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The Many Faces of Carbohydrate Metabolism in Male Germ Cells: From Single Molecules to Active Polymers

Las Diferentes Caras del Metabolismo de los Carbohidratos en las Células Germinales Masculinas: desde Moléculas Individuales a Polímeros Activos

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SUMMARY: Spermatogenesis is a complex physiological process that involves cell proliferation, meiotic division and a final cell differentiation of post-meiotic cells into spermatozoa. During this process male germ cells also undergo a metabolic differentiation process, in which post-meiotic spermatogenic cells (spermatids) but not meiotic spermatogenic cells (spermatocytes) respond differentially to D-glucose metabolism, glucose transporters (GLUTs) distribution and utilization of non-hexose substrates, such as lactate/pyruvate or dihydroxyacetone. These differences might be explained by the requirement for a specific metabolic process to support cell differentiation or in some cases, cell viability. In addition, though glycogen is considered to be the main glucose store, in male germ cells this polymer may play a novel role in cell proliferation, acting as a new marker for apoptotic events in testicular tissue via a yet unknown mechanism. In this article, we summarize the main metabolic changes that occur during male germ differentiation, with a specific focus on metabolic sources during spermatocyte to spermatid transition. The latter considering that these cells come from the same cell linage as specialized cells, but are not isolated from their environment, describing the roles from single molecules to polymers on the viability of male germ cells.

KEY WORDS: Testis; Carbohydrates; GLUTs; Glycogen; Glucose; Gluconeogenesis; Spermatogenesis.

INTRODUCTION

The testis is a complex organ that performs two crucial functions: steroidogenesis and production of competent spermatozoa (Amann & Schanbacher, 1993; Amann, 2008). Spermatogenesis is a complex process in which stem spermatogonia become mature spermatozoa through a series of events involving mitosis, meiosis and cell differentiation. Developing germ cells are anchored to and supported by sustentocytes, which are described as 'nurse-like' epithelial cells that give rise to the basic structure of the seminiferous

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epithelium. The junctions between adjacent sustentocytes form the blood-testis barrier (BTB), which separates the seminiferous epithelium into the adluminal and basal compartments. This barrier has three main roles: 1) creating a specialized environment; 2) regulating the passage of molecules; and 3) serving as an immunological barrier (Mruk & Cheng, 2004). In addition, sustentocytes produce and release different metabolic products, many of which are important for germ cell survival. These products include amino acids, carbohydrates, lipids, vitamins, and metal ions (Mruk & Cheng). In fact, other molecules released from somatic cells, such as hormones (testosterone, dihydrotestosterone, and estradiol) and cytokines, have been described as factors that can enhance the viability of germ cells *in vivo* and *in vitro* assays, confirming the vital relationship between germ cells and somatic cells (Lie *et al*., 2013). However, there is a limit for the cooperation. sustentocytes can efficiently sustain up to fifty developing germ cells (Weber *et al*., 1983: Wong *et al*., 1983), without affecting the effective glucose metabolization to lactate (the preferred energy source of germ cells) or the secretion of other vital factors for spermatogenesis.

Certainly, sustentocytes play a crucial role on the nutritional and structural requirements during spermatogenesis, but the metabolism of germ cells continues to be poorly understood, and its impact on the differentiation or viability of the same cell. Germ cell death, involved in cell proliferation as well as in differentiation, is also conspicuous during normal spermatogenesis in various mammalian species, including humans, and plays a critical role in determining the quantitative degree of sperm output (Shaha *et al*., 2010). For both spermatogenesis and apoptosis, metabolic status and energy demand are necessary to drive normal progress. Both processes are responsible for supporting normal testis development, and subsequently, male fertility.

