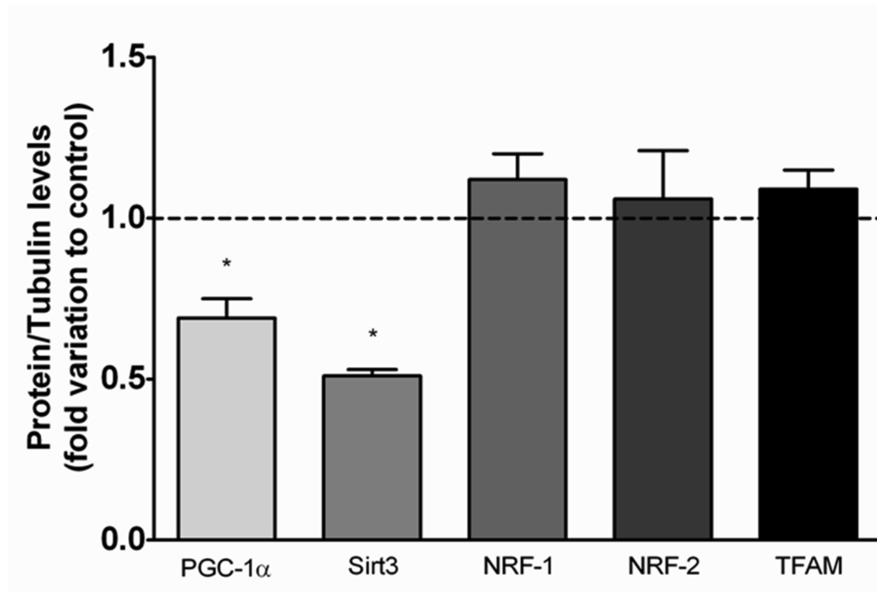
Figure 7.1. A) Blood glucose levels of Control group and HED group animals after 30 days of treatment. B) AUCg of the intraperitoneal glucose tolerance test performed in Control group and HED group animals. C) Insulin levels of Control group and HED group animals after 30 days of treatment. Results are the means ± SEM of six independent experiments, corresponding to six animals/group. *P<0.05 vs. control group. HED – high-energy diet. AUCg – area under the curve for glucose tolerance test.

Pre-diabetes compromises testicular mitochondrial biogenesis by decreasing PGC-1α protein levels

High-energy diets are known to downregulate PGC-1α protein levels [23], thus we evaluated the effects of pre-diabetes in testicular PGC-1α protein levels and we observed a 1.53-fold reduction in HED animals when compared to control group (Figure 7.2A and B). As PGC-1α is considered the key regulator of mitochondrial biogenesis, the decreased levels of PGC-1α may compromise this cellular process, so we quantified the protein levels of its downstream targets: NRF-1, NRF-2 and TFAM. The obtained results showed no differences between groups

for both nuclear transcriptors NRF-1 and NRF-2, as well as, the key activator of mitochondrial transcription and mtDNA replication, TFAM (Figure 7.2A and B).

A



B

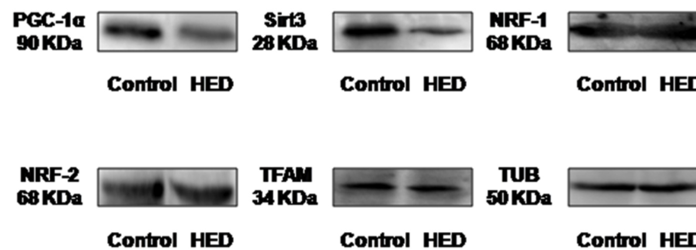


Figure 7.2. Effect of high-energy diet (HED) on testicular PGC-1α, SIRT3, NRF-1, NRF-2 and TFAM protein (Part A) levels. Part B represents an illustrative Western blot experiment. Results are the mean ± SEM of five independent experiments, corresponding to five animals/group and performed in triplicate. *P<0.05 vs. control group.

Pre-diabetes significantly decreased testicular levels of Sirtuin 3

In addition, since PGC-1α is crucial for the expression of SIRT3, we further quantified the protein levels of this mitochondrial deacetylase. SIRT3 is the most important deacetylase that modulates mitochondrial metabolism and oxidative stress [24]. SIRT3 protein levels were decreased by 2-fold in HED animals when compared to control group (Figure 7.2A and B). The decreased levels of testicular SIRT3 may contribute to a compromised testicular mitochondrial function and increased oxidative stress, so we further assessed mitochondrial function and oxidative stress parameters.

Pre-diabetes significantly decreased mtDNA relative copy number

mtDNA comprises genes encoding for polypeptides that constitute the multi-subunit enzyme complexes of the respiratory chain. Alterations in mtDNA content have been associated to disturbances in the activity of ETC [43]. Therefore, we determined the effects of HED on testicular tissue mtDNA content. We observed a 38.8% decrement in mtDNA content in HED rat testicles compared to control group (Figure 7.3A). This suggests that mitochondrial respiratory function may be affected in these conditions. Thus, we determined ETC complexes activity in order to evaluate possible testicular mitochondrial bioenergetics alterations induced by pre-diabetes.

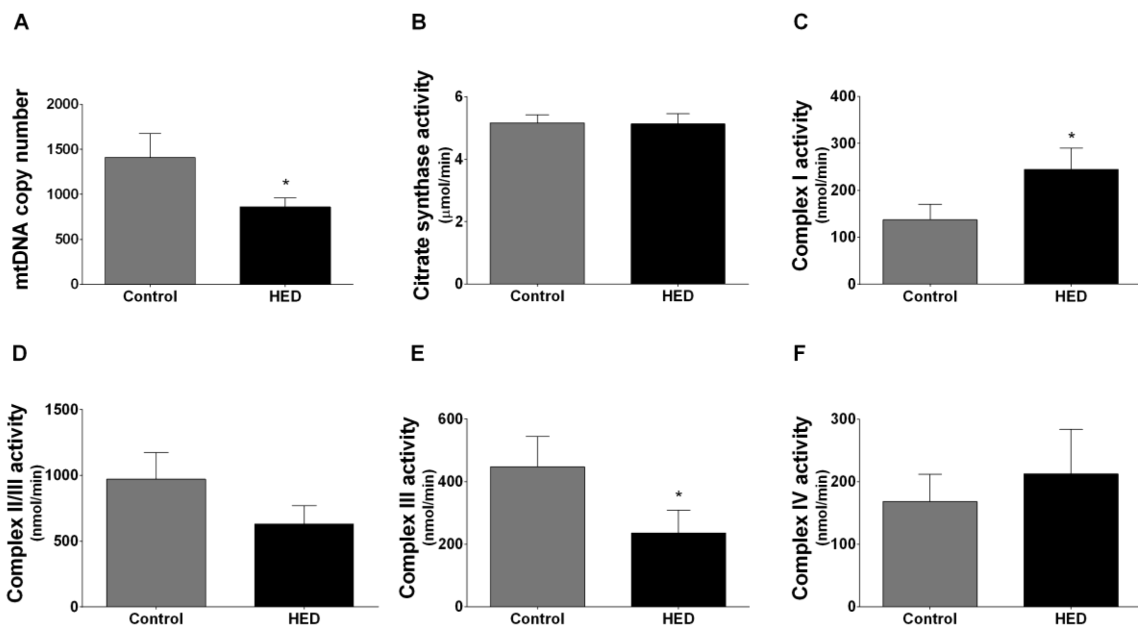


Figure 7.3. A) Relative mtDNA copy number in control group and HED group. B) Citrate synthase activity; C) NADH reductase (Complex I) activity; D) succinate cytochrome c reductase (Complex II/III) activity; E) cytochrome c reductase (Complex III) activity; F) cytochrome c oxidase (Complex IV) activity. Results are the mean ± SEM of five independent experiments, corresponding to five animals/group and performed in triplicate. *P<0.05 vs. control group.

Mitochondrial complex I and complex III activities were altered in pre-diabetic rats

Mitochondrial function is highly dependent the activities of several mitochondrial enzymes, such as citrate synthase and respiratory complexes I to IV. Citrate synthase activity is a marker for testicular mitochondrial content and integrity [31]. In this regard, we did not find significant differences in testicular tissue from animals of both groups (Figure 7.3B).

We also evaluated the enzymatic activities of mitochondrial respiratory complexes I to IV (Figure 7.3C-7.3F). Despite no significant differences in both succinate cytochrome c reductase (complex II-III) and cytochrome c oxidase (complex IV) activities in testicular tissue from animals of both groups (Figure 7.3D and 7.3F), NADH reductase (complex I) activity was increased by 79% in HED rat testicles compared to control (Figure 7.3C). Testicular cytochrome c reductase (complex III) activity of HED rats was decreased from 448 ± 97 nmol/min (in control rats) to 236 ± 73 nmol/min (Figure 7.3E).

Testicular adenylate energy charge was significantly decreased by pre-diabetes

Testicles are organs with high-energy demands and the AEC is often used to evaluate overall energetic status of cells and tissues [44, 45]. Thus, we analyzed the HED effect in adenine nucleotides metabolism and in testicular AEC. ATP content was 39% lower in testicles of HED than in control rats (Figure 7.4A). Likewise, ADP content in testicular tissue of HED rats showed a significant decrease (by 56%) when compared with control animals (Figure 7.4B). Conversely, testicular AMP content was increased by 89% in HED rats (Figure 7.4C). As a consequence of the lower levels of ATP and the significant increase in AMP content, lower ATP/AMP ratio in testicles of HED rats when compared with control animals (Figure 7.4D) was detected.

As expected, the overall testicular AEC was 3.4-fold decreased in HED rats when compared to control animals (Figure 7.4E). The lower testicular AEC level in HED rats points to low energy levels in testicular milieu, with a high accumulation of AMP.

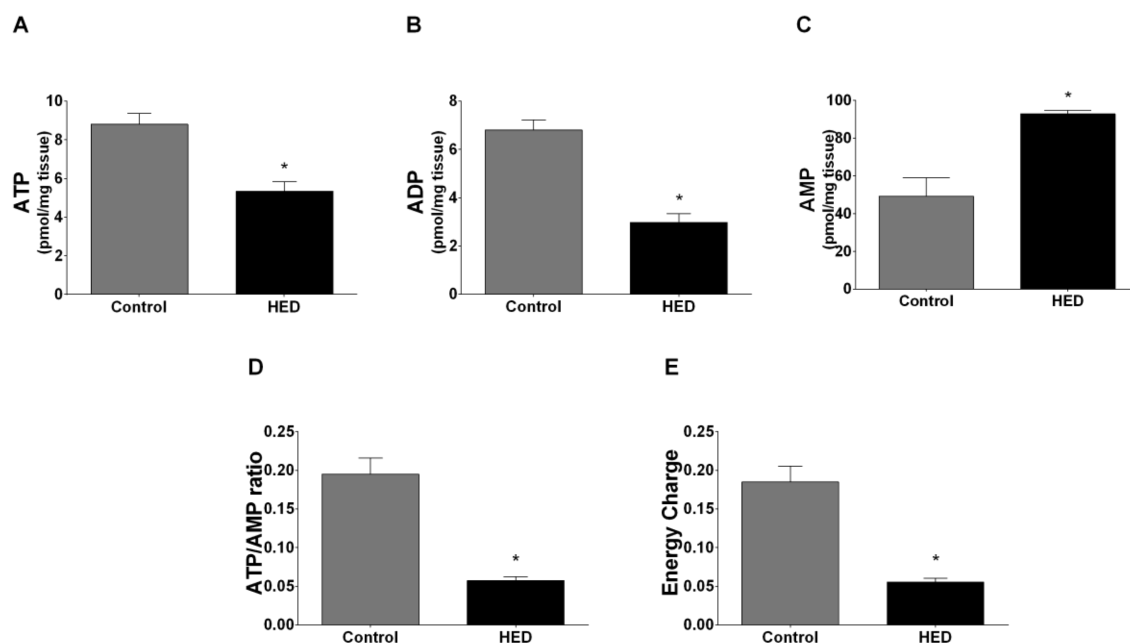


Figure 7.4. Testicular adenine nucleotides levels in control group and HED group. A) ATP; B) ADP; C) AMP; D) ATP/AMP ratio; E) Testicular adenylate energy charge. Results are expressed as mean ± SEM of five independent experiments, corresponding to five animals/group and performed in duplicate. *P<0.05 vs. control group.

