Molecular Human Reproduction, Vol.23, No.11 pp. 725–737, 2017
Advanced Access publication on September 28, 2017 doi:10.1093/molehr/gax049

molecular human reproduction

REVIEW

Glycerol and testicular activity: the good, the bad and the ugly

Luís Crisóstomo[1,2,3], Marco G. Alves[1], Giuseppe Calamita[4], Mário Sousa[1,5], and Pedro F. Oliveira[1,2,3,4,*]

[1]Department of Microscopy, Laboratory of Cell Biology, and Unit for Multidisciplinary Research in Biomedicine (UMIB), Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Porto, Portugal [2]Department of Genetics, Faculty of Medicine, University of Porto, Porto, Portugal [3]i3S—Instituto de Investigação e Inovação em Saúde, University of Porto, Porto, Portugal [4]Department of Biosciences, Biotechnologies and Biopharmaceutics, University of Bari 'Aldo Moro', Bari, Italy [5]Centre for Reproductive Genetics Professor Alberto Barros, Porto, Portugal

*Correspondence address: Department of Microscopy, Laboratory of Cell Biology, Institute of Biomedical Sciences Abel Salazar (ICBAS), Rua de Jorge Viterbo Ferreira no. 228, 4050-313 Porto, Portugal. Tel: +351-220-428000; E-mail: pfobox@gmail.com

Submitted on April 21, 2017; resubmitted on August 7, 2017; editorial decision on September 4, 2017; accepted on September 5, 2017

Over the past decades, there have been several studies suggesting that semen quality is declining. Interestingly, these observations are paired with a significant increase in the number of individuals diagnosed with metabolic diseases, including obesity and diabetes mellitus. Hence, it is tempting to hypothesize that obesity and its associated comorbidities and risk factors (such as a hypercaloric diets) impair the homeostasis of the male reproductive health, with a possible direct effect on the testes. The blood and interstitial fluids of obese individuals usually have increased levels of glycerol, notably due to triglyceride and phospholipid catabolism and high fructose intake. Glycerol is metabolized via intermediary metabolism by a group of reactions centred at the glycerol-3-phosphate shuttle, which links the metabolic pathway of glucose, lipids and oxidative phosphorylation, illustrating its high relevance for biological systems. Glycerol enters and exits the cells by the action of specialized carriers, known as aquaglyceroporins, whose functional importance for male reproductive health has emerged in the last few years. Notably, glycerol has antispermatogenic properties. When present in high concentration in the testis, it causes blood–testis barrier disruption, impairing tubular fluid homeostasis. Nevertheless, glycerol metabolism in testicular cells remains a matter of debate. Herein we discuss previous and current research concerning the role of glycerol and its metabolism in testicular cells, and how it can influence testicular activity.

Key words: aquaglyceroporins / glycerol / male fertility / metabolism / obesity / spermatogenesis

Introduction

Glycerol is an important metabolite in the human body, connecting the metabolic pathways of carbohydrates (glycolysis and gluconeogenesis) and lipids (glyceroneogenesis). In addition, after phosphorylation, it transfers reducing equivalents from mitochondria to cytosol, via the glycerol phosphate shuttle (Meyerhof, 1919; Euler *et al.*, 1937; Lin, 1977; Dulermo and Nicaud, 2011; Mráček *et al.*, 2013). The main source of plasma glycerol is the hydrolysis of ester bonds between the glycerol backbone and the acyl groups (fatty acids (FAs)) of triacylglycerols (TAGs) (Lin, 1977). The glycerol needed for lipid biosynthesis is mostly obtained from dietary intake of TAG and carbohydrate metabolism. However, it can also be directly ingested without adverse effects, due to its low toxicity (LD_{50} > 25 000 mg/kg for oral glycerol intake on rats) (Hine *et al.*, 1953; Bartsch *et al.*, 1975). Still, it is a banned substance by the World Anti-Doping Agency (WADA), because it is used to mask doping substances in athletes due to its hyperhydrating properties (Nelson *et al.*, 2011).

Glycerol concentrations in the human body are controlled within narrow intervals, but there are significant differences between different tissues and according to age and lifestyle. The average bloodstream concentration in adults ranges from 0.05 to 0.1 mM (Hagstrom-Toft *et al.*, 1997; Nelson *et al.*, 2011; Vestergaard *et al.*, 2013), but newborns can present up to 0.4 mM in their first 48 h of life (Persson and Gentz, 1966), and obese or diabetic adults can show persistent glycerol concentrations up to 0.3 mM (Hagen *et al.*, 1963; Bortz *et al.*, 1972; Lin, 1977). Conditions that promote lipid mobilization, such as physical exercise, fasting or high levels of catecholamines and theophylline temporarily increase glycerol serum levels (Nilsson-Ehle *et al.*, 1975; Nelson *et al.*, 2011; Schmidt *et al.*, 2014). The highest extracellular concentration of glycerol (almost three times more concentrated than in bloodstream) is found in adipose tissue, its main source (Hagstrom-Toft *et al.*, 1997). Thus, metabolic modulation of adipose tissue dynamics, particularly by insulin, plays a decisive role in glycerol regulation. Insulin resistance, a condition that occurs in type 2 diabetes mellitus (T2DM), promotes glycerol release into the bloodstream, due

to increased lipid mobilization (Suganami *et al.*, 2005; Lafontan and Langin, 2009; Girousse *et al.*, 2013; Ertunc *et al.*, 2015; Morigny *et al.*, 2016). Increased lipid mobilization stimulates mitochondria to break down FAs through β-oxidation. This overstimulation boosts ATP production and triggers a negative feedback over insulin-induced glucose uptake, through AMP-activated protein kinase (AMPK) inhibition (Minokoshi *et al.*, 2004; Kahn *et al.*, 2005; Ye, 2013). In obesity, insulin resistance may arise from hyperstimulation of β-islet cells, which release more insulin, while its clearance is reduced in liver and kidney, causing hyperinsulinaemia (Michael *et al.*, 2000; Farris *et al.*, 2003; Ye, 2013). Hence, obesity and the associated free fatty acid (FFA) release into the bloodstream may be implicated in the development of insulin resistance (a key feature of T2DM onset). Still, glycerol does not seem to induce insulin resistance in human cells, *per se* (Hoeks *et al.*, 2012; Nowotny *et al.*, 2013). Although glycerol is a structural element of TAGs, along with FAs, it does not stimulate insulin secretion (Noel *et al.*, 1997). Its serum concentrations rather reflect lipogenesis or lipolysis rate.