VERSATILITY OF GLUCOSE TRANSPORTERS IN TESTIS

Different members of the facilitative glucose transporters (GLUTs) protein family are expressed in testis; and GLUT transporter expression has been associated with the metabolism of male germ cells and their nutritional requirement during spermatogenesis (Angulo *et al*., 1998; 2011; Rauch *et al*., 2006; Galardo *et al*., 2008), such as, GLUT1, GLUT2, GLUT3, GLUT5 and GLUT8, which have been previously described in rat, mouse and human testis (Angulo *et al*., 1998; 2011; Rauch *et al*.; Galardo *et al*., 2008). These transporters can be classified by their structural characteristics, kinetic properties and main substrate transported; or by their levels of expression and localization (Uldry & Thorens, 2004). Structurally, these molecules have been divided in three classes: GLUTs1–4 (class 1); GLUTs5, 7, 9 and 11 (class 2) and GLUTs6, 8, 10, 12 and HMIT (class 3). GLUTs1–4 are the initially characterized glucose transporters. GLUT5 is a fructose transporter, and the functions of the other transporter-like proteins from class 2 are not yet firmly established. The class-2 proteins lack the tryptophan equivalent to W388 of GLUT1. Among the class-3 family, the function of GLUT8 and 10 as glucose transporters have been clearly established, and HMIT is an H+/myo-inositol symporter (Uldry & Thorens). The most abundant glucose transporter expressed in testicular tissue is GLUT1, which is found with varied levels of expression in different cell types. The expression level usually correlates with the rate of cellular glucose metabolism. It is also highly expressed in blood-tissue barriers, particularly in the endothelial cells forming the blood-brain barrier (Maher *et al*., 1994). GLUT2 is a low-affinity transporter for glucose (Km ~17 mM) (Johnson *et al*., 1990), galactose (Km ~92 mM), mannose (Km ~125 mM) and fructose (Km ~76 mM), but a high-affinity transporter for glucosamine (~0.8 mM) (Uldry *et al*., 2002). This GLUT has been described as a transporter able to modulate the uptake of glucose in polarized cells, which allows rapid equilibration of glucose between the extracellular medium and the cell's cytoplasm (Johnson *et al*.). GLUT3 mRNA expression is almost ubiquitous in humans and other mammals, although protein distribution is restricted mainly to brain and testis. However, it is possible to detect the protein in the membrane of spermatozoa, skeletal muscle and platelets (Haber *et al*., 1993; Heijnen *et al*., 1997; Kayano *et al*., 1988). GLUT5 does not exhibit glucose transport activity and plays an important role in fructose absorption (Km ~6 mM) by the intestine

and other polarized cells and is mostly located in the apical membrane of epithelial cells forming barriers (Uldry & Thorens). GLUT5 has been also detected in the testis of humans and rats, as well as in bull spermatozoa (Angulo *et al*., 1998). Finally, GLUT8 (formerly GLUTX1) mRNA is expressed at high level in the testis, at a lower level in the cerebellum, adrenal gland, liver, spleen, brown adipose tissue and lung. In situ hybridization and immunofluorescence detection studies show GLUT8 to be expressed strongly in differentiating spermatocytes of the type-1 stage in the testis, but to be weakly detected in mature spermatozoa (Ibberson *et al*., 2002). GLUT8 is entirely retained in the intracellular compartment, and reveals a relatively high affinity for glucose with a Km of ~2 mM. However it is able to transport fructose and galactose (Uldry & Thorens).

We focus our studies in the most abundant glucose transporters in testicular tissue and we have demonstrated that GLUTs are expressed with a differential cell distribution in spermatocytes and spermatids from rats, showing a compartmentalization of GLUTs in these cells during spermatogenesis. Specifically, GLUT1 and GLUT3 have been described in spermatocytes, spermatids and spermatozoa, with GLUT3 found to be highly expressed in spermatids (Rauch *et al*.). This suggests that the high-affinity glucose uptake by these cells may play a role during cell differentiation. In addition, GLUTs are able to transport non-hexose substrates, such as dehydroascorbic acid (the oxidized intermediate of ascorbic acid) (Vera *et al*., 1993; 1995; Rumsey *et al*., 1997), specifically GLUT1 and GLUT3 transport L-dehydroascorbic acid (DHA), and this new substrate competes with glucose uptake, showing a low affinity. However it has been demonstrated that a higher concentration of glucose (10 mM) is required to reduce DHA uptake in rat astrocytes (Wilson, 2002). Once transported into the cell, dehydroascorbic acid (DHA) is immediately reduced to ascorbic acid (AA) allowing its recycling (Guaiquil *et al*., 1997; May, 1998; Wilson, 2002; 2005), suggesting that GLUTs may contribute with not only an energetic substrate but also with an antioxidant precursor. In fact, the reduced form of ascorbic acid is specifically transported with a high affinity by SVCT (sodium-vitamin C co-transporters) and contributes directly to the total intracellular vitamin C or AA concentration (Wilson, 2005; May; Daruwala *et al*., 1999; Tsukaguchi *et al*., 1999; Godoy *et al*., 2007). AA is accumulated and concentrated within male germ cells and sustentocytes by transport of both reduced and oxidized forms, reaching a concentration of 200 μM of this antioxidant in the testis (Wilson, 2002; 2005; Moser, 1987; Angulo *et al*., 2008). The relevance of this machinery is summarized by the regeneration of the glutathione metabolism and the synthesis of many metabolic intermediaries, such as collagen and catecholamine. All of them are required to support the normal testis development.