Pre-diabetes alters testicular adenosine metabolites

The lower testicular ATP/AMP observed in HED rats lead us to hypothesize that ATP metabolism should be progressing to adenosine formation. In fact, the conversion of AMP to adenosine is a final common step in the catabolism of the adenine nucleotides [46]. Herewith, we did not observed significant differences on the testicular adenosine levels between animals from both groups (Table 7.2). Interestingly, we found that inosine content was decreased by 54% in testicles of HED rat compared with the animals from the control group (Table 7.2), while hypoxanthine content raised by 24% in HED animals compared to control

animals (Table 7.2). These results suggest that pre-diabetes favors adenine nucleotides metabolism in which AMP is converted to adenosine subproducts, namely hypoxanthine, suggesting an oxidative state.

Table 7.2. Average values of the testicular adenosine metabolites measured in Control group and in HED group. HED: high-energy diet. Results are expressed as means ± SEM (n = 5 for each condition). *Significantly different relative to control ($p < 0.05$).

Adenosine Metabolites	Control Group	HED group
Adenosine (pmol/mg tissue)	7.34 ± 0.72	6.74 ± 0.26
Inosine (pmol/mg tissue)	49.89 ± 2.21	23.19 ± 4.88*
Hypoxanthine (pmol/mg tissue)	137.07 ± 3.17	170.97 ± 5.25*

Pre-diabetes favors testicular stress oxidative environment

To evaluate the effects of pre-diabetes on testicular antioxidant capacity we utilized the FRAP assay. The FRAP assay measures the potential to reduce ferric (III) to ferrous (II) in a redox-linked colorimetric reaction that involves single electron transfer [38]. This reducing power serves as a significant indicator of the potential antioxidant activity (FRAP value). The results showed that antioxidant capacity was decreased by 55% in testicles of HED animals when compared to control group (Figure 7.5A). The decreased antioxidant capacity in HED rat testicles may favor a high testicular ROS activity.

Testicles present high lipid content, which are known to be a major target of ROS. Increased testicular ROS activity may lead to lipid peroxidation, therefore we evaluated the TBARS content in testicular homogenates as a measure of lipid peroxidation. We observed a significant increase of testicular TBARS content from 0.0166 nmol/mg tissue (in control group) to 0.0498 nmol/mg tissue in HED group (Figure 7.5B). These results demonstrate a 3-fold increase on testicular lipid peroxidation levels in testicles of HED-fed rats.

As it occurs for lipids, proteins are also sensitive to oxidation by ROS. The evaluation of protein carbonyl groups content has been widely used as biomarker of protein damage by oxidative stress [47]. As pre-diabetic animals presented an alteration on testicular antioxidant capacity, we evaluated the effect of pre-diabetes on testicular protein oxidation. Hence, animals from the HED group exhibited a 26% increase of testicular carbonyl content when compared to control group (Figure 7.5C and 7.5D).

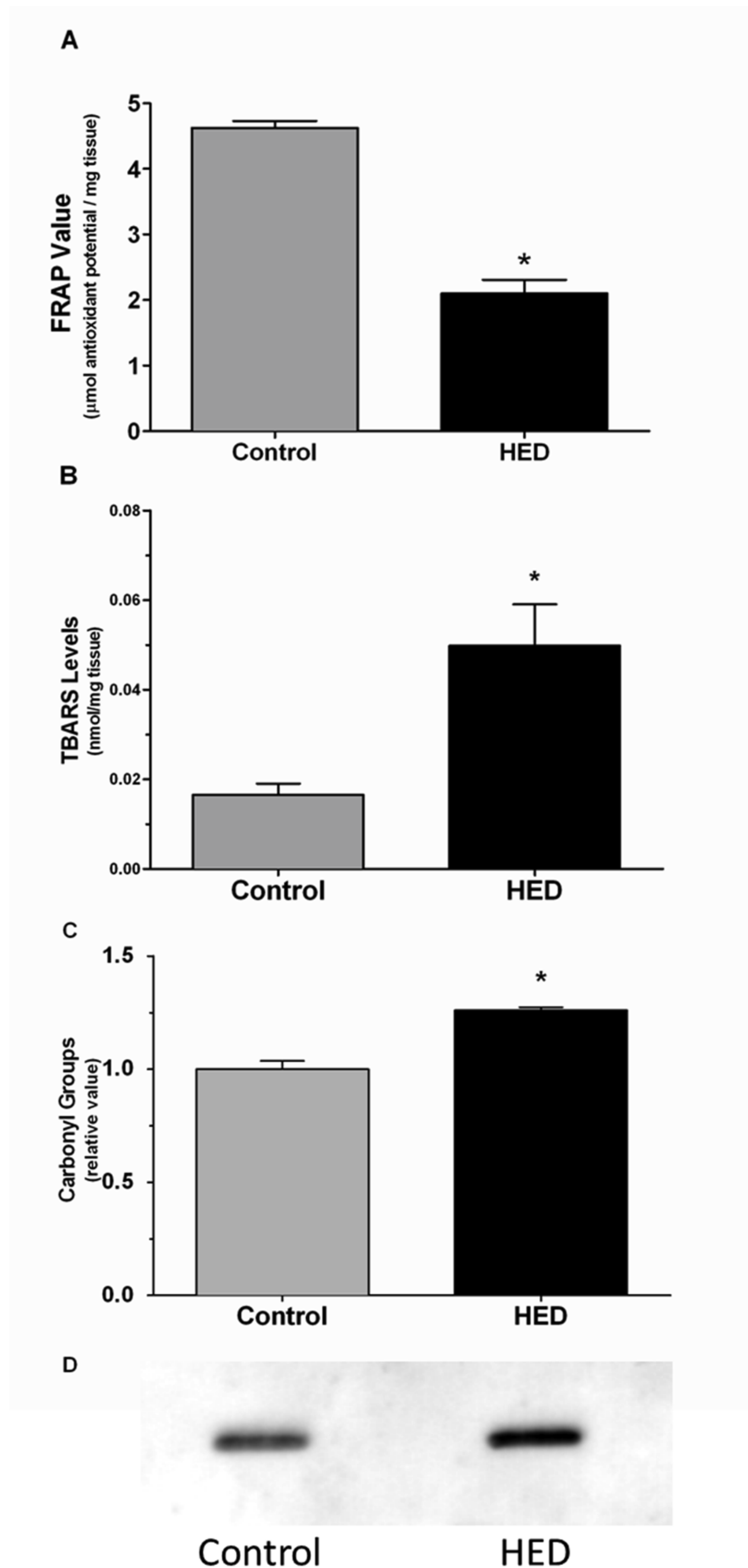


Figure 7.5. A) Testicular antioxidant capacity in control group and HED group. B) Testicular lipid peroxidation in control group and HED group. C) Testicular carbonyl content in control group and HED group. D) Illustrative representation of Slot blot experiment. Results are expressed as mean ± SEM of five independent experiments, corresponding to five animals/group and performed in duplicate. *P<0.05 vs. control group.

Discussion

Pre-diabetes is a pathological state that includes some (but not all) diagnostic criteria for DM [1], being characterized by IFG and/or IGT and by mild hyperglycemia [2]. In the present study, a HED-induced rodent model was used to evaluate the effects of this prodromal stage of DM in testicular PGC-1α/SIRT3 axis, as well as in mitochondrial bioenergetics and oxidative stress. As described by our team, this HED-fed rodent model displayed the characteristics of a pre-diabetic state as reported in other studies using the same animal model [14, 26]. As expected, the HED fed animals developed mild hyperglycaemia and glucose intolerance, which are the two main characteristics of a pre-diabetic state. The mild hyperglycaemia observed in the HED animals did not meet the criteria for established DM [48]. However, those conditions together with the lower insulin levels, point toward a higher risk for DM [49, 50]. The observed decrease in insulin levels may be caused by β-cell pancreatic failure. This hypothesis is supported by studies from Blazquez and Quijada [51], which observed hypoinsulinemia in high-fat fed animals. In fact, β-cells may present certain susceptibility to lipids [52], thus the dietary fats used in HED may induce β-pancreatic cell failure, decreasing insulin secretion. Furthermore, impaired insulin secretion has also been described in adolescents with pre-diabetes, particularly those with a higher BMI, presenting an higher risk for progression to T2DM [53]. The well-functioning of the PGC-1α/SIRT3 axis has been reported as essential for the regulation of mitochondrial metabolism, biogenesis and oxidative stress [16, 54]. PGC-1α arises as a key regulator of mitochondrial function, since it co-activates several nuclear transcriptors, which in turn regulate the expression of nuclear encoded mitochondrial proteins. PGC-1α is also essential for the expression of SIRT3. Recently Kong and collaborators [16] showed that PGC-1α knockdown effectively reduces SIRT3 expression in in muscle cells and hepatocytes, which regulates important mitochondrial functions by deacetylating several metabolic and respiratory enzymes [19, 22]. Disturbances of PGC-1α levels have been linked to the development of DM [55]. Furthermore, it has also been reported that SIRT3 is decreased by high-fat consumption [56, 57], as well as under diabetic conditions [24], which is concomitant with PGC-1α decrease [23]. In our work we identified the expression of PGC-1α and SIRT3 on the testicles of 3-month old Wistar rats. Importantly, we found that at testicular level both the PGC-1α and SIRT3 protein levels were significantly decreased on the animals of the HED group. This decrease in PGC-1α levels may be deleterious for testicular mitochondrial physiology, particularly because this effect can be exacerbated by downregulation of SIRT3. Our results suggest that the reduced expression of PGC-1α compromises the expression of SIRT3 in the testicles of HED-fed rats. Although, to the date, there are no direct reports demonstrating that down-regulation of PCG-1α leads to reduced SIRT3 levels within testicular milieu, Kong and collaborators recently described a molecular mechanism for Sirt3 expression, where PGC-1α functions as an upstream activator of Sirt3 gene expression in muscle cells and hepatocytes, having a stimulatory effect on Sirt3 promoter [16]. The results obtained herein suggest that the reduced expression of PGC-1α compromise the expression mechanism of SIRT3, thus decreasing testicular SIRT3 content. So,

in these conditions the expression of testicular SIRT3 may be downregulated at transcriptional level, leading to a decreased SIRT3 protein levels.

Decreased levels of PGC-1α and SIRT3 are known to modulate mitochondrial metabolic activity and oxidative stress regulatory pathways activation [19, 56]. We have previously reported that the high glycolytic flux evidenced by HED animals favored an oxidative environment within testicles [14], which could compromise cellular integrity. In this context, PGC-1α is required to the induction of ROS-detoxifying enzymes under oxidative stress conditions [16], whereas PGC-1α knockdown blunts antioxidant capacity [58]. Recently Kong and collaborators [16] showed that PGC-1α acts as a ROS suppressor by increasing the expression of gluthatione peroxidase 1 and superoxide dismutase 2 [54, 59]. Additionally, it has also been reported that SIRT3 induces the expression of antioxidant defenses [60], thus mediating the effects of PGC-1α on ROS levels. This highlights the synergistic role between PGC-1α and SIRT3 in the control of the oxidative stress status [16]. Indeed, HED-fed rats presented a deficient testicular antioxidant capacity, which may result in part from the significant deregulation of the PGC-1/SIRT3 axis, thus enhancing testicular ROS overproduction. Our results show an increased level of carbonyl content and TBARS in testicles of HED rats, evidencing a higher testicular susceptibility to oxidation associated with pre-diabetes. In fact the decreased levels of SIRT3 may contribute to this, since it has been reported that SIRT3 knockout animals present highest levels of hepatic and neuronal lipid peroxidation and protein carbonylation [61]. Additionally, we observed a significant decrease in testicular mtDNA content in HED-fed rats, further evidencing that the pre-diabetic condition is deleterious for the mitochondrial function. The mtDNA content loss in this case is likely related to mitochondrial biogenesis, and PGC-1α is connected with the co-activation of several nuclear transcriptors such as NRF-1, NRF-2 and TFAM, directly involved in the signaling pathways that lead to mtDNA replication [62]. Despite of NRF-1, NRF-2 and TFAM are PGC-1α responsive genes, the unaltered levels of testicular NRF-1, NRF-2 and TFAM between HED-fed and control rats might involve compensatory mechanisms that are known to be triggered under oxidative stress conditions in order to maintain mitochondrial biogenesis as those described in hepatic oxidative conditions [63]. Furthermore, though protein levels were not altered, it is expectable that their activities are downregulated, since it has also been suggested that SIRT3 is capable of regulating the activities of NRF1 and TFAM, which localize in the mitochondria [16]. Additionally, mtDNA is also highly susceptible to oxidative stress, partly due to its localization. mtDNA is packaged into a protein-DNA complex known as a mitochondrial nucleoid, however due to the imbalance between ROS scavenging and overproduction, which is exacerbated by disruption of PGC-1α/SIRT3 axis, the composition of mtDNA nucleoid may be severely compromised thus contributing to mtDNA degradation within the testicular milieu of pre-diabetic rats. This is supported by the fact that PGC-1α and SIRT3 are essential for ROS-detoxifying system and downregulation of both proteins decrease the antioxidant defenses leading to compromised mtDNA integrity [23, 64]. It has also been described that DM also affects mitochondrial function by impairing ETC complexes enzymatic