There are, however, consequences of high extracellular glycerol concentrations. One of the best-described effects is the transient arrest of spermatogenesis (Wiebe and Barr, 1984a,b; Igdoura and Wiebe, 1994). This effect was shown after intratesticular administration of a glycerol solution, which destabilized the tight-junctions between Sertoli cells (SCs), leading to a leaky blood–testis barrier (BTB) (Wiebe *et al.*, 2000; Wong and Cheng, 2005). This phenomenon looked promising for years as a means to develop a 'male contraceptive pill'. However, it was later reported that such glycerol injections produced several undesirable secondary effects, including long-term irreversible spermatogenic arrest (Eng *et al.*, 1994; Cheng and Mruk, 2002; Igdoura and Wiebe, 1994). Thus, research on this topic has been abandoned. Nevertheless, although Wiebe *et al.* (2000) had injected a supraphysiological glycerol concentration on the subjects (>1 M), it was already demonstrated that the negative effects of acute testicular exposure to environmental toxicants might be similar to those observed after lower-dose, chronic exposure to the same agent (Cardoso *et al.*, 2017) and thus, it cannot be disregarded the relevance of those findings concerning glycerol toxicity in the testis.

During the last decades, the global prevalence of metabolic disorders, such as T2DM, has increased (WHO, 2000), and semen quality has been reported to have declined over a similar time scale (Hamilton and Ventura, 2006; Rato *et al.*, 2012b; Skakkebaek *et al.*, 2016). Notably, the two most prevalent metabolic pathologies (obesity and T2DM) increase glycerol concentrations in tissues and bloodstream, and high extracellular glycerol concentrations in testicular tissue may result in spermatogenesis arrest (Eng *et al.*, 1994; Wiebe *et al.*, 2000). The exact same pathologies are related to poor fertility outcomes, particularly in males, a relation already supported by laboratorial and clinical evidence (Anderson and Thliveris, 1986; Cai *et al.*, 2000; Alves *et al.*, 2013a, 2016). Therefore, we speculate that high blood glycerol concentrations may be associated with male (in)fertility promoted by metabolic disorders and associated comorbidities, such as overweight, obesity and T2DM. In this review, we discuss the effect of glycerol on testicular activity and male fertility to shed light, who might be the good, the bad and the ugly on this complex metabolic script. This review comprises bibliographic records searched on the first trimester of 2017, from Pubmed and Google Scholar databases. We included all scientific communications published after 1900. The main search terms were 'glycerol metabolism', 'glycerol and fertility', 'glycerol in testicular cells', 'testes metabolism', 'aquaglyceroporins' and 'intermediary metabolism'. MeSH term search was done when applicable.

Testicular cells and testicular metabolism: brief overview

When discussing testicular metabolism, we must account for the pivotal role played by SCs. They are essential for spermatogenesis, granting physical and nutritive support to developing germ cells (Alves *et al.*, 2013b; Boussouar and Benahmed, 2004; Dias *et al.*, 2014; Fijak *et al.*, 2011; Oliveira and Alves, 2015; Zheng *et al.*, 2015). In addition, they are also responsible for maintaining the integrity of the BTB (Dimitriadis *et al.*, 2015; Fijak *et al.*, 2011; Griswold, 1998; Oliveira and Alves, 2015), having exocrine and paracrine activities (Chemes *et al.*, 2008; Kumanov *et al.*, 2005; Takase and Nusse, 2016) and being a main hormonal target and regulation point of male fertility (Alves *et al.*, 2012; Bernardino *et al.*, 2016a; Crespo *et al.*, 2016; Guma *et al.*, 1997; Samy *et al.*, 2000) (Fig. 1).

In rodent models, developing germ cells are unable to metabolize glucose rather depending upon the lactate provided by the SCs (Boussouar and Benahmed, 2004; Jutte *et al.*, 1981; Rato *et al.*, 2012b), whereas, in humans, lactate has demonstrated anti-apoptotic effects on germ cells (Erkkilä *et al.*, 2002). Nonetheless, the dependence on lactate is particular to each differentiation stage (Bajpai *et al.*, 1998b). Mature sperm cells use glucose and other hexoses as main substrate (Dias *et al.*, 2014; Miki, 2006), and rely also on lipid β-oxidation, as suggested by the expression of a significant proportion of enzymes related to lipid metabolism (Amaral *et al.*, 2013).

SCs do not rely on glucose oxidation to overcome their energetic needs. Instead, they mainly rely on β-oxidation for internal energy consumption, while the pyruvate generated by glycolysis is directed for lactate production, the main metabolic substrate for germ cells (Oliveira *et al.*, 2015a; Rato *et al.*, 2012b; Xiong *et al.*, 2009). Only about a quarter of the pyruvate synthetized from glucose by SCs enters the Krebs cycle (Grootegoed *et al.*, 1986; Rato *et al.*, 2012b).

Sex steroid hormones modulate SC metabolism, 5α-dihydrotestosterone (DHT) and 17β estradiol (E_2) inhibit lactate production and export, decreasing lactate dehydrogenase (LDH) and monocarboxylate transporter isoform 4 (MCT4) expression (Dimitriadis *et al.*, 2015; Rato *et al.*, 2012a, b). E_2 stimulates acetate secretion by SCs at the expense of lactate, whereas insulin deprivation decreases acetate secretion (Rato *et al.*, 2012a). This hormonally regulated production of lactate is particularly crucial in overweight, obese and diabetic men, as their sex steroid hormone levels are frequently altered towards a greater estrogen:androgen ratio, in comparison to normal values (Rato *et al.*, 2015). SCs metabolism is also affected by drugs prescribed to T2DM patients. (Alves *et al.*, 2014; Meneses *et al.*, 2016). Pioglitazone increases the expression of several glucose transporters (GLUT1, GLUT2 and GLUT3) and enzymes of the glycolytic pathway in SCs, increasing glycolytic flux and lactate production (Meneses *et al.*, 2016). Metformin, despite decreasing glucose transporter mRNA expression in SCs, increases alanine and lactate production, improving SCs oxidative status (Alves *et al.*, 2014).