In addition, DHA could have diverse roles in mammalian cells that explain the versatility of glucose transporters especially in male germ cells. DHA *in vitro* and *in vivo* has been shown to be a competitive inhibitor of GLUTs, hexokinase, glyceraldehyde-3-phosphate dehydrogenase and glucose-6-phosphate dehydrogenase. These inhibitory activities can be reverted at higher doses of the specific substrate for each enzyme (Wilson, 2002; Vaulont *et al*., 2000; Fiorani *et al*., 1998), modulating directly or indirectly the sugar metabolism in these cells. DHA can also modulate the progression of the cell cycle or promotion of apoptosis, limiting ascorbic acid availability by its recycling or by the impairing the use of other metabolites during nutritional limitation or oxidant conditions. In fact, ascorbic acid concentrations above the normal serum level of 50 μM induce oxidative stress and apoptosis of lymphoid and myeloid cells (Fiorani *et al*.; Sakagami & Satoh, 1997; Podmore *et al*., 1998; Amano *et al*., 1998). In blood cells, DHA may have dual properties according its concentration. At 800 μM DHA, after 48 h of treatment, it has the capacity to indirectly stimulate NADPH, via the pentose phosphate pathway, and subsequent glutathione synthesis (Puskas *et al*., 2000). These activities of DHA molecules (or ascorbic acid oxidation) suggest that in other tissues or cells, these molecules may promote an additional mechanism to revert the oxidative stress induced by cell proliferation and differentiation during spermatogenesis. The intracellular presence of DHA may not only represent an accumulation of the oxidized form of vitamin C, but may have a role in influencing GSH levels through the

pentose phosphate pathway (PPP). Previously, it has been reported that products of DHA metabolism, 2,3-diketo-L-gulonate and its decarboxylation products, L-xylonate and L-lyxonate, can enter the non-oxidative branch of PPP (Kanfer *et al*., 1960; Banhegyi *et al*., 1997; Doiron *et al*., 1996). On the other hand, DHA induces elevations of G6PD activity by an increase of G6PD protein levels with respect to actin levels, suggesting that DHA may stimulate gene expression (Puskas *et al*.). Carbon flux via the PPP has been implicated in regulating expression of other glucose-metabolizing enzymes, pyruvate kinase, glucose 6-phosphatase, and phosphoenolpyruvate carboxykinase (Massillon *et al*., 1998).

Furthermore, the expression of functionally-active vitamin C transporters (SVCT-1 and -2) in spermatogenic cells suggests that these cells can adapt to the seminiferous tubule environment, situation that contributes to supporting the redox potential of the germ cell during differentiation and the journey from the germinal epithelium to the tubular lumen as a sperm (Angulo *et al*., 1998, 2008, 2011). In osteoblasts, it has been reported that insulin and insulin-like growth factor I can increase the maximal rate of DHA transport by GLUTs, thereby raising the intracellular AA concentration (Qutob *et al*., 1998). This suggests that in these cells and other insulin-sensitive cells these hormones could modulate the REDOX status by DHA or AA uptake.

In both cases, GLUTs and SVCTs may promote a quick detoxification of extracellular DHA, increasing antioxidant power, enabling uptake of both forms of vitamin C, and then accumulated as active form (Wilson, 2002, 2005; Augustin, 2010). In insulin-sensitive cells, it has been proposed that an excess of glucose during uncontrolled diabetes may impair DHA uptake into cell types where DHA transport is mediated largely by facilitative glucose transporters. Hence, the slowing of DHA uptake may lead to impairment regeneration of AA and weakening of antioxidant defenses in diabetes or other conditions that promote a hyperglycemic state (Wilson, 2002, 2005).

In brief, the expression of different glucose transporter GLUT isoforms has been largely visualized as an adaptation to the requirement of different substrates for cell metabolism. GLUTs have been associated physically with hexokinase I and caveolae, representing a new mechanism for the regulation of signaling cascades in which glucose participates as signaling molecule (Rauch *et al*.; Vaulont *et al*.; Augustin *et al*.). Most relevant is the specific colocalization of hexokinase I with GLUT1 in spermatocytes and with GLUT3 transporters in spermatids. This evidence could confirm the participation of GLUTs and sugar uptake in the differentiation stage. The capacity of GLUT to transport glucose or other sugars and the oxidized form of vitamin C, suggests an important role for these molecules in normal testis metabolism. Therefore, they could be essential for the maintenance of spermatogenesis and fertility.