activities [65, 66]. Although it has been reported that citrate synthase activity is diminished under diabetic conditions [65, 67], we failed to observe significant differences on testicular citrate synthase activity in HED-fed rats and thus suggesting that testicular mitochondrial integrity may be unaltered. This may be explained by the fact that our animal model was in a prodromal stage of DM, not suffering the co-morbidities related with the later stages of the disease [68]. On the other hand, we found that HED fed rats presented a significant increase in testicular NADH reductase (complex I) activity. This observation is inconsistent with previous report, using a knockout model for SIRT3, that demonstrated reduced complex I activity [21]. Indeed, knockout models fail to show the molecular and cellular adaptations that might occur in wild type animals. Testicles are organs with high-energy demands and sperm are highly dependent on aerobic metabolism [69], so the increased mitochondrial complex I activity might be a compensatory mechanism by testicular mitochondria in response to metabolic changes caused by pre-diabetic state, in order to maintain a correct electron transport chain function and guarantee an adequate energy supply to the spermatogenic event. Furthermore, the observed increase in mitochondrial complex I activity in HED animals is consistent with the reported increase of mitochondrial oxidative capacity observed in other diet-induced animal models [70]. After NADH oxidation, the electrons flow sequentially through cytochrome c reductase (complex III), which funnels electrons from the coenzyme Q pool to cytochrome c [71]. The significant decrease observed in mitochondrial complex III activity of HED-fed rat testicles may be caused by the decreased levels of SIRT3, since complex III is one of the several main targets of this deacetylase protein [22]. The decoupling in complex I and complex III activities reverse the electron flow, favoring ROS production and impairing ATP synthesis, which could further exacerbate the pro-oxidative environment previously described in the testicles of HED rats by our team [14]. Disruption of PGC-1α/SIRT3 axis presented by pre-diabetic animals induces important changes in testicular ETC function, particularly at the level of complex I and complex III. The observed changes in ETC is a consequence of both PGC-1α and SIRT3 deregulation and whole testicular metabolic fluctuations [14], since testicles are compartmentalized organs presenting special metabolic characteristics and energy demands that confer a special microenvironment to testicular milieu [72-75]. Although it has been reported that DM can severely affect testicular ATP production [15], to our knowledge the testicular energetic status in pre-diabetic individuals remains undisclosed. The significant decrease in testicular ATP levels of HED-fed rats reported herein results from the disruption of PGC-1α/SIRT3 axis with a consequent impairment of ETC function. Moreover, such decrement in testicular ATP content may arise from its hydrolysis to subsequent adenine nucleotides (ADP and AMP) and adenosine metabolites. This hypothesis is further supported by the lower ADP levels we observed in HED-fed rats, suggesting that adenylate kinase reaction could be operating towards AMP production. Accordingly, testicular AMP levels were significantly increased in HED rats. Noteworthy, the imbalance in adenine nucleotides underlies a lower testicular ATP/AMP ratio, evidencing a decreased testicular AEC in HED rats. In these conditions, AMP may arise

as a key regulatory molecule, enhancing catabolic pathways, such as glycolysis [76]. Regarding the hypoxanthine accumulation reported in testicles from HED rats, our previous work suggested that it could result from the high testicular glycolytic flux [14]. Indeed, high hypoxanthine levels have been correlated with an increased lactate/pyruvate ratio [77], which may also reflect the redox state of cells and tissues [78]. Altogether, our results show that HED-induced pre-diabetes downregulates both PGC-1α and SIRT3 protein expression, disrupting this axis with a consequent impairment on testicular mitochondrial bioenergetics (especially in complex I and complex III) and testicular AEC. It leads to hypoxanthine accumulation that may culminate in increased oxidative damage to testicular cells. Clearly, testicular function of HED animals is severely affected even in a prodromal stage of diabetes. Furthermore, this is strongly supported by previous observations by our team using this HED model [14, 42], where we showed decreased testicular weight and compromised testicular function, including lower testicular testosterone content and altered sperm quality. To our knowledge, this is the first report giving new insights in the role of PGC-1α/SIRT3 axis in overall testicular bioenergetics at this prodromal "silencing stage" of DM. This topic should deserve special attention, as the subtle changes in testicular glucose metabolism and mitochondrial bioenergetics occurring earlier in the pathology may have a later severe negative impact in male fertility.

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Chapter 8

Testicular metabolic reprogramming in neonatal streptozotocin-induced type 2 diabetic rats impairs glycolytic flux and promotes glycogen synthesis

This chapter was adapted from the submitted work:

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Testicular metabolic reprogramming in neonatal streptozotocin-induced type 2 diabetic rats impairs glycolytic flux and promotes glycogen synthesis

Abstract

Defects in testicular metabolism are directly implicated with male infertility, but most of the mechanisms associated with type 2 diabetes (T2DM)-induced male infertility remain unknown. We aimed to evaluate the effects of T2DM on testicular glucose metabolism by using a neonatal-streptozotocin (n-STZ)-induced T2DM animal model. T2DM state in rats was induced by STZ intraperitoneal injection (40 mg/kg) freshly diluted in citrate buffer (0.1 M, sodium citrate, pH 4.5). Plasma and testicular hormonal levels were evaluated by using kits. mRNA and protein expression levels were assessed by real-time PCR and Western blot, respectively. Testicular metabolic profile was assessed by ^{1}H-NMR spectrometry. T2DM rats showed hyperglycaemia, impaired glucose tolerance and hyperinsulinemia. Both testicular and serum testosterone levels were decreased. Testicular glycolytic flux was not favored in T2DM rats, since despite the increased expression of both glucose transporters 1 and 3 and the enzyme phosphofructokinase 1, lactate dehydrogenase activity was severely decreased contributing to lower lactate content. However, T2DM enhanced testicular glycogen accumulation, by modulating the availability of its precursors. T2DM also affected the reproductive sperm parameters. These results indicate that T2DM induce a testicular metabolic reprogramming by enhancing alternative metabolic pathways and such alterations are associated with impaired sperm parameters.

Keywords: Type 2 diabetes mellitus; Testicular metabolism; Glucose metabolism; Testicular glycogen; Spermatogenesis

Introduction

Diabetes mellitus (DM) is characterized by hyperglycaemia, resulting from defects in insulin secretion, insulin action, or both. The most prevalent form of DM is type 2 diabetes mellitus (T2DM), comprising up to 95% of all diagnosed diabetic individuals in developed countries [1]. T2DM induces metabolic alterations, disrupting the endocrine system, with a subsequent dysfunction of the hypothalamus-pituitary testicles (HPT) axis [2], that may end-up in impairment of the male reproductive health. Although not all diabetic men are infertile, evidences strongly associate T2DM with high prevalence of male subfertility/infertility [3-6] and decreased birth rates [7, 8].

Recently our team reported that diet-induced pre-diabetes, which is a prodromal stage of T2DM, compromises sperm quality and promotes marked alterations in overall testicular metabolism and ionic homeostasis [9, 10]. The testicles comprise a heterogeneous cell

population consisting of both somatic and germ cells. Testicular cells present unique metabolic characteristics, in part due to the existence of the blood-testis-barrier. The Sertoli cells (SCs) are responsible for the production of metabolic precursors essential for germ cell's development [11, 12]. These cells show a "Warburg-like metabolism" [13], using the external glucose to produce lactate, which is the preferred substrate of developing germ cells [14-17]. Testicular cells in particular SCs, take up extracellular glucose via specific glucose transporters (GLUTs), which is then oxidized to pyruvate and promptly reduced to lactate by lactate dehydrogenase (LDH), with a concomitant oxidation of NADH. Once produced, lactate is exported through the monocarboxylate transporter 4 (MCT4) to the intratubular fluid to become available for the developing germ cells. Thus, SCs and germ cells establish a tight metabolic cooperation that is highly dependent on glucose uptake and lactate production [9, 17]. At the physiological point of view the metabolism of carbohydrates, specially glucose, is vital for male reproductive health, so the maintenance of testicular glucose metabolism homeodynamics is of particular relevance otherwise spermatogenesis is arrested [18, 19]. Hence, the full enlightenment of testicular glucose metabolism and the molecular mechanisms that control it is of extreme importance, since alterations in these mechanisms may be on the basis of male infertility [9, 20]. It has been suggested a role for alternative substrates during diabetic conditions (for review see [18]). The use of these alternative substrates, such as glycogen, may act as a compensatory mechanism for fluctuations in insulin and glucose concentrations. Glycogen is a readily mobilized fuel store, which provides the body with a readily available source of energy if blood glucose availability decreases. The presence of glycogen and glycogen metabolism-related enzymes in testicular tissue has already been reported [21-23]. In fact, glycogen seems to play a pivotal role within the testicles, particularly during testicular development [23], where it acts as modulator of germ cell survival [23]. However, the role of glycogen within testicular milieu has been overlooked, particularly under abnormal physiological conditions such as T2DM. Apart from a study showing that diabetic animals present increased testicular levels of uridine diphosphoglucose (UDP-glucose), a glycosyl donor for the initial step of glycogen synthesis [24], no other studies were performed to evaluate the impact of T2DM on testicular glycogen. Herein we aimed to evaluate the effects of T2DM on testicular metabolism, with particular emphasis on glycolytic and glycogen metabolism. We hypothesized that T2DM may impair male fertility by acting in key glycolytic-associated enzymes and transporters, altering testicular metabolism. We also hypothesized that T2DM induces crucial changes in testicular glycogen metabolism.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A) unless specifically stated. Moloney Murine Leukemia Virus Reverse Transcriptase and deoxynucleotides triphosphates were purchased to NZYTech (Lisbon, Portugal).

Animals

Twelve three-months old male Wistar rats (*Rattus norvegicus*) (Charles River Laboratories, Barcelona, Spain) were used in this study. The animals were housed in our accredited animal colony and maintained with food and water ad libitum in a constant room temperature (20 ± 2ºC) on a 12-hour cycle of artificial lighting. All experiments were performed according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the European directives for the care and handling of laboratory animals (Directive 86/609/EEC).

Experimental design

Animals were randomly distributed in a control and neonatal-streptozotocin (n-STZ)-induced T2DM group. Animals from T2DM group were injected with a low-dose of STZ to achieve a T2DM model, according to the method described by Iwase and collaborators [25]. In brief, two-days old male Wistar rats were intraperitoneally injected with STZ (40 mg/kg) freshly diluted in citrate buffer (0.1 M, sodium citrate, pH 4.5). The control group received the vehicle solution in an equivalent volume. All animals were fed with standard chow diet (4RF21 certificate, Mucedola, Italy) and water. Animals' glycaemias were weekly monitored between the 30th and 90th days of age using a glucometer (One Touch® Ultra® Lifescan-Johnson, Milpitas, CA, USA). After treatment, animals were killed by decapitation. Blood was collected to heparinized tubes for further analysis and testicles were removed, weighed and stored at -80° C. The levels of glycated hemoglobin (HbA1C) were also determined using A1cNow$^{+®}$ meter (Bayer Diabetes Care, USA).

Glucose and insulin tolerance test

At 3 months of age, animals were submitted to a glucose tolerance test, as described by Rato and collaborators [9]. In brief, 14 h before the test, food was removed and the animals kept in fast. An intraperitoneal (IP) injection with 6 mL of glucose 30% (w/v) per kg of body weight was given to each animal. Blood samples were obtained from the tail and glucose levels measured every 30 min during 2 h. The area under the curve for the glucose tolerance test (AUC_{GTT}) was calculated using the trapezoidal rule, as described previously in [20].

The animals were also subjected to an insulin tolerance test as described by Holmes and collaborators [26]. In brief, 4 h before the test, food was removed and animals were kept in fast. An IP injection with 0.75 U insulin per kg of body weight was given to each animal. Blood

samples were obtained from the tail and glucose levels measured every 30 min during 2 h. The area under the curve for the insulin tolerance test (AUC_{ITT}) was calculated using the trapezoidal rule, as described previously in [20]

Testosterone, 17ß-estradiol and Insulin measurement

Hormonal levels were measured as described by Rato and collaborators [9]. In brief, testosterone (T), 17β-estradiol (E_2) and insulin levels were determined using commercial rat EIA kits according to manufacturer instructions. T and E_2 EIA Kits were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Insulin ELISA measurement kit was purchased from Mercodia (Uppsala, Sweden). The EIA kits used had detection limits of approximately of 40 µU/mL (for insulin), 6 pg/mL (for T) and 20 pg/ml (for E_2).