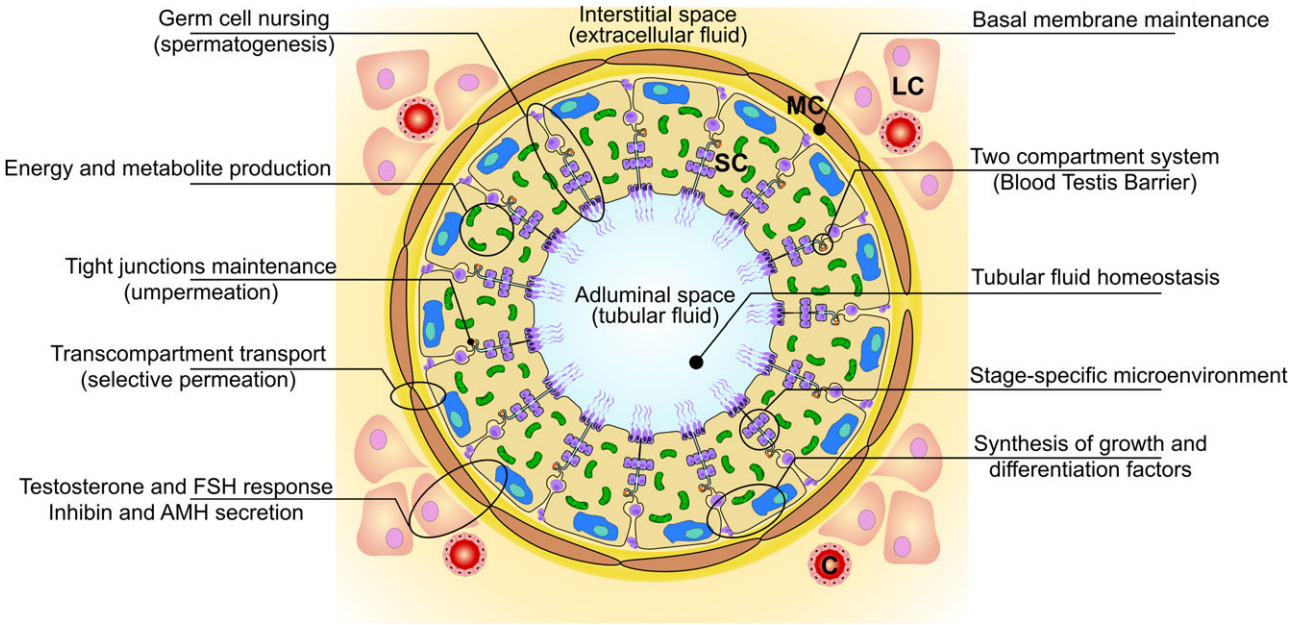

Figure 1 The role of Sertoli cell on spermatogenesis and on testicular tissue physiology. Schematic representation of a cross-sectional cut from a seminiferous tubule (SeT), to illustrate the crucial role of Sertoli cells (SCs) in the testicular morphology and physiology. Germ cells differentiate in the microenvironment created by SC plasmatic membrane invaginations. This microenvironment is specific to the differentiation state of the germ cells. SCs also maintain the highly impermeable blood–testis barrier (BTB), enabling the creation of two separated compartments: the immune-privileged adluminal space, and the extracellular interstitial space. SCs are physically part of this barrier, and the tight junctions between SCs are one of the most important features to guarantee BTB's tightness. However, since few substances can cross BTB, germ cells depend upon the nutritional support and selective substance transport by SCs. Besides those functions, SCs function is modulated by steroid hormones and have themselves endocrine activity. C—capillary, LC—Leydig cell, MC—Myoid cell.

SC metabolism is also regulated by hormones linked to metabolic homeostasis such as insulin, ghrelin and leptin (Alves *et al.*, 2016, 2015, 2013b). As demonstrated by Oliveira *et al.* (2012), using an insulin-deprived SC model, its absence causes decreases in initial glucose consumption and lactate production, linked to altered expression of LDHA, MCT4 and GLUT3 in SCs. In SCs, ghrelin a hormone linked to satiety and disrupted in obesity, stimulates GLUT1 and LDH expression and activity, and glucose consumption, whilst decreasing alanine and acetate production. Thus, ghrelin apparently directs SC glucose metabolism further towards lactate production (Martins *et al.*, 2016, 2015b). Leptin, another hormone linked to satiety and produced by adipocytes, modulates SC metabolism, resulting in increased LDH activity whilst decreasing acetate production (Martins *et al.*, 2015a). Some of these effects are dose dependent and GLUT2 mRNA and protein levels are up-regulated in lean-like leptin concentrations, but not in obese-like concentrations (Martins *et al.*, 2015a).

Glycerol metabolism

Glycerol is a central intermediary in cellular metabolism and it is metabolized by the liver (Peroni *et al.*, 1995). This polyol is at a 'crossroads' between several metabolic pathways, due to the role of its phosphorylated form, glycerol-3-phosphate (G3P), in intermediary (or central) metabolism (Euler *et al.*, 1937; Lin, 1977; Meyerhof, 1919; Seidler, 2013). Glycerol is activated into G3P by glycerol kinase (GK) (Boyer and Krebs, 1987; Hirschmann, 1960; Kosuga *et al.*, 2011). This reaction was thought to be irreversible, but a recent study reports the existence of a glycerol-3-phosphate phosphatase (G3PP) in mammals, capable of the inverse reaction (Mugabo *et al.*, 2016). After being converted to G3P, glycerol may be used for gluconeogenesis (Mohammad *et al.*, 2015), further oxidized for ATP generation (glycolysis) (Hibuse *et al.*, 2009), enter the pentose-phosphate pathway (Jin *et al.*, 2014) or be incorporated into lipids (lipogenesis and glyceroneogenesis) (Christoffersen *et al.*, 2003; Lin, 1977; Mugabo *et al.*, 2016; Nye *et al.*, 2008; Seidler, 2013). The balance between those pathways is determined by a well-regulated chemical equilibrium depending on substrate abundance, enzyme availability, enzyme activation or inhibition, allosteric regulation and co-factors (Boyer and Krebs, 1987; Chaves *et al.*, 2012; Euler *et al.*, 1937; Garrib and McMurray, 1986; Hellung-Larsen *et al.*, 1987; Hirschmann, 1960; Meyerhof, 1919; Mráček *et al.*, 2013; Mugabo *et al.*, 2016).

The main reactions of glycerol metabolism are condensed in the glycerol-3-phosphate shuttle (Fig. 2), a cycle centred at two different isoforms of glycerol-3-phosphate dehydrogenase (GPD) (Dipple and McCabe, 2001; Green, 1936; Hopkinson *et al.*, 1974; Lin, 1977; Mráček *et al.*, 2013). For years, the physiological importance of this shuttle was underestimated. It was postulated that it would need equimolar concentrations of the two GPD isoforms, the cytosolic