UPTAKE AND AVAILABILITY OF GLUCOSE IN NORMAL OR PATHOLOGICAL CONDITIONS

Many authors have discussed that male reproductive health is highly dependent on glucose or other non-sugar metabolite uptake and its metabolization by testicular cells (Amann & Schanbacher; Jutte *et al*., 1981; Mita & Hall, 1982; Angulo *et al*., 1998; Augustin; Alves *et al*., 2013a; Bucci *et al*., 2011; Mueckler & Thorens, 2013). As we described previously, there are different GLUTs in somatic and germ cells from the seminiferous tubules (Angulo *et al*., 2008; Augustin). On other hand, the identity and distribution of these glucose transporters can determine the availability of glucose as a carbon source for specific metabolic processes during germ cell differentiation, or to support the integrity of the seminiferous epithelium by functional sustentocytes. Some authors have described that glucose uptake by GLUT1 and GLUT3 in sustentocytes directly supports lactate production as a metabolic strategy involved in the metabolic cooperation established between these cells and germ cells (Augustin). GLUTs are also detected in male germ cells, and the identity and distribution of these transporters have shown significant differences, suggesting that the high-affinity glucose uptake by these cells may play a role during cell differentiation in these cells (Jutte *et al*.). GLUT1 has the ability to transport glucose and other sugars, such as galactose, mannose and glucosamine. The GLUT3 expression has been reported in tissues with high

demand for glucose, and this protein can transport galactose, mannose, maltose, xylose, and dehydroascorbic acid (Jutte *et al*.). In summary, the presence of these transporters confirm that germ cells, including spermatozoa, may use glucose or other sugars as a carbon substrate. Nevertheless, there are many studies indicating that glucose is not a good energetic substrate for male germ cells (Jutte *et al*.; Mita & Hall; Rato *et al*., 2012; Eddy & O'Brien, 1994; Mueckler & Thorens). Glucose may not be necessary during germ cell differentiation; through the spermatocytes to spermatids transition the glycolytic pathways is inhibited, but is activated in the spermatozoa (Hoshi *et al*., 1991), suggesting that glucose is required to support the spermatic function. In fact, in absence of this sugar, human spermatozoa reduce their motility and fail to penetrate the zona pellucida, and glucose (but not monocarboxylates) is necessary to support the protein tyrosine phosphorylation events during capacitation in murine spermatozoa (Travis *et al*., 2001).

To date, Oliveira´s group has contributed a wealth of information on pathologies related to metabolic disorders, such as diabetes, and its impact on male fertility (Alves *et al*., 2012; 2013a; 2013b; Rato *et al*., 2012; 2013; Oliveira *et al*., 2012). They have described in human sustentocytes that insulin deprivation promotes a reversible decrease in lactate production and glucose consumption, showing a modification in the relative levels of GLUTs expression, specifically GLUT1 and GLUT3. They demonstrated that insulin deprivation in sustentocytes induced an altered glucose consumption and lactate secretion (Oliveira *et al*.), In fact, this group has described for the first time the effects of hyperglycemia on spermatic quality parameters in rats fed a diet rich in energy, reporting a serious impairment on sperm morphology without affecting the motility or viability. The authors proposed that other metabolic changes, such as testosterone secretion and an imbalance on REDOX status in the testicular tissue could be responsible for these effects (Rato *et al*., 2013; Alves *et al*., 2013b). However, there is also indirect evidence about morphological changes associated to diabetes, such as an alteration in the testicular capillaries and lymphatic endothelia in biopsies from men with diabetes mellitus, suggesting an abnormal interstitial matrix expansion (Cameron *et al*., 1985). It has also been described that testes from streptozotocin (STZ)-induced diabetic rats showed a reduction in the seminiferous tubular diameter, an increase in the number of empty testicular tubules and also an increase in vascular density and wall thickness of small blood vessels (Anderson & Thlieveris, 1986; Cai *et al*., 2000). These authors have suggested that the higher vascular density might lead to increased scrotal temperature and a limited access to oxygen from circulation, which could be responsible for a higher rate of apoptosis in germ cells.

Additionally, it has been suggested that diabetes may cause male infertility by altering steroidogenesis, sperm motility and levels of GLUT protein expression, suggestive of an interrelation between sugar uptake and other metabolic process (Alves *et al*., 2012; 2013a; Bucci *et al*.; Cai *et al*.; Kim & Moley, 2008). There is evidence that changes involved in the presence of glucose transporters in spermatozoa from type 1 diabetic mouse models (streptozotocin treatment and Akita strain), showed a significant reduction in the relative protein levels of SLC2A8 (GLUT8) in sperm and serious deficiencies in the spermatic biology (motility, velocity and concentration). Both diabetic models showed an impairment of fertility (Kim & Moley). Recently, it was reported that human sustentocytes can export acetate to the germ cells and this molecule may support the continuous production of lipids, phospholipids, cholesterol and proteins during testis development or germ cell differentiation (Cameron *et al*.). However, during insulin-deprivation, sustentocytes completely suppress acetate production and its export to the germ cells, suggesting that in patients with diabetes type I, acetate restriction could impair normal testis development and promote male infertility. Simultaneously, the sperm may lose their ability to uptake glucose, directly affecting fertilization events (Oliveira *et al*.; Cameron *et al*.).