NMR spectroscopy

A combined extraction of polar and apolar metabolites was performed as described by Rato and collaborators [9]. The aqueous phase containing the water-soluble metabolites was lyophilized. ^{1}H-NMR spectra were acquired as previously described by Rato and collaborators [9]. Sodium fumarate was used as an internal reference δ_H [^2H_2O] 6.5 [s, 2xCH] to quantify the following metabolites: lactate δ_H [^2H_2O] 1.33 [d, J 6.9 Hz, $^3CH_3$], alanine 1.45 [d, J -14.36 Hz, $^3CH_3$], UDP-glucose 7.97 [d, J 8.1 Hz, CH]. The relative areas of ^{1}H-NMR resonances were quantified using the curve-fitting routine supplied with the NUTSpro™ NMR spectral analysis program (Acorn, CA, U.S.A.).

Testicular glycogen content

Testicular glycogen content was determined by using a commercial kit (Abnova KA0861, CA, USA) and following the manufacturer's instructions. Glycogen content was expressed as nanomoles of glycogen per milligram of tissue (wet weight).

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed to determine glucose transporter 1 (GLUT1), glucose transporter 2 (GLUT2), glucose transporter 3 (GLUT3), phosphofructokinase 1 (PFK1), lactate dehydrogenase A (LDHA), alanine aminotransferase 2 (ALT2), MCT4, muscle glycogen synthase (GYS1) and liver-type glycogen phosphorylase (PGYL) mRNA expression levels. Specific primers were designed for the amplification of target genes and for ß2-microglobulin which was used as internal control to normalize gene expression (Table 8.1). qPCR was carried out in an iQ5 system (Bio-Rad, Hercules, CA, USA). Efficiency of the amplification was determined for all primer sets using serial dilutions of cDNA (1, 1:5 and 1:25). PCR conditions were previously optimized and specificity of the amplicons was determined by melting curves. qPCR amplifications were performed with 1 µL of synthesized cDNA in a 20 µL reaction containing 10 µL Maxima™ SYBR Green/Fluorescein qPCR Master Mix (Biorad) and 300 nM of sense and antisense primers for each gene. Amplification conditions

comprised 5 min denaturation at 95°C, followed by 40 cycles at 95°C for 10 sec, a specific annealing temperature for each gene (Table 8.1) for 30 sec and 72°C for 10 sec. Samples were run in triplicate in each PCR assay. Normalized expression values were calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta Ct}$ [27].

Table 8.1. Oligonucleotides and cycling conditions for PCR amplification of GLUT1, GLUT2, GLUT3, PFK1, LDHA, MCT4, ALT2, GYS1, PYGL and β2MG. AT — annealing temperature; ALT2 – alanine aminotransferase 2; GLUT1 – glucose transporter 1; GLUT2 – glucose transporter 2; GLUT3 – glucose transporter 3; GYS1 – muscle glycogen synthase; LDHA – lactate dehydrogenase A; MCT4 – monocarboxylate transporter 4; PFK1 – phosphofructokinase 1; PYGL – liver-type glycogen phosphorylase; β2MG – beta 2 microglobulin.

Gene	Sequence (5'-3')	AT (C°)	Accession number
GLUT1	Sense:TCCGGCGGGAGACGCATAGT Antisense:CCCGCATCATCTGCCGACCC	61	NM_138827.1
GLUT2	Sense:GGGTTCCTTCCAGTTCGGAT Antisense:TCGTATGTGCTGGTGTGACT	60	NM_012879.2
GLUT3	Sense:GCGCAGCCCTTCCGTTTTGC Antisense:CCCCTCGAAGGCCCGGGTAA	63	NM_017102.2
PFK1	Sense:GAGTGCTGACAAGCGGCGGT Antisense:GTGGCCCAGCACGGTCACTC	61	NM_013190.4
LDHA	Sense:GCGCAGCCCTTCCGTTTTGC Antisense:CCCCTCGAAGGCCCGGGTAA	63	NM_017025.1
ALT2	Sense: TGAGGTAATCCGAGCCAACA Antisense: CACGTCCTCTCGGATACAGT	60	NM_001012057.1
MCT4	Sense:ATGTGGGCATGGCGTGTGCC Antisense:CCCAGCCATGGCAGCTCGAA	66	NM_001013913.1
GYS1	Sense:CAGCTATGGGACACAGCCAA Antisense:TTCGTCGGATGGTGGTCAAG	60	NM_001109615.1
PYGL	Sense:CTCCCAATCAGCCAGACCTC Antisense:GGAAGGCTCCATGTTCCAGA	60	NM_022268.1
β2MG	Sense: ATGAGTATGCCTGCCGTGTG Antisense: CAAACCTCCATGATGCTGCTTAC	60	NM_012512.2

Western blot

Western Blot procedure was performed as previously described by Simões and collaborators [28]. The resulting membranes were incubated with rabbit anti-GLUT1 (1:500, SC-7903, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-GLUT2 (1:1000, SC-9117, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-GLUT3 (1:1000, ab41525, Abcam, Cambridge, MA, USA), rabbit anti-PFK1 (1:500, SC-67028, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-MCT4 (1:1000, SC-50329, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-LDH (1:10000, ab52488, Abcam, Cambridge, MA, USA), rabbit anti-ALT (1:500, SC-99088, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-GYS1 (1:100, SC-81173, Santa Cruz Biotechnology, Heidelberg, Germany) and rabbit anti-PYG (1:500, SC-66913, Santa Cruz Biotechnology, Heidelberg, Germany). Mouse anti-tubulin

(1:5000, T9026, Sigma-Aldrich, Roedermark, Germany) was used as protein loading control for testicular tissue. The immuno-reactive proteins were separately detected with goat anti-rabbit IgG-AP (1:5000, Sc-2007, Santa Cruz Biotechnology, Heidelberg, Germany) or goat anti-mouse IgG-AP (1:5000, Sc-2008, Santa Cruz Biotechnology, Heidelberg, Germany). Membranes were reacted with enhanced chemifluorescence detection system (GE Healthcare, Webling, Germany). The densities from each band were obtained using the Quantity One Software (Bio-Rad, Hemel Hempstead, UK), divided by the respective tubulin band density and then normalized against the respective control.

Enzymatic assays

LDH activity was determined using a commercial assay kit (Promega, Madison, USA) and following the manufacturer's instructions. PFK activity was determined as previously described by Alves and collaborators [29]. ALT was determined by a colorimetric assay as previously described by Mohun and Cook [30]. The attained activities were expressed as fold variation versus the control group.

Epididymal sperm parameters

Evaluation of epididymal sperm parameters was performed as previously described by Dias and collaborators [31]. In brief, cauda epididymis were isolated and placed in pre-warmed (37ºC) Hank's Balanced Salt Solution (pH 7.4), minced with a scalpel blade and the suspension incubated for 5 minutes (37ºC). Sperm motility was evaluated by assessing the percentage of motile sperm in 10 random fields, and the average value was used as the total sperm motility. Sperm viability was assessed examining eosin-nigrosin stained epididymal sperm smears. Epididymal sperm concentration was determined using a Neubauer counting chamber. For the assessment of sperm morphology we used standard methods [32]. Sperm morphology was evaluated using Diff-Quick® (Baxter Dale Diagnostics AG, Dubinger, Switzerland) stained smears according to the manufacturers' instructions. To be classified as normal, a sperm cell must have a hook-shaped head and no defects of head, neck or tail. Otherwise, sperm were considered abnormal.

Statistical analysis

The statistical significances of differences of all experimental data were assessed by Student-t test (Graph Pad Software 6.0, San Diego, CA, USA). All experimental data are shown as mean ± standard error of the mean; $P<0.05$ was considered significant.

Results

Streptozotocin-treated rats developed type 2 diabetes mellitus exhibiting mild hyperglycaemia, glucose intolerance and insulin resistance

After three months of age average glycaemic values were significantly increased (by 26%) in n-STZ-treated animals (126 ± 1 mg/dL), when compared to control group (99 ± 1 mg/dL; Table 8.2). Blood HbA1c levels were also significantly increased (by 17%) in n-STZ-treated animals (5.60 ± 0.07 %) when compared to control group (4.80 ± 0.02 %; Table 8.2).

Table 8.2. Average values of the animals weight, glycaemia, Hb1Ac and insulin levels measured in animals from the Control (CTR) and T2DM group after three months of age. CTR – control; Hb1Ac – glycated hemoglobin; T2DM – Type 2 diabetes mellitus. Results are expressed as mean ± SEM (n=6 for each condition). * Significantly different relative to Control (P<0.05).

Parameters	CTR	T2DM
Body weight (g)	347 ± 8.0	363 ± 11.0
Average Glycaemia (mg/dL)	99 ± 1.0	126 ± 1.0*
Hb1Ac (%)	4.80 ± 0.02	5.60 ± 0.07*
Insulin (μU/mL)	4.3 ± 0.3	5.2 ± 0.4*

Together, these results prefigure a prolonged state of hyperglycaemia and subsequent impaired glucose metabolism. Indeed, the results attained for the glucose tolerance test show that n-STZ-treated animals showed a significant increase in blood glycaemia during the 120 min of the IP glucose tolerance test (Figure 8.1A) and developed significant glucose intolerance, as can be seen by the significantly increased (by 29%) AUC_{GTT} values in n-STZ-treated animals (23364 ± 2231 arbitrary units (a.u.)) when compared to animals from the control group (18153 ± 735 a.u.) (Figure 8.1B). These results led us to investigate the insulin responsiveness status, so we performed an IP insulin tolerance test. Our results showed that n-STZ-treated animals did not respond to insulin (Figure 8.1C) as observed by the significant increase of AUC_{ITT} (by 30%) in n-STZ-treated animals (10570 ± 1054 a.u.), when compared with rats from the control group (7420 ± 657 a.u.) (Figure 8.1D), illustrating that these rats developed insulin resistance. Consistent with these results, are the higher levels of fasting insulin (increased by 21%) observed in n-STZ-treated animals (Table 8.2), corroborating that these rats developed insulin resistance. Altogether these characteristics clearly illustrate that the n-STZ-treated rats developed type 2 diabetes (T2DM group).