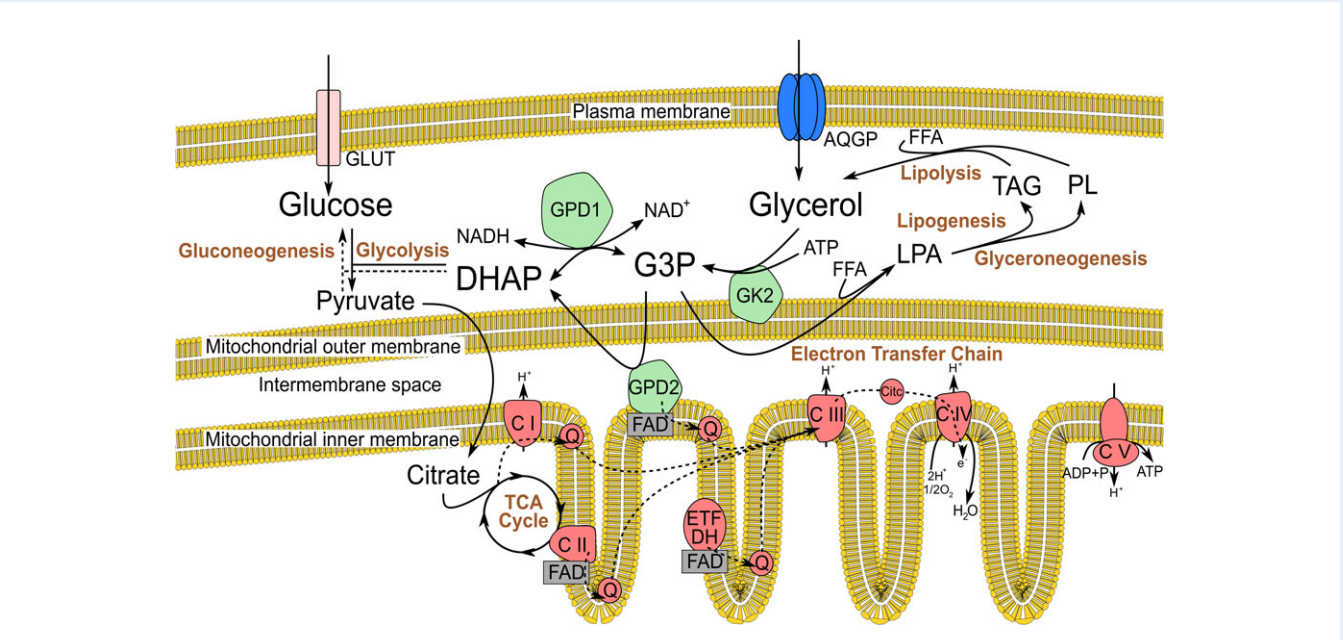

Figure 2 The glycerol-3-phosphate shuttle in Sertoli cells, and its relation to other metabolic pathways. Glycerol kinase 2 (GK2) phosphorylates glycerol into glycerol-3-phosphate (G3P), initiating the shuttle reaction cycle. G3P may be oxidized to dihydroxyacetone phosphate (DHAP) by glycerol-3-phosphate dehydrogenase 1 (GPD1), the cytosol enzyme, or glycerol-3-phosphate dehydrogenase 2 (GPD2), the mitochondrial enzyme. Although GPD2 catalysed reaction is irreversible, from both reactions result reducing equivalents as by-products. Reducing equivalents from the GPD2-mediated reaction are collected by the FAD co-factor linked to it, which then feed the ubiquinone pool. FAD co-factor is not freely available in mitochondria's matrix, so its reduction and oxidation happens at the enzyme interaction site. This is valid for GPD2 and other enzymes having FAD as co-factor. DHAP may follow the upstream route to glucose (gluconeogenesis) or be further oxidized into pyruvate (glycolysis). G3P may not be converted to DHAP and follow the lipid pathway (glyceroneogenesis, lipogenesis). Glycerol is obtained from extracellular medium thanks to aquaglyceroporins (AQGPs), or from the intracellular TAG hydrolysis (lipolysis).

NAD-dependent GPD1 and the mitochondrial FAD-dependent GPD2, as the former is scarce in most mammalian tissues (Houštěk *et al.*, 1975; Mráček *et al.*, 2013; Ohkawa *et al.*, 1969). When it was found that the G3P shuttle is active in rat brown adipocyte tissue (BAT), it was hypothesized that it could have a critical role in mammalian metabolism, rerouting it according to the energetic needs (Chaves *et al.*, 2012; Houštěk and Drahota, 1975; Mráček *et al.*, 2013; Ohkawa *et al.*, 1969).

Deficiencies of glycerol metabolism have severe impacts on human health, because they affect every metabolic pathway where this polyol is involved. Genetic mutations that affect enzymes intervening in G3P shuttle (GPD1, GPD2) and glycerol activation (GK) are rare but, if left uncontrolled, they cause serious neuronal damage and death at early age, or arise as chronic metabolic diseases (Dionisi-Vici *et al.*, 2016; Rahib *et al.*, 2007; Wikiera *et al.*, 2012; Wu *et al.*, 2015).

Molecular basis of glycerol transport by aquaglyceroporins

One key determinant of glycerol metabolism is its intracellular availability. Although glycerol is a small, uncharged molecule, its transport depends upon facilitation by transmembrane proteins, the aquaglyceroporins (AQGPs), which regulate substrate availability for glycerol metabolism (Bernardino *et al.*, 2016b), also known as glycerol facilitation-like proteins (GLP), a protein subfamily from the aquaporin (AQP) family (Kruse *et al.*, 2006). From the 13 aquaporin classes identified thus far in humans, four AQGPs (AQP3, AQP7, AQP9 and AQP10), and three more AQPs from other families (AQP6, AQP8 and AQP11) transport glycerol (Calvanese *et al.*, 2013; Finn and Cerdà, 2015).

AQPs are tetrameric proteins, formed by four proteinaceous transmembrane pores placed in parallel, which creates a fifth pore on the centre of the tetramer (Fu *et al.*, 2000; Murata *et al.*, 2000; Walz *et al.*, 1997) (Fig. 3). Aquaporin-mediated transport of glycerol and other solutes was first described as a channel-driven mechanism, but its dependence upon concentration and temperature, plus the competitive inhibition by glycerol-like compounds, are more related to a carrier-mediated transport (Ishii *et al.*, 2011; Katano *et al.*, 2014; Ohgusu *et al.*, 2008).

AQGP play a crucial role on FFA and glycerol homeostasis. Their expression is mediated by the metabolic status of the individual, and is concerted on liver and adipose tissue to maintain the balance between lipolysis and lipogenesis, by the action of insulin and leptin (Fig. 4) (Méndez-Giménez *et al.*, 2014; Gena *et al.*, 2017; Rodríguez *et al.*, 2011a). The importance of this cooperation to avoid pathological states has been already studied. Murine knock-out models for AQP9 show that its absence increased blood levels of glycerol and FFAs, a fertile ground to induce insulin resistance (Rojek *et al.*, 2007). In humans, AQP7 expression was found to be down-regulated in a lean