GLUCONEOGENESIS AND METABOLIC CHANGES DURING SPERMATOGENESIS

Much research currently focuses on somatic cell metabolism and its participation in male germ viability (Amann & Schanbacher;

Mruk & Cheng; Angulo *et al*., 2011; Bucci *et al*.; Rato *et al*., 2012. While it is imperative that germ cells have access to an adequate level of energy substrates to avoid degeneration and apoptosis, male germ cells have a very complex metabolism. During spermatogenesis, many metabolic and structural changes occur. It is well known that the spermatogonia from rats (less differentiated germ cells or male stem cells) may utilize glucose as the major energy substrate (Nakamura *et al*., 1981, 1984). But, spermatocytes and spermatids (transitional cells and more differentiated cells respectively) suffer a rapid decline in their ATP content in glucose-supplemented media and require lactate/pyruvate for maintenance of their ATP concentrations (Mueckler & Thorens; Jutte *et al*.; Mita & Hall). Bajpai *et al*. (1998) showed a greater activity of glycolytic and pentose phosphate pathway enzymes in spermatocytes than in spermatids from rats, with spermatids exhibiting greater tricarboxylic acid (TCA) cycle enzyme activity than spermatocytes. Spermatozoa exhibited markedly greater glycolytic enzyme activity and a significantly lower activity of pentose phosphate pathway and TCA cycle enzymes than did testicular germ cells (Bajpai *et al*.). These authors concluded that the unusual and exclusive dependence on lactate in spermatids may be due to their lower glycolytic potential, whereas spermatocytes with a comparatively greater glycolytic activity have an intermediate dependence on lactate, and are therefore able to utilize lactate, pyruvate, or both, while retaining their better ability to utilize glucose. Spermatozoa with the greatest glycolytic potential and the lowest TCA cycle activity appear to be 'programmed' to exclusively utilize glucose/fructose for energy (Mann, 1975; Jones *et al*., 1992). It has also been reported that the sensitivity of intracellular Ca2+ levels in response to carbohydrate metabolism is higher in round spermatids than in pachytene spermatocytes isolated from adult rats (Reyes *et al*., 2002; Brauchi *et al*., 2005).

In addition to the metabolic changes during spermatogenesis, the less differentiated cells, such as type-B spermatogonia and spermatocytes, experience a strong increase in apoptotic rate (Kierszenbaum, 2001; Sinha Hikim & Swedloff, 1999). This phenomenon does not affect the spermatid or the more differentiated cells. Germ cell death plays a critical role in determining the quantitative degree of sperm output. However, the mechanism regulating the rate of spontaneous apoptosis during the first spermatogenic wave is unknown (Shaha *et al*.; Kierszenbaum; Sinha Hikim & Swedloff). Thus, differentiation-related changes in carbohydrate metabolism in spermatogenic cells may determine a dynamic and differential modulation of cell viability and correct development of cells into mature spermatozoa. Our previous studies done in adult rats have shown that pachytene spermatocytes expressed mainly MCT1 and MCT4 isoforms (intermediate- and low-affinity forms, respectively) of the monocarboxylate transporter. Meanwhile round spermatids, as well as expressing MCT1 and MCT4, also express the MCT2 isoform (high-affinity transporter). These differences reflect the ability of these cells to switch between the generation of glycolytic L-lactate in the presence of external glucose, and the use of L-lactate when this substrate is available in the external environment (Reyes *et al*.; Brauchi *et al*.).