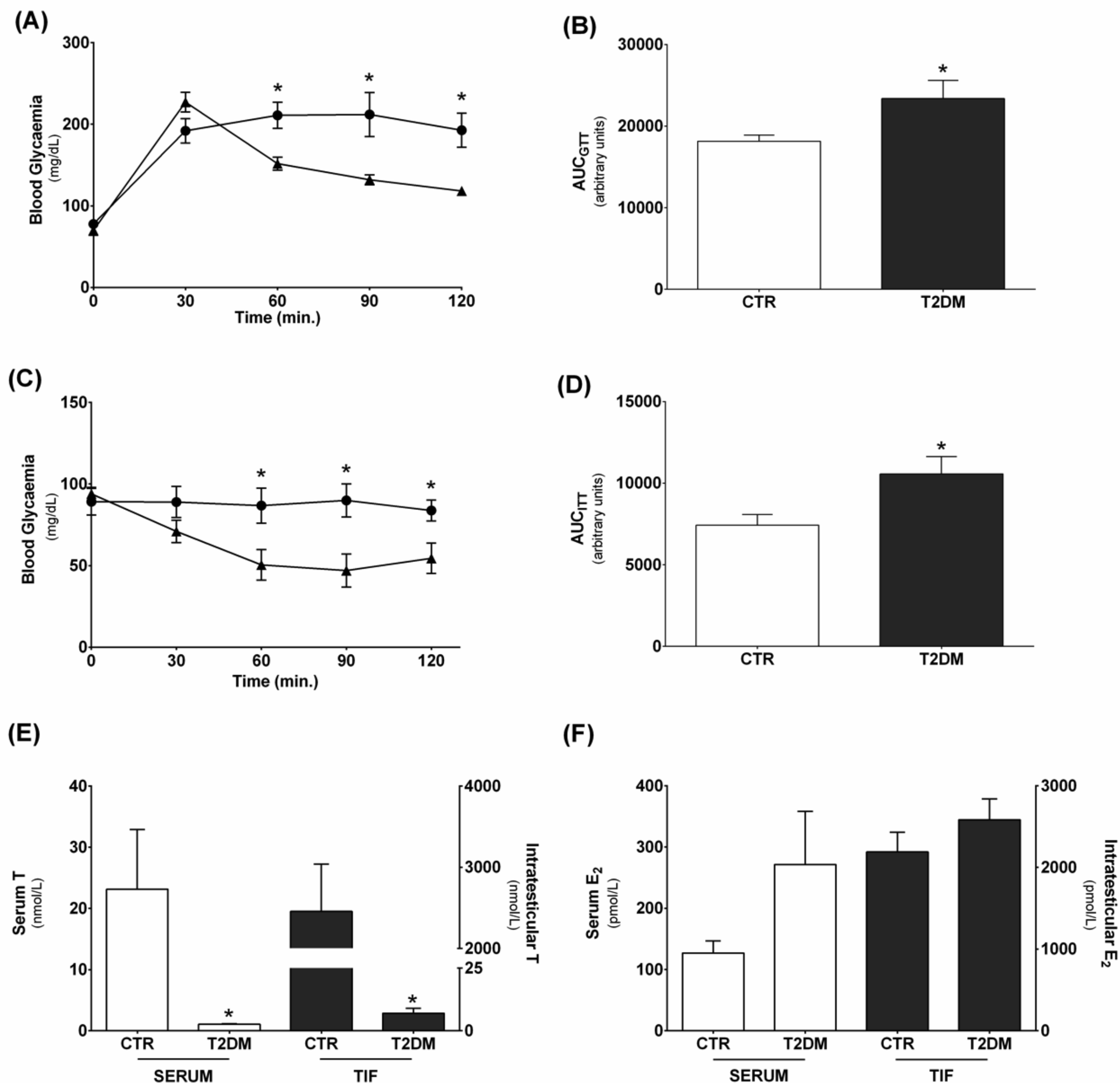

Figure 8.1. Type 2 diabetes mellitus (T2DM) induces (A and B) glucose intolerance and (C and D) insulin resistance and significantly alters both (E) serum and testicular testosterone levels. (A) Blood glucose levels of the control group (▲) and T2DM group (●) measured during intraperitoneal glucose tolerance test. (B) Area under the curve for the intraperitoneal glucose tolerance test (AUCGTT) performed in control and T2DM group rats. (C) Blood glucose levels of the control group (▲)and T2DM group (●) measured during intraperitoneal insulin tolerance test. (D) Area under the curve for the intraperitoneal insulin tolerance test (AUCITT) performed in control and T2DM group rats. (E) Testosterone levels in testicular interstitial fluid and serum of control and T2DM group rats. (F) 17β-estradiol levels in testicular interstitial fluid and serum of control and T2DM group rats. Results are presented as mean ± SEM of six independent experiments, corresponding to six animals/group. * Significantly different relative to control ($p < 0.05$).

T2DM decreases serum and testicular testosterone levels

We evaluated the levels of the sex steroid hormones (T and E_2) in serum and testicular environment, since they are pivotal for several events that control spermatogenesis, including testicular metabolism [17]. T serum concentration was significantly decreased (by 95%) in T2DM group (1.06 ± 0.09 nmol/L) when compared with the control group (23.16 ± 9.76

nmol/L) (Figure 8.1E). On the other hand, serum E_2 levels were not altered in the animals that developed T2DM (272 ± 87 pmol/L) when compared to control (127 ± 20 pmol/L) (Figure 8.1 F). Testicular cells are bathed by testicular interstitial fluid (TIF) and the establishment of an appropriate fluid is crucial for an adequate hormonal control of spermatogenesis. In this fluid, sex steroid levels are 100 to 1000-fold higher than in serum, as observed in our results [9]. T levels present in TIF were significantly decreased by 348% in T2DM group animals (7 ± 2 nmol/L) when compared with animals from the control group (2457 ± 583 nmol/L) (Figure 8.1E). Contrastingly, T2DM animals did not exhibit significantly altered E_2 concentrations in TIF (2585 ± 255 pmol/L) when compared to control (2192 ± 239 pmol/L) (Figure 8.1F).

T2DM increases GLUT1/GLUT3 levels and PFK activity in rat testicles

Glucose metabolism is pivotal for the normal occurrence of spermatogenesis. Its uptake from the extracellular medium is a rate-limiting step for glucose metabolism. Hence we evaluated the effects of T2DM on the expression of the most relevant glucose membrane transporters in testicles (GLUT1, GLUT2 and GLUT3). No significant differences were observed on testicular GLUT1 transcript levels between animals from both groups (Figure 8.2A), while GLUT1 protein in animals from T2DM group were significantly increased in 13% (1.13 ± 0.07 fold variation), when compared with the control group (Figure 8.2B). Concerning GLUT2 expression, no alteration was observed on both transcript and protein levels of T2DM animals when compared with the control group (Figure 8.2 A, B). GLUT3 mRNA expression was significantly increased by 40% in T2DM group (1.40 ± 0.17 fold variation to control) (Figure 8.2A) and was followed by a 31% increase in protein levels (1.31 ± 0.13 fold variation to control) (Figure 8.2B). After being internalized, glucose is metabolized via glycolysis, in which the irreversible conversion of fructose 6-phosphate to fructose 1,6-bisphosphate by PFK is a key control point. We evaluated the effects of T2DM in PFK1 levels (widely expressed in testicular tissue) and observed no alteration in both transcripts and protein levels of PFK1. Nevertheless, when we assessed the activity of PFK it was significantly increased by 44% in the testicular tissue of T2DM rats (1.44 ± 0.14 fold variation), when compared with testicular tissue of rats from the control group (Figure 8.2D).

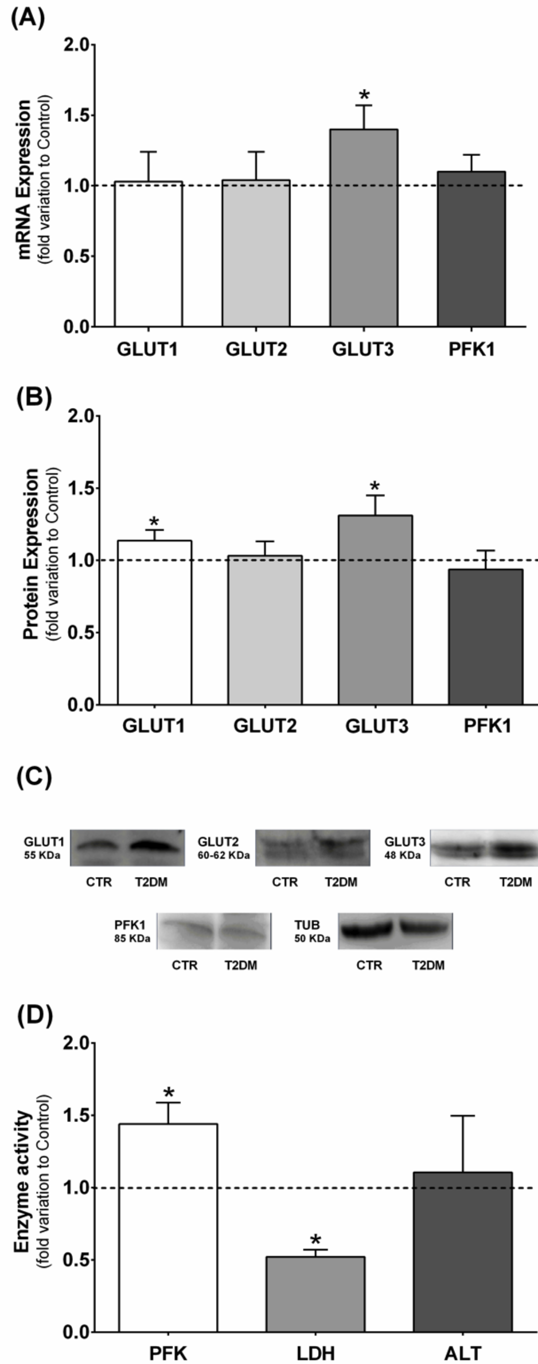

Figure 8.2. Type 2 diabetes mellitus (T2DM) modulates the (A) mRNA, (B) protein levels and the (C) activity of glycolytic enzymes and transporters. (A) Pooled data of independent experiments, indicating the fold variation of glucose transporters (GLUT1, GLUT2 and GLUT3) and of phosphofructokinase 1 (PFK1) mRNA levels found in testicles of T2DM rats when compared with the control rats (dashed line). (B) Pooled data of independent experiments, indicating the fold variation of GLUT1, GLUT2, GLUT3 and PFK1 protein levels found in testicles of T2DM rats when compared with the control rats (dashed line). (C) Illustrative Western Blot experiment. (D) Pooled data of independent experiments, indicating the fold variation of PFK1, lactate dehydrogenase (LDH) and alanine transaminase (ALT) enzymatic activities in testicles of T2DM rats when compared with the control rats (dashed line). Results are expressed as mean ± SEM (n=6 for each condition). * Significantly different relative to control ($p < 0.05$).

T2DM causes a reduction of testicular lactate levels by decreasing LDH activity

In testicular tissue the majority of glucose is converted into lactate, which is not a "waste" end product of glycolysis, but a critical "fuel" for germ cells development. Thus, we evaluated the effects of T2DM on the testicular glycolytic metabolic profile. Our results show that testicular lactate content was significantly decreased by 50% in rats from T2DM group (1.45 ± 0.30 nmol/mg tissue), when compared to rats from the control group (2.90 ± 0.60 nmol/mg tissue) (Table 8.3).

Table 8.3. Metabolite content of testicular tissue in animals from the Control (CTR) and T2DM group. CTR – control; T2DM – Type 2 diabetes mellitus. Results are expressed as mean ± SEM (n=6 for each condition). * Significantly different relative to control (P<0.05).

Metabolites (nmol/mg tissue)	CTR	T2DM
Lactate	2.90 ± 0.60	1.45 ± 0.30*
Alanine	0.70 ± 0.20	0.73 ± 0.20
Glycogen	0.25 ± 0.03	0.41 ± 0.10*
UDP-Glucose	0.48 ± 0.01	0.54 ± 0.01*
Lactate/Alanine ratio	3.70 ± 0.30	2.70 ± 0.40*

Since testicular lactate content was significantly reduced, we evaluated the effects of T2DM on the expression and activity of LDH. When we assessed the LDHA transcript levels (which is highly expressed in the lactate-producing SCs), we observed a significant reduction (by 19%) in the testicles of rats from the T2DM group (0.81 ± 0.07 fold variation) when compared with rats from the control group (Figure 8.3A). However, the overall protein levels were not altered in the testicles of rats from both groups (Figure 8.3B). We further evaluated LDH activity on the testicular tissue of animals from both groups and observed a significant decrease (by 48%) in the testicles from T2DM rats (0.52 ± 0.05 fold variation; Figure 8.2D), as compared with rats from the control group. Once produced, in order for lactate to reach the developing germ cells it must be exported from SCs by monocarboxylate transporter MCT4. When we evaluated the testicular expression of MCT4, we observed no differences between animals from T2DM and control groups concerning mRNA levels (Figure 8.3A). However, MCT4 protein levels were significantly increased (72%) in the testicular tissue of T2DM rats (1.72 ± 0.35 fold variation) when compared with rats from control group (Figure 8.3B).