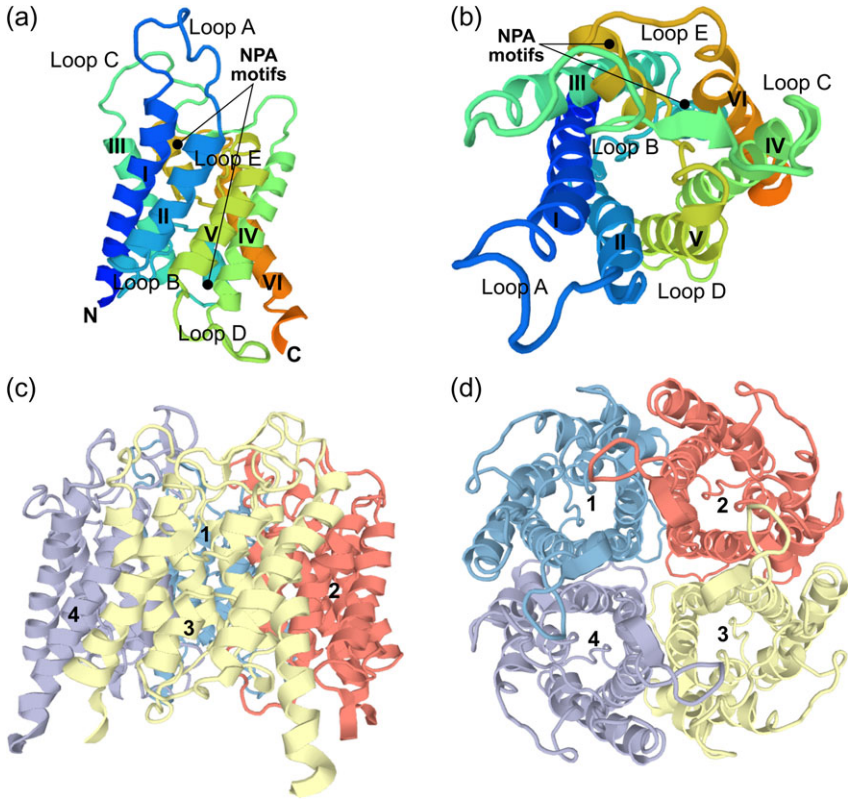

Figure 3 Structural details of the classical aquaporin (AQP). A classic-aquaporin monomer weights from 24 to 35 kDa and consists of six α-helix (I–VI) linked by five loops (A–E). Helixes confer hydrophobic and liposoluble properties to AGPs, enabling their transmembrane location. Loops A, C and D are located out of the cell membrane, due to their higher hydrophilicity. Loops B and E present small helixes thanks to the NPA motif (from the single letter aminoacid code: N—asparagine, P—proline, A—alanine), which confer them hydrophobic properties crucial for pore formation. This monomeric structure resembles an hourglass, both in shape and functioning. However, AQPs usually become fully functional only when arranged in a tetramer. This arrangement permits the formation of five pores, one per monomer plus a fifth pore in the middle of the monomers (top view). (**a**) AQP monomer, lateral view. N—amino-terminus; C—carboxyl-terminus. (**b**) AQP monomer, top view. (**c**) AQP homotetramer, lateral view. (**d**) AQP homotetramer, top view.

population, comparing to an obese population, which may indicate it is a susceptibility factor towards obesity (Marrades *et al.*, 2006). On the opposite trend, AQ7 expression is up-regulated in the white adipose tissue of streptozotocin-induced diabetic mice, eliciting its role on glycerol metabolism (Skowronski *et al.*, 2007).

Concerning human reproductive function, several studies have highlighted the role of AQGPs (or glycerol transporting AQPs) on male reproductive tissues and cells (Jesus *et al.*, 2014; Saito *et al.*, 2004; Yeung, 2010; Yeung *et al.*, 2010). AQP7, AQP8 and AQP11 were identified in spermatozoa and germ cells (Yeung, 2010; Yeung *et al.*, 2010). AQP3, AQP9 and AQP10 expression is described in testicular tissue, including in SCs and LCs (Elkjaer *et al.*, 2000; Huang *et al.*, 2006; Jesus *et al.*, 2014). A study by Chen *et al.* (2011) suggested that AQP3 is essential for spermatozoa to tackle the progressive osmotic decrease through the female reproductive tract. AQP3-deficient mice showed impaired sperm motility, due to spermatozoa inability to regulate its cytoplasmic volume, which led to tail deformations (Chen *et al.*, 2011). AQP7 is expressed on human spermatozoa and is crucial for sperm motility (Saito *et al.*, 2004). In this case, and similarly to findings of Chen *et al.* (2011) regarding the function of AQP3, AQP7 transports water against the osmotic gradient, during spermiogenesis, to control sperm cell volume for proper motility in the mature spermatozoa (Saito *et al.*, 2004). Jesus *et al.* (2014) found that AQP9 interacts with the cystic fibrosis transmembrane conductance regulator (CFTR) in rat SCs, and their molecular interaction might be essential for the homeostasis of seminiferous tubular secretion. HCO_3^- transport by CFTR is critical in normal male reproductive function to maintain a good ionic balance and pH in tubular fluid for proper spermatogenesis, in sperm capacitation and even for egg fertilization (Bernardino *et al.*, 2013). Those studies highlighted the relevance of AQPs for male fertility but the possible role for the transport of glycerol by these transporters remains overlooked and needs further elucidation.

Biological relevance for glycerol metabolism: from basic aspects to male (in)fertility

Glycerol is potentially a substrate for gluconeogenesis, glycolysis, lipogenesis and glyceroneogenesis (Chaves *et al.*, 2012; Jin *et al.*, 2014;