Several observations have indicated that lactate/pyruvate may represent a preferential energy substrate for male germ cells in seminiferous tubules (Amann & Schanbacher; Angulo *et al*., 1998; Augustin; Bucci *et al*.; Mita & Hall; Bajpai *et al*.; Bustamante-Marin *et al*., 2012). It has been shown that lactate is a preferential energy substrate produced by sustentocytes and transported to germ cells by monocarboxylate transporters (MCT1 and MCT2). MCT1 is immunolocalized to all the different germ cell types, but MCT2 has been exclusively detected in elongated spermatids (Bustamante-Marin *et al*.; Herrera *et al*., 2000). Spermatocytes (meiotic cells) and spermatids (post-meiotic cells) use lactate rather than glucose as their primary substrate for producing ATP and to support their viability. Spermatocytes from rats cultured without an energy source died by necrosis, while spermatocytes cultured with physiological glucose concentration showed a significant increase in apoptosis. However, apoptotic signals were not observed in spermatocytes cultured in lactate or deoxyglucose, suggesting that the availability of glucose and/or lactate would affect the type of death or the survival of primary spermatocytes. In this scenario, glucose can induce apoptosis,

while lactate could be a protective factor (Herrera *et al*.). In other studies done in cells isolated form rats, glucose and lactate were shown to modulate intracellular free calcium Ca2+i and pHi levels in pachytene spermatocytes and spermatids (Brauchi *et al*.; Mishra *et al*., 2006). Both intracellular parameters, Ca2+i and pHi, have been also shown to play crucial roles in cell death and differentiation in somatic cells and male germ cells (Pinton *et al*., 2008; Galardo *et al*., 2014). Thus, lactate could provide germ cells with the metabolic and redox status of its environment and other accessory signals for spermatogenesis (such as hormones and molecules released by sustentocytes), and it could modulate the expression of genes during differentiation of post-meiotic cells. To evaluate these hypothesis, cultured germ cells from rats exposed to 10 or 20 mM lactate showed an increase in lactate dehydrogenase (LDH) C and monocarboxylate transporter (MCT2) expression [Albarracin *et al*., 2004]. The authors suggest that the effects induced for L-lactate taken up by germ cells depend on ROS production by NADPH oxidase-4 (NOX4) activity during lactate oxidation to pyruvate. In this case, lactate could be a precursor for intracellular signalling in testis, and its availability may modulate these roles, including ROS scavengers and molecules able to act as inhibitors or modulators of NADPH oxidase or NADH synthesis (Albarracin *et al*., 2004).

We have demonstrated the existence of functional gluconeogenesis in testis and sperm in different species (human, dog and rats) by highlighting the presence of key enzymes in this metabolic pathway, such as the liver isoform of fructose 1,6-bisphophatase (FBPase), phosphofructose-1 kinase (PFK) and aldolase B (Albarracin *et al*.; Yañez *et al*., 2007, 2003). Rat spermatids were also able to convert lactate to fructose- and glucose-6-P, indicating that both glycolytic and gluconeogenic fluxes are present in these cells (Yañez *et al*., 2007. We have reported that mRNA and PFK protein levels are relatively similar in spermatogenic cells, but are more strongly expressed in human sustentocytes. In contrast, expression of FBPase is stronger in round and elongating spermatids as compared to other spermatogenic cells (Yañez *et al*., 2003). Unpublished data from our laboratory indicate by real-time PCR that liver FBPase mRNA is mainly expressed in testis after two weeks post-birth, when spermatids cells are present in seminiferous epithelium from mice and rats. These results suggest that a coordinated expression of key enzymes, at the level of FBPase, appears in the last stages of spermatogenic cell differentiation, indicating that perhaps this enzyme is required to keep these cells viable and support their cellular differentiation into sperm. Germ cells experience differentiation-related changes in their carbohydrate metabolism, where meiotic (spermatocytes) and post-meiotic cells (spermatids) use lactate, rather than glucose, as their energy source (Nakamura *et al*.; Bajpai *et al*.).

Glucose 6-phosphate has been described as a potent allosteric activator of glycogen synthase activity (Bouskila *et al*., 2010) and its availability and accumulation into germ cells could modulate glycogen synthesis. In this case, a scavenger mechanism of excess of gluconeogenic substrates, such as lactate, might promote cell survival at the last stage of cellular differentiation during spermatogenesis when glycogen may reach dangerous levels or the (necessary) lactate concentration is not able to be metabolized by oxidative processes in the mitochondria. At the same time, pyruvate-lactate conversion and the eventual accumulation of pyruvate and its effects in TCA cycle could be avoided indirectly through pentose phosphate pathway and the FBPase activity. Previously, dog spermatozoa incubated in the presence of glucose or fructose exhibit an increase in glycogen deposition in a time- and concentration-dependent manner suggesting active gluconeogenic metabolism in these cells (Palomo *et al*., 2003; Ballester *et al*., 2000). In addition, in dog spermatozoa it has been reported that the gluconeogenesis-linked glycogen metabolism is important during *in vitro* capacitation under glucose deprivation (Albarracin *et al*.). The authors demonstrated the liver FBPase and aldolase B expression and that the (14C) lactate uptake was followed by an accumulation of radioactive glycogen confirming an active gluconeogenic metabolism. Our data by real time PCR analysis and histochemical studies (Yañez *et al*., 2007, 2003), suggest that FBPase is absent in pre-meiotic cells (spermatogonia and primary spermatocytes) and sustentocytes, but it is strongly expressed in

round and elongated spermatid and spermatozoa, confirming the relative impact of this enzyme and other enzymatic components during cell differentiation.