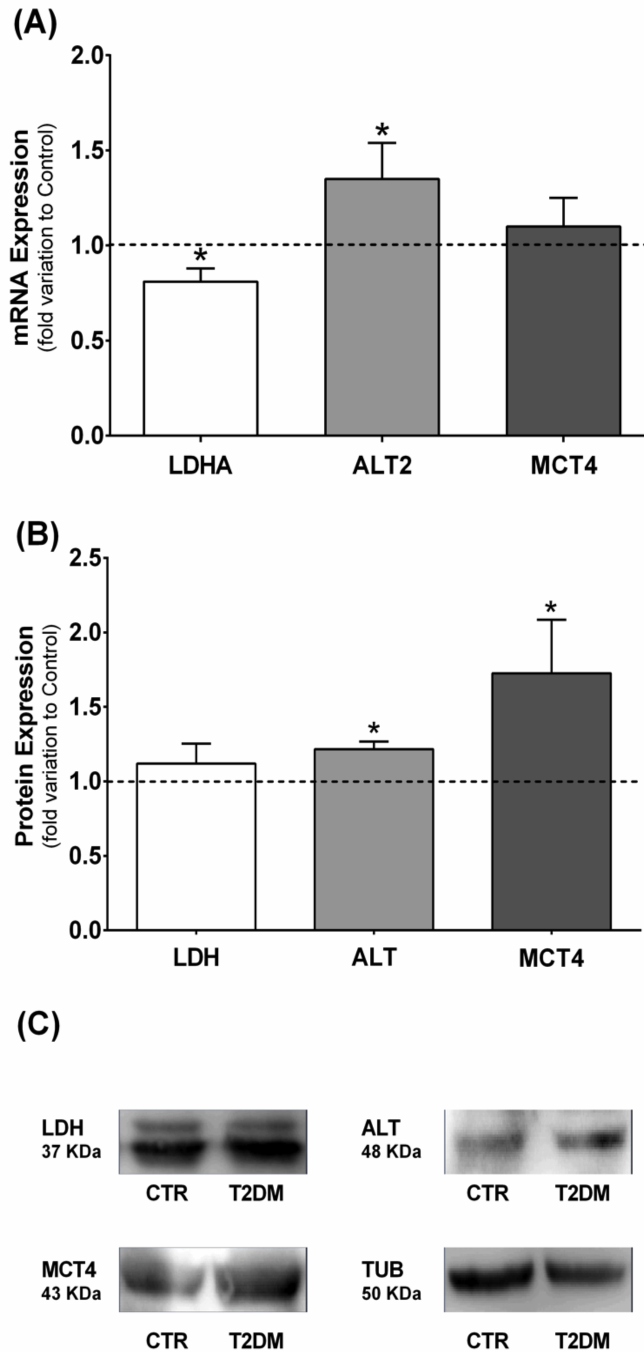

Figure 8.3. Type 2 diabetes mellitus (T2DM) modulates the expression of lactate production-related enzymes and transporter. (A) Pooled data of independent experiments, indicating the fold variation of lactate dehydrogenase (LDH), alanine aminotranferase 2 (ALT2) and monocarboxylate transporter 4 (MCT4) mRNA levels found in testicles of T2DM rats when compared with the control rats (dashed line). (B) Pooled data of independent experiments, indicating the fold variation of LDH, ALT and MCT4 protein levels found in testicles of T2DM rats when compared with the control rats (dashed line). (C) Illustrative Western Blot experiment. Results are expressed as mean ± SEM (n=6 for each condition). * Significantly different relative to control ($p < 0.05$).

T2DM slightly increase the testicular content of alanine, altering the lactate/alanine ratio

Lactate is converted from pyruvate, which is at a crossroad of several metabolic pathways. Pyruvate is an intermediary metabolite that can be reversibly converted either to lactate (by

LDH) or to alanine (by alanine aminotransferase (ALT)). Following the observed decrease in testicular lactate content of T2DM rats, we further evaluated testicular alanine levels. We found it to be slightly (but not significantly) increased in T2DM rats (0.73 ± 0.20 nmol/mg tissue; Table 8.3), as compared with rats from the control group (0.70 ± 0.20 nmol/mg tissue).

We then assessed the mRNA levels of the main testicular ALT isoform (ALT2). Our results show a significant increase (by 35%) in T2DM rats (1.35 ± 0.19 fold variation to Control) (Figure 8.3A). Additionally, we observed that the overall testicular protein levels of ALT were also significantly increased by 21% in T2DM rats (1.21 ± 0.05 fold variation to Control) (Figure 8.3B). However, when we evaluated the activity levels of ALT in testis we found no significant differences between both groups (Figure 8.2D). Importantly, the lower levels of lactate together with the slight increase in the alanine levels detected in the testicular tissue of T2DM rats, illustrate a decrease of the lactate/alanine ratio (Table 8.3) (2.70 ± 0.40), as compared with the control group (3.70 ± 0.30).

T2DM enhances testicular glycogen deposition, modulating the expression of glycogen-associated enzymes

We evaluated the testicular content of glycogen and of its precursor monomers UDP-glucose. We observed that glycogen levels in the testicles of T2DM rats were significantly increased by 26% (0.41 ± 0.10 nmol/mg tissue), as compared with the control group (0.25 ± 0.03 nmol/mg tissue; Table 8.3). We also observed that UDP-glucose testicular content in T2DM rats was significantly increased by 12.5% when compared with the control group, from 0.48 ± 0.01 nmol/mg tissue to 0.54 ± 0.01 nmol/mg tissue (Table 8.3). We further evaluated the effects of T2DM on testicular glycogen metabolism by assessing the expression levels of key rate-limiting enzymes involved in testicular glycogen metabolism. The mRNA levels of GYS1 (the main GYS isoform expressed in the testicles) were significantly decreased by 20% in the testicles of T2DM rats (0.80 ± 0.01 fold variation) when compared to rats of the control group, while its protein levels were not significantly altered (Figure 8.4A, B).

On the other hand, when we evaluated the testicular expression levels of glycogen phosphorylase (PYG), a key enzyme responsible for glycogen degradation, we found that these were significantly altered by T2DM. We assessed PYGL transcript levels (the isoform preferentially expressed in the testicles) and overall PYG protein levels. Our results show that PYGL mRNA levels were significantly increased by 44% in T2DM rats (1.44 ± 0.20 fold variation) when compared with rats from the control group (Figure 8.4A). The overall PYG protein levels were also significantly increased by 48% in the testicles of T2DM rats (1.48 ± 0.06 fold variation to control) (Figure 8.4B).

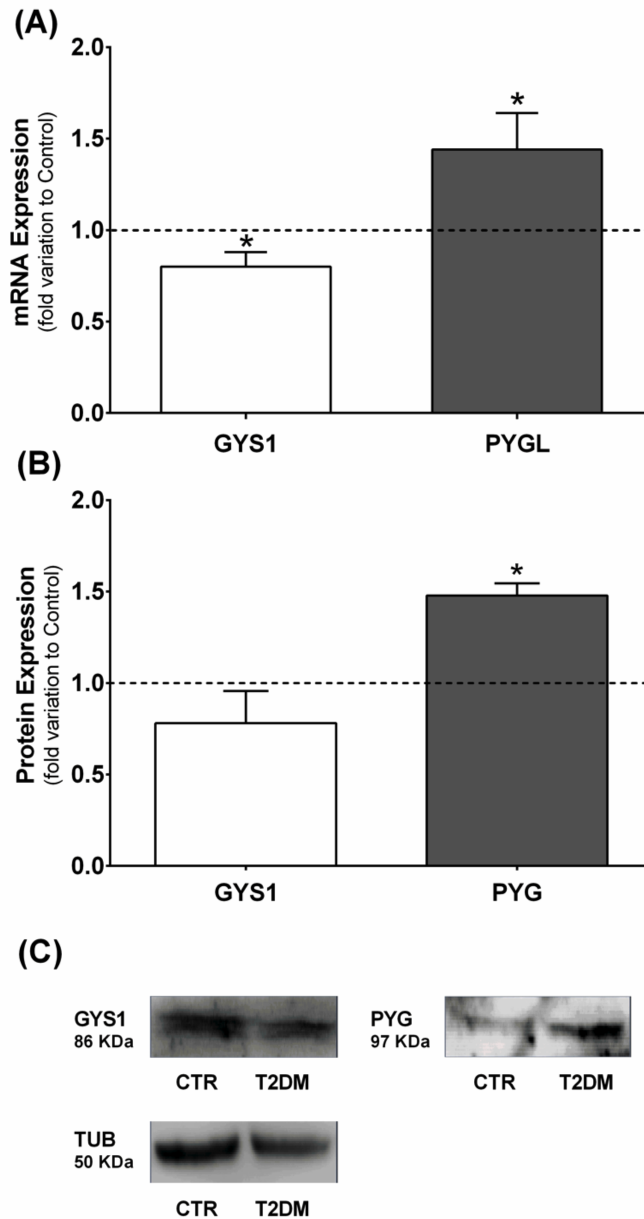

Figure 8.4. Type 2 diabetes mellitus (T2DM) modulates the expression of glycogen metabolism-related enzymes. (A) Pooled data of independent experiments, indicating the fold variation of glycogen synthase 1 (GYS1) and glycogen phosphorylase L (PYGL) mRNA levels found in testicles of T2DM rats when compared with the control rats (dashed line). (B) Pooled data of independent experiments, indicating the fold variation of GYS1 and PYG protein levels found in testicles of T2DM rats when compared with the control rats (dashed line). (C) Illustrative Western Blot experiment. Results are expressed as mean ± SEM (n=6 for each condition). * Significantly different relative to control ($p < 0.05$).

T2DM affects epididymal sperm quality

T2DM is known to affect sperm quality parameters, such as concentration, motility, viability and morphology [5]. As could be expected, our results showed that T2DM rats presented alterations in specific epididymal sperm parameters when compared with rats from the control group. Although the sperm concentration in control rats was ($4.10 \pm 0.40 \times 10^7$

cell/mL), which was not significantly different from T2DM rats (4.70 ± 0.40 x10^7 cell/mL), spermatozoa total motility of T2DM rats was significantly lower (73.2 ± 1.0 %) relatively control rats (78.2 ± 0.9 %). Furthermore, when we evaluated epididymal spermatozoa viability, control rats showed a significantly higher viability (74.0 ± 0.2 %) than T2DM rats (51.0 ± 1.0 %; Table 8.4). When assessing sperm morphology, our results showed that the percentage of abnormal spermatozoa in control group was (38.0 ± 3.0 %) and T2DM rats presented a significantly higher percentage of abnormal spermatozoa (49.0 ± 3.0 %; Table 8.4).

Table 8.4. Epididymal sperm concentration, motility, viability, and morphology in animals from the Control (CTR) and T2DM group. CTR – control; T2DM – Type 2 diabetes mellitus. Results are expressed as mean ± SEM (n=6 for each condition). * Significantly different relative to control ($P<0.05$).

Sperm Parameters	CTR	T2DM
Concentration (x10^7 cell.mL^{-1})	4.10 ± 0.40	4.70 ± 0.40
Motility (%)	78.20 ± 0.90	73.20 ± 1.00*
Viability (%)	74.00 ± 0.20	51.00 ± 1.00*
Morphology (% abnormal spermatozoa)	38.00 ± 3.00	49.00 ± 3.00*

Discussion

T2DM is the most prevalent form of DM, characterized by insulin resistance and impairment of whole-body metabolism [1]. Several animal models have been developed to study the mechanisms involved in the pathophysiology of the various co-morbidities associated with T2DM [33]. In this study, we used a n-STZ-induced T2DM animal model that displays the typical characteristics of the early stages of T2DM. STZ is a widely used diabetogenic agent, which produces a selective toxic effect on pancreatic ß-cells [34]. When administrated to rats at low doses during postnatal age they are reported to develop T2DM [25]. In our study the n-STZ-treated animals showed mild-hyperglycaemia and impaired glucose tolerance, as observed by the significant increase in the AUC_{GTT} values. In fact, when subjected to a glucose tolerance test, their glycaemia levels remained within the interval 140-200 mg/dL 2 hours post-glucose load. This is consistent with the observed increase of HbA1c values in T2DM rats, which were in accordance with others [25, 35]. HbA1c is a marker of cumulative glycaemic exposure over a preceding of 8 to 12 weeks and a strong predictor of the development of DM [1]. Insulin intolerance (resistance) is also closely associated to T2DM [1] and was also observed in our animal model. Furthermore, plasma insulin levels were also increased. This may be explained as a compensatory mechanism in order to maintain normoglycemia to face the detected insulin intolerance in T2DM rats [36]. Hyperinsulinemia presented by T2DM rats is also concomitant with the marked insulin intolerance observed in these animals. Altogether, the displayed characteristics of STZ-treated animals clearly indicate that a T2DM condition was attained in these rats.

T2DM rats presented insulin intolerance that has been associated with disruption of the HPT axis, leading to an imbalance in the levels of sex steroid hormones [37]. Insulin is essential for the normal function of the reproductive axis. Deficiency on this hormone has been directly associated with a decrease in T secretion by Leydig cells [38, 39]. In this context, we evaluated sex steroids levels and observed a significant decrease in both testicular and serum T levels in T2DM rats. Undoubtedly, insulin intolerance compromises testicular T production and consequently whole body T levels, as previously observed in insulin resistant individuals [38]. Concerning the E_2 levels, we did not observe significant differences at both, testicular and serum levels, between groups, which is in accordance with other reports [40]. Unaltered E_2 levels may be explained not only by the unchanged levels and/or enzyme activity of aromatase observed in early stages of DM [40], but also by the contribution of peripheral tissues, such as adipose tissue, bone and skin [41]. Therefore the T2DM promoted by STZ might not be sufficiently advanced to induce significant differences in E_2 levels that are known to occur in more severe stages of the disease [37, 42].