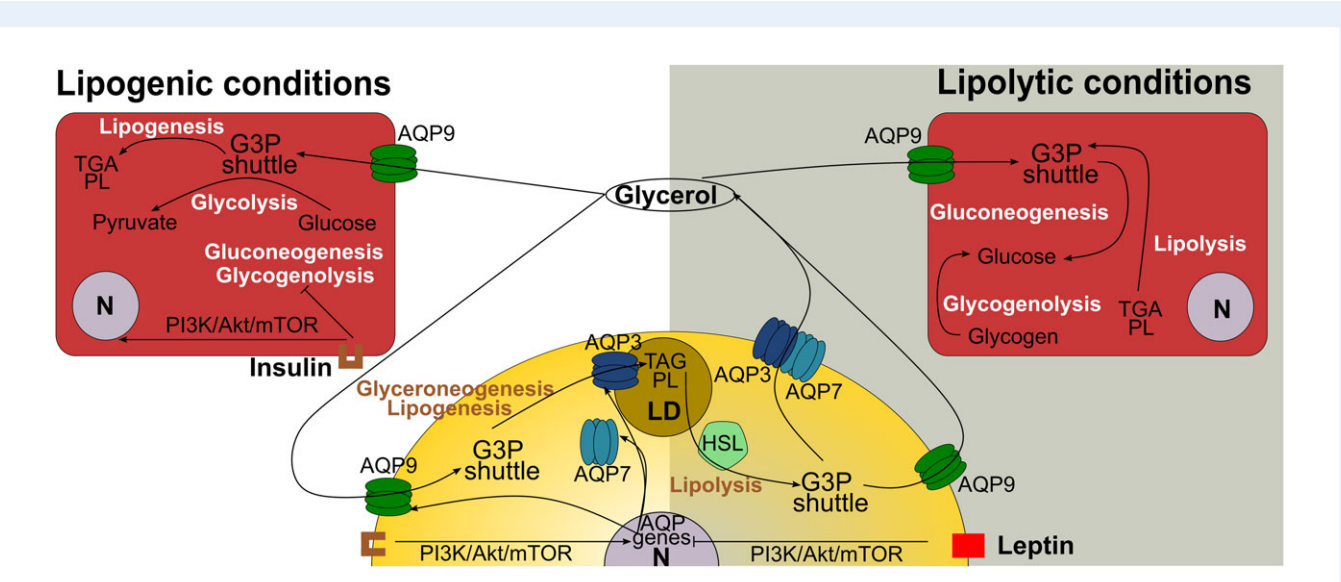

Figure 4 The role of adipocyte and hepatocyte aquaglyceroporins (AQGPs) in glycerol serum concentration. Under lipogenic conditions (left), insulin is secreted by pancreas and stimulates AQGPs gene expression in both adipocytes and hepatocytes, via mammalian target of rapamycin (mTOR) signalling. AQP9 protein is expressed at both cells plasmatic membrane to promote glycerol intake, which then enters glycerol-3-phosphate (G3P) shuttle. Glycerol is used or stored by these cells, reducing its serum concentration. Under lipolytic conditions (right), adipocytes release leptin, which causes an autocrine effect. It inhibits AQGPs gene expression via mTOR signalling, but promotes AQP3 and AQP7 migration to the plasmatic membrane, whose expression was promoted by insulin in lipogenic conditions. There, they enhance adipocyte's capacity to export glycerol, increasing its serum levels. Meanwhile, due to insulin absence, hepatocytes are promoted to obtain *de novo* glucose. However, its ability to import glycerol is not inhibited, and it is only affected by glycerol availability in extracellular medium. This mechanism permits glycerol usage as substrate for gluconeogenesis in liver, while it is being released by adipocytes.

Mohammad *et al.*, 2015), and indirectly influences other metabolic pathways, such as lipolysis, pentose-phosphate pathway (PPP), glycogenesis, tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) (Jin *et al.*, 2014; Wu *et al.*, 2015). The cross-talk between those metabolic pathways is centred on G3P, and it is referred as intermediary metabolism (Dulermo and Nicaud, 2011; Euler *et al.*, 1937; Green, 1936; Lin, 1977; Mráček *et al.*, 2013; Mugabo *et al.*, 2016; Schlossman and Bell, 1977).

Being at the crossroads of so many metabolic pathways, 70–90% of the whole body glycerol metabolism takes place in the liver (Peroni *et al.*, 1995). However, to be metabolized, glycerol needs a previous activation by addition of a phosphate group, a phosphorylation catalysed by GK (Kosuga *et al.*, 2011; Wikiera *et al.*, 2012), which presents two known isoforms, GK1 and GK2 (Sargent *et al.*, 1994). GK1 activity is ubiquitously found on adult somatic cells, whilst GK2 activity seems to be restricted to testes and foetal tissues (Sargent *et al.*, 1994). In the opposite direction, G3P can be dephosphorylated back to glycerol, in rat hepatocytes and pancreatic β-cells, by the glycerol-3-phosphate phosphatase (G3PP) (Mugabo *et al.*, 2016).

One of the possible outcomes of the intermediary metabolism is the reversible dehydrogenation of G3P into dihydroxyacetone phosphate (DHAP) by the cytosol enzyme GPD (cGPDH or GPD1), having NAD^+ as electron acceptor (Green, 1936; Houštěk *et al.*, 1975; Wu *et al.*, 2015). DHAP is a substrate for both gluconeogenesis or glycolysis (Houštěk *et al.*, 1975; Wu *et al.*, 2015). G3P dehydrogenation can also be performed by the mitochondrial FAD-linked isoform of GPD (mGPDH or GPD2). However, this enzyme is unable to catalyse the reverse reaction (Bukowiecki and Lindberg, 1974; Hopkinson *et al.*, 1974; Lin, 1977; Mráček *et al.*, 2013; Wu *et al.*, 2015).

The conversion from G3P to DHAP (and vice-versa) by GPD1 and GPD2 is known as the G3P shuttle (Mráček *et al.*, 2013). This shuttle summarizes the metabolic crossroads of the intermediary metabolism into three outcomes: (i) DHAP, to 'feed' glucose metabolism (Hibuse *et al.*, 2009; Mohammad *et al.*, 2015); (ii) G3P, to store reserves from lipid metabolism (Chaves *et al.*, 2012; Christoffersen *et al.*, 2003; Nye *et al.*, 2008); and (iii) electron transfer to ubiquinone and then to Complex III of the electron transfer chain (ETC), to generate energy by OXPHOS (Bukowiecki and Lindberg, 1974; Garrib and McMurray, 1986; Mráček *et al.*, 2014).

The reaction catalysed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an important metabolic regulation point in glucose metabolism, since it is competitively inhibited by its end-product, 1,3-biphosphoglycerate (1,3-BPG), diverting glycerol from entering the glycolytic pathway (Seidler, 2013; Williamson, 1965). In addition, it may be considered as part of the G3P shuttle, since the NAD^+ formed by the reaction mediated by GPD1 is used as electron receptor to this reaction (Seidler, 2013; Wick *et al.*, 1957). Further eliciting the complexity of glycerol metabolism, G3P shuttle links to yet another shuttle, the malate-aspartate shuttle, as the NADH resulting from GPD1 reaction may donate electrons to the cytosolic malate dehydrogenase (Mráček *et al.*, 2013; Sato *et al.*, 2015; Wick *et al.*, 1957).