Other authors have described negative effects on germ cells and testicle development induced by lower levels of oxygen. They have suggested that oxygen is necessary to support the complete oxidation of monocarboxylates and the mitochondrial functionality, especially in post-meiotic cells (Farias *et al*., 2005a). Under oxygen restriction, such as hypoxia conditions, the authors described an increase in testicular temperature by an increase of blood irrigation and local number of blood vessels; these adaptations might be responsible for several histological alterations in the seminiferous tubule, affecting normal spermatogenesis (Farias *et al*., 2005a, 2005b). In addition, during an intermittent chronic hypobaric hypoxia the sperm parameters measured in rats are diminished respect to control animals, showing that oxygen restriction can negatively affect the most differentiated cells from the seminiferous tubules, such as spermatids and spermatozoa. These alterations might influence offspring, promoting subfertility under those conditions (Cikutovic *et al*., 2009). In fact, hypoxia can also promote an alteration at the level of the hypothalamus-hypophysis-gonadal axis, decreasing the levels of LH and testosterone in serum (Farias *et al*., 2008). This suggests that oxygen is not only a metabolite to support mitochondrial activity, but also to support the androgenic equilibrium during spermatogenesis.

These antecedents reinforce the role of testicular metabolism, considering this tissue and its cells as independent compartments with special and individual nutritional needs during proliferation and differentiation. It is possible that the metabolic substrates supplied by sustentocytes could regulate spermatogenesis, not only in terms of energy-dependent processes, but also by tilting the balance between survival and death of germ cells.

TESTICULAR GLYCOGEN AND ITS POSSIBLE FUNCTIONS

In most mammals, quantities of testicular glycogen vary during normal testis development, from being very abundant in the intratubular portion of seminiferous tubules in the pre-puberal stage, to diminished numbers at the onset of puberty (Arzac, 1950, 1953; Ewing *et al*., 1966; Fabbrini *et al*., 1969; Fouquet & Guha, 1969; Datta *et al*., 1988). In pathologies, glycogen storage is modified. In cryptorchid or varicocele patients, glycogen is less abundant than in normal testis (Arzac, 1950; Seilicovich & Pérez-Lloret, 1973; Re *et al*., 1983; Reddy *et al*., 1983; Sultan Sheriff, 1984). This evidence leads to the assumption that pathological conditions in testis seems to alter availability of the energy substrate required for normal functioning of germ and non-germ cells, resulting in defective spermatogenesis and affecting other metabolic processes, leading finally to degeneration of the seminiferous epithelium (Arzac, 1950). Studies in rats indirectly suggest that an imbalance in glycogen homeostasis in adult testes could induce apoptosis and degeneration of germ cells (Thakur *et al*., 2003; Kuramori *et al*., 2009). Administration of lithium, an indirect activator of glycogen synthase activity (Kuramori *et al*.; Vilchez *et al*., 2007; Graham *et al*., 2010; Roach *et al*., 2012), induces desquamation of seminiferous tubules and vacuolization of sustentocytes and consequent cell death (Thakur *et al*.). Inhibition of the glucano-transferase activity of glycogen debranching enzyme (GDE) by phthalate esters induces a strong increase in the apoptotic rate of germ cells in adult testes. This suggests that glycogen homeostasis is important for maintaining the integrity of male germinal epithelium in rats (Kuramori *et al*.). Thus, the glycogen molecule may modulate the survival of germ cells and may also affect fertility in mammals.

Recently we reported in mice and other species that the muscle isoform of glycogen synthase is responsible for testicular glycogen synthesis (Villarroel-Espíndola *et al*., 2013). In whole tissue from mice testis, this enzyme showed a specific pattern of expression and activity at five days after birth, suggesting fine regulation of this enzyme during testis development. However, in the seminiferous epithelium, germ cells show marked differences compared to somatic cells, such as sustentocytes, with respect to glycogen stores and glycogen synthase activity, with germ cells demonstrating lower glycogen levels than

sustentocytes. A spermatogonium-like cell line (GC-1) stores glycogen at levels 10 times lower than sustentocytes (42GPA9 cell line and primary culture), demonstrating that different levels of glycogen synthesis occur among different cell types in seminiferous tubules, which may be dependent on cell physiological function and stage of differentiation (Villarroel-Espíndola *et al*., 2013). Moreover, each cellular type (somatic or germinal) showed a differential amount of enzyme and grade of phosphorylation, showing a specific availability of active enzyme to glycogen synthesis (unpublished data on cell lines from adult mice).