The endocrine disruption observed in T2DM rats is reflected in whole body metabolism, and may also disturb testicular energy metabolism and male reproductive function [17, 43, 44]. Testicular tissue consists of a heterogeneous population of somatic and germ cells, where germ cells are dependent on the nutritional support provided by SCs and DM alters this metabolic cooperation. It is has been discussed that the altered metabolism within testicular environment is closely associated with decreased male fertility (for review see [45]). Previous studies from our team showed that in a prodromal stages of T2DM, known as pre-diabetes, testicular cells are able to adapt their metabolism to promote an adequate environment for germ cell development [9]. Glucose uptake is enhanced in testicular tissue of pre-diabetic animals, as well as PFK activity, favoring a high glycolytic flux [9]. Moreover, the testicular expression levels of proteins involved in lactate production and transport were also found to be enhanced in pre-diabetic animals, resulting in higher amounts of testicular lactate [9]. Like pre-diabetic rats, our T2DM rats presented increased testicular expression levels of GLUT1 and GLUT3, favoring the glucose uptake. This was accompanied by increased activity of PFK1, thus showing that the two rate-limiting steps of glycolysis are not affected by T2DM which illustrates that the high testicular glycolytic flux observed in the testicles of pre-diabetic animals may also occurs in the testicles of T2DM. In sum, testicles metabolize glucose at high rates not only in prodromal stages but also when T2DM is already established. However, we observed that testicular lactate production is reduced by T2DM. In the present study, the main contributor to the lower lactate content detected in testicles of T2DM rats seems to be the decrease of LDH activity. Moreover, lactate export is not impaired by T2DM, since MCT4 levels were increased in T2DM rats. Concurrent results have been described in a previous study using a spontaneous T2DM animal model [46]. Those authors reported that 3 months old Goto-Kakizaki rats, which constitute an important model to study the initial events of DM development (since at early age these animals do not exhibit the severe complications associated with the disease) [47], showed reduced testicular lactate

production, illustrating the existence of testicular metabolic adaptations according to the degree of severity of T2DM.

The present data illustrates that testicular glycolytic flux, which ends up in lactate production, is compromised in T2DM rats. Glucose taken up by testicular cells is not converted into lactate as efficiently, being most probably redirected to other metabolic pathways. Pyruvate, the end-product of glycolysis, can be used as a precursor metabolite for several metabolic pathways. Nevertheless, within the testicular *milieu* it is preferentially converted to lactate by LDH or alanine by ALT [17]. We found that alanine levels were slightly (but not significantly) increased in T2DM rats, which was associated with a slight (non-significant) increase of ALT activity. This slight increase in alanine levels together with the lower levels of lactate, led to a decrease in testicular lactate/alanine ratio in T2DM rats. This lactate/alanine ratio is often used as an index of the redox state of tissues [48], because it reflects the NAD^+/NADH ratio, since the conversion of pyruvate or its conversion to alanine is coupled with re-oxidation of NADH into NAD^+. The decreased testicular lactate/alanine ratio, illustrates that, in the present conditions, the feed-forward pathway in which LDH fuels glyceraldehyde 3-phosphate dehydrogenase with NAD^+ is not favored, which may eventual end up in compromised glycolytic pathway.

Kim and collaborators [49] have described an association between alterations in glucose metabolism and decreased sperm quality in diabetic mice models. Although those authors used a distinct type 1 diabetes rodent model, they observed a decrease in sperm parameters that they correlated with an abnormal metabolic activity, which could also be exacerbated by the local autoimmune mechanism known to occur in this pathological state [5, 50]. In that study, Kim and collaborators [49] reported an alteration of expression of specific GLUTs involved either the supplying of substrates to the pentose phosphate pathway known to occur in the sperm head (for review see [51]) or in the uptake of fructose from the seminal plasma (see review [52]), pointing towards possible impairment in the maturation and fertilization events and not in spermatogenesis [49]. No information was given concerning the other rate-limiting steps of the glycolytic flux and production of lactate.

Still, our results demonstrate that T2DM condition altered testicular GLUT's expression, favoring the glucose uptake, while the production of lactate, the key substrate for developing germ cells, was compromised. These evidences suggested that, in our animal model, T2DM induced a testicular metabolic reprogramming, promoting distinct glucose metabolic pathways and/or substrate preferences. Bearing in mind that glycogen metabolism plays a preponderant role in T2DM, contributing to high glucose disposal (for review see [9, 53]), and that testicles readily synthetize and store glycogen [23] we hypothesized that, under T2DM conditions, testicular glycogen may represent an alternative metabolic pathway and contribute to adaptive metabolic mechanisms. We evaluated testicular glycogen content and observed a significant increase in T2DM rats. T2DM animals present several conditions that favor glycogenesis such as: (1) elevated levels of insulin, which stimulate glycogen synthesis through inactivation of glycogen synthase kinase 3 (GSK-3) that is known to maintain GYS1 in

an activated form; (2) hyperglycaemia, which increases the blood-to-testicles glucose availability, exerting an allosteric activation on GYS1 via G-6-P (accelerating glycogen synthesis) [54, 55]; (3) increased testicular levels of UDP-glucose, an active intermediate in the synthesis of glycogen [24]. As glycogen synthase is the rate-limiting enzyme in glycogen synthesis, we evaluated testicular GYS1 levels. We found no significant alterations in GYS1 protein levels of T2DM rats, although a significant decrease was observed in GYS1 mRNA levels. These divergent results concerning mRNA and protein levels may be due to factors associated with regulatory mechanisms in gene expression, such as mRNA retention in the nucleus, processing, stability and half-life time of mRNA molecule [56]. The simultaneous increase in testicular glycogen amount and overall PYG levels seems to be somewhat contradictory. A possible explanation may rely on the increased levels of sugar nucleotides, such as UDP-glucose, which is the immediate precursor in glycogen synthase reaction. Increased levels of UDP-glucose were detected in the testicles of T2DM rats illustrating the ability of testicular tissue to synthesize glycogen. This increase of UDP-glucose and glycogen content in tissues promoted by T2D was also reported in the heart [57, 58]. The regulation of glycogen metabolism is complex and several enzymes taking part in this process allosterically respond to metabolites and/or hormones. It has been proposed that the increased cardiac glycogen synthesis observed in STZ-induced diabetic rats [59] is due to a metabolic reprogramming of cardiac tissue, in which the increased glucose influx together with the reduced glucose oxidation augments the cardiac content of UDP-glucose [60]. This effect, with the combined activation of glycogen synthase increases cardiac glycogen storage. Our results support that this phenomena may also occur in testicles. The increased GLUT1 and GLUT3 expression, in the opposition to the low lactate production and together with the higher levels of UDP-glucose in T2DM rats illustrate that T2DM stimulates glycogen accumulation.

Despite the ability of testicles to adopt alternative pathways, the altered glucose utilization and the glycogen accumulation observed in testicles of T2DM rats was concurrent with the impairment of sperm parameters. In fact, although o changes were observed in sperm concentration of T2DM rats, the percentage of abnormal sperm was significantly increased. A side effect of hyperglycaemia and altered testicular bioenergetics is the increase of oxidative stress and reactive oxygen species overproduction within testicular environment [20], which contributes to abnormal sperm morphology. Thus, our results are consistent with previous studies [46, 61], illustrating that testicular metabolic alterations induced by T2DM are closely associated with a decrease in male fertility.

In conclusion, our results clearly suggest that T2DM significantly alters testicular glycolytic profile and glycogen metabolism and such alterations are associated and lead to the impairment of sperm quality, nevertheless the underlying molecular mechanisms that lead to decreased sperm parameters must be fully elucidated. Further knowledge on the functioning and regulation of these mechanisms will be essential to provide new insights on effects of T2DM on male fertility.

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Chapter 9

General Discussion and Conclusion

General Discussion

The incidence of metabolic diseases in modern societies has been progressing alarmingly over the last decades. Eating habits, as well as current lifestyle behaviours, are major contributors for this increased incidence and for the deleterious effects observed in human health.

Diets based on sugars and fats (high-energy diets, HED) are more affordable and palatable, with their consumption being increasingly favored to that of low caloric diets. Recent statistics gave alert that individuals from a very early age (even in an early infancy) tend to adhere to these erroneous eating behaviors. Therefore, the long-term effects of HED consumption (and its associated chronic diseases, such as DM and obesity) should be discussed and thoroughly investigated.

Infertility is one of the silent outcomes that may arise from HED overconsumption. Formation of competent spermatozoa is an intricate and complex process initiated within the seminiferous epithelium and is highly dependent on whole body metabolism. Current eating habits negatively impact male fertility, not only through indirect effects mediated by changes in hormone levels, but also by direct changes in the metabolic cooperation established between testicular cells. The success of spermatogenesis is on the basis of male fertility, and part of this success is dictated by the metabolic performance of SCs. One of the major functions of SCs is to ensure the transport and metabolism of glucose to produce the metabolic precursors needed by developing germ cells [1].

Insulin is a peptide hormone that regulates the metabolism of carbohydrates and, hence, whole body metabolism. Deregulation of insulin levels or sensitivity has been closely associated with HED consumption and metabolic diseases. Insulin links whole body metabolism and reproduction, via the reproductive axis, but it also seems to play an important role within the seminiferous epithelium, since SCs express insulin receptors [2]. In fact, it has been reported that insulin stimulates DNA and protein synthesis [3] and increases *in vitro* production of lactate in SCs from immature rats [4].

This work illustrates that hSCs cultured under insulin-deprived conditions decreased the consumption of glucose, which resulted in compromised lactate production. In fact, in those conditions both LDHA and MCT4 were regulated at transcriptional level, since the respective mRNA levels were downregulated. For the first time, pivotal molecular mechanisms concerning to metabolic behavior of SCs under diabetic-like conditions were revealed. These results evidenced the relevance of insulin for lactate production by hSCs, illustrating how metabolic diseases, and particularly DM, may hamper spermatogenesis by modulating the metabolism of SCs. Previous works have shown that insulin levels are linked to T levels. In addition, another effect of DM in the testicular secretory function is the inhibition of T synthesis by LCs. Spermatogenesis is dependent on the presence of a suitable testicular level of these sex steroid hormones. Given their location and function, SCs are constantly targeted by these hormones and the so called “male hormones” (such as T and its non aromatizable derivate 5α-DHT) exert their effects through its specific receptors present on SCs [5]. In hSCs exposed to 5α-DHT we observed a metabolic reprogramming from a Warburg-like metabolism

to an oxidative metabolism. In those conditions, SCs presented an increased glucose uptake, which was not followed by an increased production of lactate. This metabolic behavior may result from the impairment of key mechanisms involved in lactate production, such as decreased lactate synthesis by LDH enzyme and/or decreased lactate transport via MCT4, observed in 5α-DHT-exposed SCs. T is an important metabolic regulator and it was evidenced that at testicular level modulates metabolism of SCs via 5α-DHT, showing that the role of this hormone goes far beyond of spermatogenic-related events (*e.g.*: BTB remodeling, spermiation) where SCs metabolism is on the fulcrum of spermatogenesis. So pathological conditions that induce large fluctuations of T levels, directly affect the metabolism of SCs and consequently may contribute to the decline of male reproductive clinical outcomes. However, little is known how SCs metabolically respond under T deficiency induced by progressive stages of T2DM (pre-diabetes and T2DM). In this context, SCs were cultured in different concentrations of T induced by progressive states of T2DM and showed significant metabolic alterations with the more pronounced effects being concurrent with the lower levels of T. T deficiency associated to different diabetic stages seems to compromise rate-limiting steps of glycolysis. However, we showed that under severe conditions of DM, SCs are still able to adapt their metabolism in order to enhance glucose uptake. Indeed, when exposed to the T levels associated with the prodromal stage of DM, SCs were not able to uptake glucose as efficiently as those cells exposed to T2DM-like conditions. Notably, the culture conditions of T deficiency might be responsible for the substrate preference, since SCs of the T-PreD conditions preferentially consumed pyruvate, whereas SCs of the T-T2DM conditions largely consumed glucose. Still, SCs cultured under T deficiency levels associated to T-PreD and T-T2DM conditions did not produce higher amounts of lactate, as part of the pyruvate was used to produce alanine. Our results showed that T deficiency induced by the different diabetic development stages did not favor glycolytic flux of SCs. Glucose taken up by SCs was not efficiently converted into lactate, being partly redirected to alternative metabolic pathways, such as glycogen synthesis, in order to ensure endogenous reserves of glucose that allow them to face the energy requirements of germ cells. In fact, SCs are able to adapt and use glycogen since they express enzymes involved in the glycogen metabolism [6, 7]. Moreover, testicles of diabetic animals present an increased accumulation of glycogen precursors, such as uridine diphosphate glucose [8], thus supporting the hypothesis that glycogenesis may be of high relevance within the testicular *milieu* particularly under diabetic conditions. These data point towards the physiologic role that androgens (particularly T and 5α-DHT) may have on the glucose metabolism of SCs. Still, one must take into account that the conditions used in these cell culture-based systems may differ among the several studies and the media used in cultures are very complex and may exert a stoichiometric pressure towards some metabolic pathways in detriment of other rendering some careful to make definitive assumptions of the physiological situations.