Another possible outcome of intermediary metabolism is the lipid pathway, the glycerolipids/FFA cycle (Chaves *et al.*, 2012; Christoffersen

et al., 2003; Nye et al., 2008). The G3P shuttle receives metabolites from lipolysis and provides intermediaries for lipogenesis and glyceroneogenesis (Chaves et al., 2012; Christoffersen et al., 2003; Mráček et al., 2013; Mugabo et al., 2016; Nye et al., 2008). Acyl-CoA esters and FFAs are effective (but reversible) inhibitors of the G3P shuttle, by inhibiting GPD2 (Bell and Coleman, 1980; Bukowiecki and Lindberg, 1974; Houštěk and Drahota, 1975; Mráček et al., 2013). Acyl-CoA synthetase (ACS) catalyses the first step of FFA lipolysis and has a major importance for glycerol metabolism, as it originates free glycerol upon the removal of the acyl groups (Hellung-Larsen et al., 1987; Li et al., 2009; Stadtman and Kornberg, 1953). Human and mice present an isoform of this enzyme, ACSBG2, specifically expressed on brain and testes (Pei et al., 2006). Interestingly, in mice, mRNA for ACSBG2 was only found in post pubertal subjects, particularly in SCs and germ cells (Fraisl et al., 2006; Zheng et al., 2005). It is also missing in azoospermic mice (Zheng et al., 2005), suggesting an involvement of this isoform in spermatogenesis, possibly permitting a suitable lipid metabolism necessary for germ cell membrane remodelling (Fraisl et al., 2006; Pei et al., 2006). Bajpai et al. (1998a) refer that ACSBG2 might be involved on the 'lipid cycle' between SCs and germ cells, as spermatocytes and spermatids totally lack this enzyme, and therefore rely on SCs to mobilize FAs from its lipid droplets partially metabolized for their own utilization.

GK2 is the testis-specific isoform of GK, catalyst of glycerol phosphorylation (Sargent et al., 1994; Siva et al., 2010). The GK gene family is ancestral from the chromosome X, and then it was copied by retrotransposition events to chromosome 4, where the two active forms of the enzyme are preserved (Sargent et al., 1994). GK2 is bound to mitochondria. Its transcripts present a domain on exon 18 which is thought to direct GK2 to mitochondria (Dipple and McCabe, 2001; Sargent et al., 1994). It is postulated that the autosomal replicates of the GK family compensate the inactivation of the X chromosome on germ line cells (Lifschytz and Lindsley, 1972; Sargent et al., 1994). GK2 is presented in its phosphorylated active form in capacitated hamster spermatozoa, and in the acrosome and mid piece of spermatozoa of several mammals, including humans (Kota et al., 2009). Adults suffering the benign form of GK deficiency present higher glycerol concentrations in the blood and are prone to develop T2DM (Dipple and McCabe, 2001). Interestingly, asthenozoospermic individuals show up to a 5-fold increase in GK2 protein expression in the testicular tissue comparing to normozoospermic individuals (Siva et al., 2010), highlighting that this specific isoform may have a significant role for sperm motility in humans. Since GK2 is bound to mitochondria, interacting with the porin–adenine nucleotide carrier complex that exchanges cytosol ADP with mitochondrial ATP (Fiek et al., 1982), defects on the interacting surface may be responsible for impairing the energy homeostasis in spermatozoa, and thus its mobility (McCabe, 1983; Siva et al., 2010).

GPD1 is the cytosolic enzyme that catalyses the reversible dehygenation of G3P into DHAP, reducing NAD^+ to NADH (Sato et al., 2015; Wu et al., 2015). This is the most expressed and active G3P dehydrogenase in most mammal tissues (Gao et al., 2011; Mráček et al., 2013; Sato et al., 2015). In testes, it is primarly found in SCs (Ratner et al., 1981), and its availability is supposely similar to GPD2 availability, since the G3P shuttle is active in SCs and it needs an equimolar proportion of both enzymes (Mráček et al., 2013; Ohkawa et al., 1969).

While higher GPD1 expression levels favours fat accumulation and T2DM insulin resistance onset (Gao et al., 2011; Kozak et al., 1991; Park et al., 2006), the absence of a functional GPD1 causes a rare autosomal recessive disorder known as transient infantile hypertriglyceridemia (Dionisi-Vici et al., 2016; Wu et al., 2015). On the other hand, mice models have shown that GPD1 deficiency can be benefitial for aerobic endurance, as it favours lipid oxidation without impairing glycolysis, and delays lactate production and glycogenolysis (Sato et al., 2015). This effect might be negative for SC function and thus, the nutritional support of spermatogenesis. Glycolysis is the preferred metabolic pathway for SCs, producing pyruvate that is used for lactate production, later directed for energetic support for the developing germ line cells (Boussouar and Benahmed, 2004; Jutte et al., 1981; Rato et al., 2012b).

GPD2 reduces G3P into DHAP. GPD2 expression varies considerably amongst mammalian tissues, being more expressed in BAT, muscle and brain, and less expressed in liver and heart (Koza et al., 1996; Mráček et al., 2013). However, GPD2 appears to have critical physiological importance on other tissues, namely the testis (MacDonald and Brown, 1996; Weitzel, 2002). This isoform exhibit three special features: (i) it is a transmembrane protein of the mitochondrial inner membrane; (ii) it is not effective in catalysing the reverse reaction; and (iii) it feeds the ubiquinone pool using a bound FAD^+ co-factor as intermediary (Houštěk et al., 1975; Bharadwaj et al., 2014; Mráček et al., 2013; Wu et al., 2015). Regarding testicular function, those features are related to (i) connecting the lipidic and glycolytic metabolism for metabolite interconversion (essential for germ cell nurturing and membrane remodelling) (Bajpai et al., 1998b; Boussouar and Benahmed, 2004; Oliveira and Alves, 2015); (ii) it allows the regeneration of the cytosolic NADH pool without complex I, which may be related to heat dissipation (important for spermatogenesis) (Lardy et al., 1989; Setchell, 2006); and (iii) it pumps electrons on the ETC feeding the ubiquinone pool (crucial for ATP production, notably for sperm motility) (Bharadwaj et al., 2014; Mráček et al., 2013).

GPD2 expression is regulated by a cAMP-response element (CRE), the transcription factor CREMτ, an important gene expression regulator during spermatogenesis (Rajković et al., 2004; Weitzel, 2002). GPD2 presents three different promotors, permiting three forms of alternative splicing, including a testis-specific 2.5 kb transcript, resulting from the testis-specific promoter C (Koza et al., 1996; Mráček et al., 2013; Rajković et al., 2004). Interestingly, Koza et al. (1996) reported a particularly high expression of GPD2 mRNA in testis, but a low level of protein expression, and hypothesize that the low-weight 2.5 kb transcripts, for being more stable, may accumulate in spermatozoa after being active during meiosis. This hypothesis is supported by Weitzel (2002) observations, who found a CRE-site recognized by CREMτ, downstream the GPD2 gene sequence, suggesting it is expressed during spermatogenesis (and meiosis). However, the function of this testis-specific fragment and its regulation throughout spermatogenesis is still unclear (Rajković et al., 2004).

The GPD2 enzyme results from the cleavage of a larger protein, the inner mitochondrial membrane peptidase 2-like (IMMP2L) protein, which contains mitochondrion inner membrane location sequences (Bharadwaj et al., 2014). Mutations in IMMP2L may impede the correct migration of these enzymes, if they affect the location sequences. In homozygous mice a mutation in one of the signalling sequences of IMMP2L resulted in infertility in females and severe subfertility in males, along with accelerated aging (Bharadwaj et al., 2014; Lu et al., 2008). Considering the male mice, Lu et al. (2008) report that this mutation hyperactivated mice mitochondria, which led to ATP and

MMP accumulation, and superoxide generation, ultimately causing erectile dysfunction by low NO availability and acelerated aging due to uncontrolled reactive oxygen species (ROS) production. Superoxide quickly depletes NO because the reaction kinetic for peroxynitrite formation is more favourable than the superoxide degradation by superoxide dismutase (Katusic, 1996). GPD2 has allosteric regulation by several bivalent ions, but the most effective of them is Ca^{2+}, since there is a calcium binding site, on the protein moiety exposed on the intermembrane space (Mráček et al., 2013).

The reaction catalysed by GPD2 is not fully protected against electron leakage, therefore, it is a 'hot spot' for the formation of ROS, that can leak from Complex I or II if semiquinone is formed, from the GPD2 itself or from ubiquinone to Complex III (Mráček et al., 2013; Mráček et al., 2014). Therefore, higher glycerol availability is likely to overcharge this weak spot, promoting ROS production. Ultimately, high ROS levels can render testicular cells inneffective to maintain a proper environment for spermatogenesis, and are linked to male infertility due to lipid peroxidation and erectile dysfunction (Bajpai et al., 1998b; Koksal et al., 2003; Lu et al., 2008; Rato et al., 2012b). Antioxidant agents, like tea phenols, are known to alleviate the deleterious effects induced by ROS (Oliveira et al., 2015b).

Concluding remarks

The good: the importance of glycerol metabolism to testicular cells

The central role of glycerol in intermediary metabolism is crucial for body homeostasis, as it acts as a metabolic rerouter, linking major pathways: glucose metabolism, lipid metabolism and OXPHOS (Houštěk and Drahota, 1975; Mráček et al., 2013; Seidler, 2013). Thanks to this property, cell metabolism is directed towards the most favourable pathway according to the conditions at the moment. AQGPs are a relevant part of this, since they are expressed in response to hormonal and environmental stimuli, and enable the intake or the release of glycerol from the cells in coordination between gluconeogenic and lipolytic tissues (Chaves et al., 2012; Rodríguez et al., 2011b). To a certain extent, our metabolic excesses are dampened by glycerol metabolism. Testes are dependent on glycolytic metabolism to provide proper nursing environment for spermatogenesis (Boussouar and Benahmed, 2004; Martins et al., 2013; Miki, 2006; Oliveira et al., 2015a). Hence, in these organs, the metabolic regulation is even more imperative (Rato et al., 2012b; Oliveira and Alves, 2015). Furthermore, the identification of testes-only enzyme isoforms (GK2) (Sargent et al., 1994) or isoforms present at higher concentrations than in other tissues (GPD2) (Weitzel, 2002), linked to glycerol metabolism and mitochondria, emphasize testicular cells' biological need to optimize glycerol energetic potential. In addition, it also shows that glycerol metabolism has a tissue-specificity that can be of great interest to maintain the metabolic homeostasis while regulating the fertility potential of the individual.

The bad: the antispermatogenic properties of high glycerol concentration in testes

The central metabolism relies on the G3P shuttle, which converts G3P to DHAP by the reversible cytosolic GPD1 reaction or by the irreversible mitochondrial GPD2 reaction (Hopkinson et al., 1974; Mráček et al., 2013). On a high-caloric fat-rich diet, glycerol metabolism gets saturated, due to rate-limiting reactions linking the different metabolic pathways (Chaves et al., 2012; Peroni et al., 1995). The increase of FFA and glycerolipids concentrations on the bloodstream, along with the needed insulin release, promotes AQGP expression on liver, adipocytes and SCs, increasing the glycerol intake (Méndez-Giménez et al., 2014; Morigny et al., 2016; Patsouris et al., 2004; Rodríguez et al., 2011b). FFA competitively inhibit the intermediary metabolism exit via OXPHOS, and due to the high energetic environment, the glycolytic pathway will be saturated (Bukowiecki and Lindberg, 1974; Patsouris et al., 2004; Seidler, 2013). The only way out is by lipogenesis and glyceroneogenesis that, in turn, promote fat deposition, leading to obesity. Meanwhile, the excess of glycerol accumulates, increasing its concentration on human tissues. However, high glycerol concentrations in testes affect the functionality of the BTB, by disrupting the tight junctions between SCs at the level of actin microfilaments, occludin and microtubules (Wiebe et al., 2000). Besides permeabilizing the BTB, germ cells are also detached from SC and may suffer apoptosis (Wiebe and Barr, 1984a). The combination of both effects may lead to temporary or permanent azoospermia.

The ugly: a hypercaloric, glycerol and fat-rich diet

Metabolic disorders are linked to male reproductive dysfunction (Alves et al. 2015, 2016; Oliveira et al., 2017; Rato et al., 2013). The intermediary metabolism exposes several mechanisms leading to this condition. When fasting, adipocytes release TAGs that are converted in the liver to FFA and glycerol, which is then exported via AQGPs to the extracellular medium (Lebeck, 2014; Patsouris et al., 2004; Rodríguez et al., 2011b). The concentration of these metabolites is proportional to the amount of body fat, so obese men reach higher concentrations (Hagstrom-Toft et al., 1997). Testes are a sensitive tissue for glycerol accumulation. The leaky BTB caused by high glycerol concentrations disrupts the homeostasis of the tubular fluid, promoting the apoptosis of germ line cells (Wiebe et al., 2000). This phenomenon causes temporary spermatogenesis arrest (Wiebe and Barr, 1984b) but, if it persists for some time, it may cause permanent oligospermia or even azoospermia (Wiebe et al., 2000).

Authors' roles

L.C. contributed to article design, bibliographic search, analysis, writing, illustration, data interpretation and critical discussion. M.G.A. contributed to article design, writing, bibliographic enrichment, data interpretation and critical discussion. G.C. and M.S. provided critical discussion of the article. P.F.O. contributed to article design, writing, bibliographic enrichment, data interpretation and critical discussion. All authors approved the final version for submission.

Funding

This work was funded by the Portuguese Fundação para a Ciência e a Tecnologia: L.C. (SFRH/BD/128584/2017); M.G.A. (IFCT2015 and PTDC/BIM-MET/4712/2014); P.F.O. (IFCT2015 and PTDC/BBB-BQB/1368/2014); and UMIB—Unit for Multidisciplinary Research in Biomedicine (Pest-OE/SAU/UI0215/2014); co-funded by the EU Framework Programme for Research and Innovation H2020 (POCI/COMPETE2020).

Conflict of interest

The authors declare that they have no conflict of interest.

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