As said, the current lifestyle has pushed us towards erroneous eating habits which are often associated with metabolic disorders, such as pre-diabetes. At a clinical point of view pre-

diabetes is classified as a pathological state with high risk of T2DM development, still is reversible to normoglycaemia. Nevertheless, as reproductive process is linked to energy metabolism any metabolic imbalance affects testicular physiology, so is essential disclose the effects of pre-diabetes on testicular metabolic pathways. Consequently, we evaluated the impact of pre-diabetes induced by HED consumption on testicular glucose metabolism. Our results showed that this prodromal stage of DM altered the overall testicular glucose metabolism, by increasing the glycolytic flux. We observed a significant increase in the expression of the glucose transporters (GLUT1 and GLUT3), as well as an increase in the activity of PFK, supporting the stimulation of testicular glycolytic process. As a result, lactate content was significantly increased, being also concomitant with an increased expression of both LDH and MCT4 protein levels.

This data show that testicles seem to develop adaptative mechanisms to ensure an adequate lactate supply for germ cells development, even in the prodromal stages of DM. However the increased glycolytic activity was associated with an augmented oxidative environment that led to sperm injuries. In fact, the metabolic alterations and oxidative status observed in the testicles of these animals might have contributed to the impairment of sperm quality and functionality. Pre-diabetic rats showed decreased antioxidant capacity and increased levels of protein and lipid peroxidation in testicles, evidencing a higher susceptibility to testicular oxidative damage associated with this condition. The testicular oxidative environment was enhanced by the downregulation of PGC-1α/SIRT3 axis, which is essential for the maintenance of mitochondrial biogenesis and mitochondrial antioxidant defense systems. PGC-1α is required for the induction of ROS-detoxifying enzymes and synergistically acts with SIRT3 inducing the expression of antioxidant defenses [9]. In addition, a significant loss in the mtDNA content, as well altered mitochondrial respiratory activity, were also observed. Disruption of PGC-1α/SIRT3 axis may favor important changes in the ETC, particularly at the level of complex I and complex III and these changes severely affected testicular ATP production. Such decrement in testicular ATP content may arise from its hydrolysis to subsequent adenine nucleotides (ADP and/or AMP) and adenosine metabolites. Noteworthy, the imbalance in adenine nucleotides underlies a lower testicular ATP/AMP ratio, evidencing a decreased testicular energy charge in pre-diabetic rats. Altogether these results showed that testicular metabolic changes induced by pre-diabetic state alter the metabolic homeodynamics of the testicles by increasing glycolytic flux with higher lactate production, but induces oxidative stress, which impairs the overall bioenergetic capacity that ultimately contribute to the decline of sperm quality and consequently male reproductive health. This point should deserve special attention, since as it was evidenced subtle metabolic alterations alter whole testicular bioenergetics favoring oxidative environment and such alterations are closely associated to decreased sperm parameters. In pre-diabetes, ROS production stimulated by hyperglycaemia exacerbates the testicular oxidative environment, thus contributing to significant changes in the physical and molecular composition of sperm, with implications not only in sperm quality, but also in the success of reproductive function.

Similar to what was observed in pre-diabetic rats, the initial steps of the glycolytic pathway were also stimulated in T2DM rats, since the increased testicular expression levels of glucose transporters (GLUT1 and GLUT3) was accompanied by increased activity of PFK. In those conditions, testicles were able to metabolize glucose at higher rates similarly to what happens in the prodromal stage of DM. However, in contrast to what was observed in the testicles of the pre-diabetic rats, the testicular lactate content was reduced in T2DM animals and the main contributor seems to be the decreased LDH activity, since the lactate export was not impaired. In these animals, the metabolism of glucose appears to be redirected to alternative metabolic pathways.

In this sense testicular glycogen content was favored, similarly to what was previously reported for the SCs exposed to T levels associated whit this T2DM conditions. In fact, these animals presented several conditions that favor glycogenesis: elevated levels of insulin, which stimulate glycogen synthesis through inactivation of GSK-3; increased blood-to-testicles glucose availability and increased testicular levels of UDP-glucose, which is the immediate precursor in glycogen synthase reaction. These results suggest that a metabolic reprogramming may happen within testicular environment, in which the increased glucose influx together with the reduced glucose aerobic fermentation augments the content of UDP-glucose, similarly to what was observed in cardiac tissue of diabetic rats [10]. The increased expression of glucose transporters, in opposition to the lower lactate production, and together with the higher levels of UDP-glucose in T2DM rats illustrate that T2DM stimulates glycogen accumulation. However, despite the ability of testicles to adopt alternative pathways, the altered glucose utilization and the glycogen accumulation observed in testicles of T2DM rats was concurrent with the impairment of sperm quality parameters. Furthermore, the effects of T2DM on sperm parameters were more marked to what was observed in the prodromal stage of DM, illustrating that the progression to T2DM is associated with more pronounced alterations of testicular glucose metabolism that are closely associated with a decline of male reproductive health.

Conclusion

Spermatogenesis is a complex process that depends on the coordinated action of different types of testicular cells. In this process, the metabolic cooperation established between SCsand developing germ cells plays an essential role. Indeed, glucose metabolism is crucial for spermatogenesis and compelling evidence show that the metabolic behavior of SCs goes far beyond to the nourishment of germ cells. In recent years, cell metabolism has been highlighted as a hot topic in research areas such as cancer, however in what concerns male fertility this subject remains somewhat neglected.

The normal reproductive function depends on the adequate nutritional state. The excessive and/or poor nutritional food intake (e.g. excessive intake of "unhealthy" fats) promotes the development of metabolic diseases and affects the male reproductive function. This work put forward new findings regarding to the testicular metabolic alterations induced by progressive

stages of DM, specifically pre-diabetes (associated to HED overconsumption) and established T2DM. The results obtained herein revealed that the success of the spermatogenic event is intimately associated with testicular metabolism, in particular glucose metabolism. It became evident that testicular glucose metabolism is differentially modulated according to the developing stage of DM and both cell culture-based systems and animal models used in this work showed that the production and export of lactate, which is the main substrate of developing germ cells, may be compromised. These effects were more pronounced with the advanced stage of disease. However, even in the more severe conditions, testicles (and particularly SCs) tend to adapt metabolically to guarantee the energy reserves for the spermatogenic event. Still, the metabolic alterations observed were associated with decreased sperm quality parameters, contributing to a decline on male fertility potential.
Hence, testicular metabolism is a control point of spermatogenesis and the full enlightenment of the molecular mechanisms that may be responsible for the disruption of the metabolic cooperation between SCs and developing germ cells should deserve a special consideration from researchers of this field. Particular emphasis and efforts should be made in order to clarify how testicular metabolic disruption associated to pathological states may end-up in infertility. There is still much work to be done in this field, since multiple potential threats for male reproductive health are encountered not only by exposure to modern dietary habits (and consequent development of metabolic diseases such as pre-diabetes, T2DM, obesity), but also to environmental hazards, which may impact testicular cells metabolism and male fertility.
Another point of concern is that these threats can induce biological alterations in male gametes, thus contributing for transgenerational amplification of subfertility and infertility. Evidences have shown that phenotypic outcomes may be attributed to the inheritance of environmentally induced functional changes of the genome, driven by epigenetic components. Molecular evidence suggests that epigenetic marks in gametes play important roles in the transmission of phenotypes from parents to offspring. Clearly these are exciting topics that researchers of the reproductive biology field will face in future.

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Annex

Supplementary Data

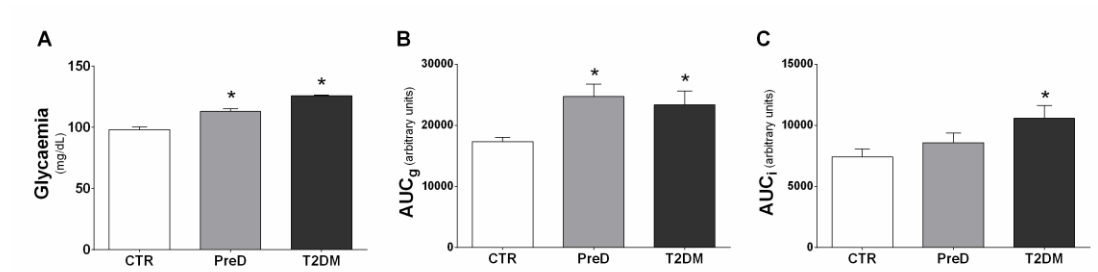

Figure 1. (A) Blood glucose levels of control group, PreD and T2DM group animals after the respective treatment. (B) AUCg of the intraperitoneal glucose tolerance test performed in control group, PreD and T2DM group animals. (C) AUCi of the intraperitoneal insulin tolerance test performed in control, PreD and T2DM group animals. Results are presented as mean ± SEM of six independent experiments, corresponding to six animals/group. * Significantly different relative to control (p< 0.05). AUCg – area under the curve for glucose tolerance test; AUCi - area under the curve for insulin tolerance test; CTR – control; PreD – pre-diabetes; T2DM – type 2 diabetes mellitus.

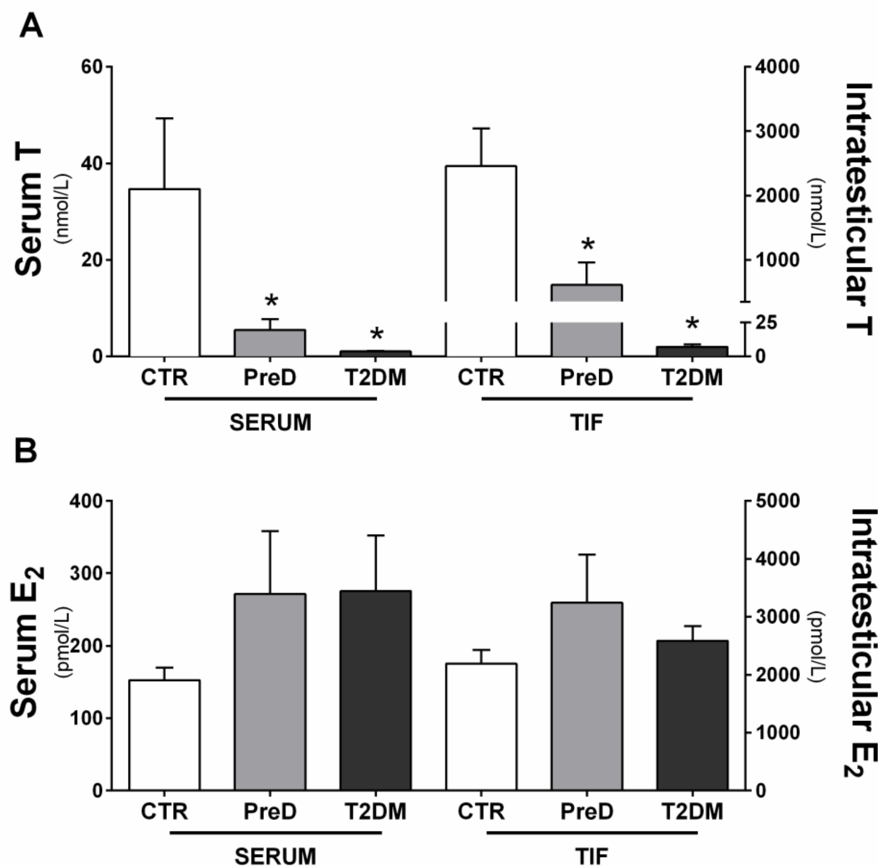

Figure 2. (A) Testosterone levels in serum and testicular interstitial fluid of control, PreD and T2DM group animals. (B) The 17β-estradiol levels (E2) in serum and testicular interstitial fluid of control, PreD and T2DM group animals. Results are expressed as mean ± SEM (n = 6 for each condition). *Significantly different relative to control (p < 0.05). CTR – control; PreD – pre-diabetes; T2DM – type 2 diabetes mellitus.