Thirty years ago, the presence and activity of glycogen phosphorylase (GP) enzyme was described (Datta *et al*.; Venkatarami Reddy *et al*., 1983), however, to date there is no evidence indicating the isoform of GP in testicular tissue. Unpublished results by qPCR suggest that the three isoforms of glycogen phosphorylase (GP) are also expressed in mice testis. The relative expression of each isoform during testis development was analysed, and muscle and liver isoforms show more relative abundant transcript levels in whole tissue, and that might suggest that each seminiferous epithelium cell type could contribute with specific GP isoforms (Villarroel-Espíndola, 2012). Glycogen branching (BE) and debranching (AGL) enzymes are also expressed in testis. Both mRNAs show moderate levels of expression during testis development [unpublished data], suggesting that glycogen (synthesis and store or its degradation) could support indirectly the spermatogenesis or other processes involved with cell proliferation and differentiation, as has been suggested by Arzac (1953). This author proposed that glycogen is not only necessarily as a source of glucose for glycolysis and ATP synthesis in testicular tissue, but that glycogen may be a metabolic intermediary in the extracellular matrix and in the synthesis of steroids in sustentocytes and interstitial endocrine cells. Arzac's observations were made in human cryptorchid tissue, which showed a strong periodic acid-Schiff (PAS) stain and an abundant fibrosis signal, leading to a possible indirect relationship between pathological glycogen accumulation and testis degeneration. However, the role of glycogen is still controversial. Recently it was described that neuronal glycogen synthesis could be a protective response during hypoxia conditions, promoting its consumption of glucose storage by the brain glycogen phophorylase and supporting a primary nutritional restriction (Saez *et al*., 2014).

Other studies indicate that glycogen accumulation can induce apoptosis in neuronal cells, though the mechanism is unknown (Vilchez *et al*.; Roach *et al*.; Saez *et al*.; Duran *et al*., 2012). Analyses in rats suggest an indirect connection between an imbalance in glycogen homeostasis in adult testes and induction of apoptosis and degeneration of germ cells (Thakur *et al*.; Kuramori *et al*.; Villarroel-Espíndola *et al*.). Thus, the glycogen molecule might modulate the survival of germ cells and may also affect fertility in mammals. Using a transgenic model over-expressing a super-active form of MGS (Valles-Ortega *et al*., 2011; Worby *et al*., 2008), we showed that higher levels of glycogen were synthesized, promoting degeneration and apoptosis in the seminiferous tubule cells from transgenic mice. Simultaneously, the transgenic testis showed an increase in extracellular matrix, suggesting that greater availability of glycogen may be associated with the fibrosis signal (Villarroel-Espíndola *et al*.). In addition, in the GC-1 germ cell line, we have demonstrated that over-expression of protein targeting to glycogen (PTG), an indirect activator of glycogen synthase (Villarroel-Espíndola *et al*.; Worby *et al*.) leads to an increase in glycogen levels and detection of the cleaved form of caspase3. This confirms that glycogen accumulation may be a pro-apoptotic signal in male germ cells, especially in spermatogonia and spermatocytes (Villarroel *et al*.). The goal of that study was to investigate glycogen synthesis during testis development, with glycogen considered to be an active molecule in spermatogenesis and germ cell selection by apoptosis. Preliminary studies by confocal microscopy using a specific antibody for glycogen, we observed a co-localization between glycogen and apoptosis markers, such as TUNEL and cleaved caspase3 in germ cells in normal animals (Villarroel *et al*.). Our results are supported by evidence of other authors, in which apoptotic cells in seminiferous tubules were positive for periodic Schiff staining (PAS), which is widely used to detect glycogen and glucose, suggesting that they have a high content of sugars (Moreno *et al*., 2006).

CONCLUDING REMARKS

We conclude that the versatility of GLUTs in male germ cells may provide a mechanism by which metabolic specialization is generated during proliferation and differentiation in seminiferous tubules (Fig. 1). In spermatogenic cells, GLUT transporters promote the differential uptake of energetic and antioxidant substrates according to cellular requirement at differentiation stage. Specifically, the differential GLUT1 and GLUT3 expression and localization in spermatocytes and spermatids respectively, alludes that the lower or higher affinity glucose

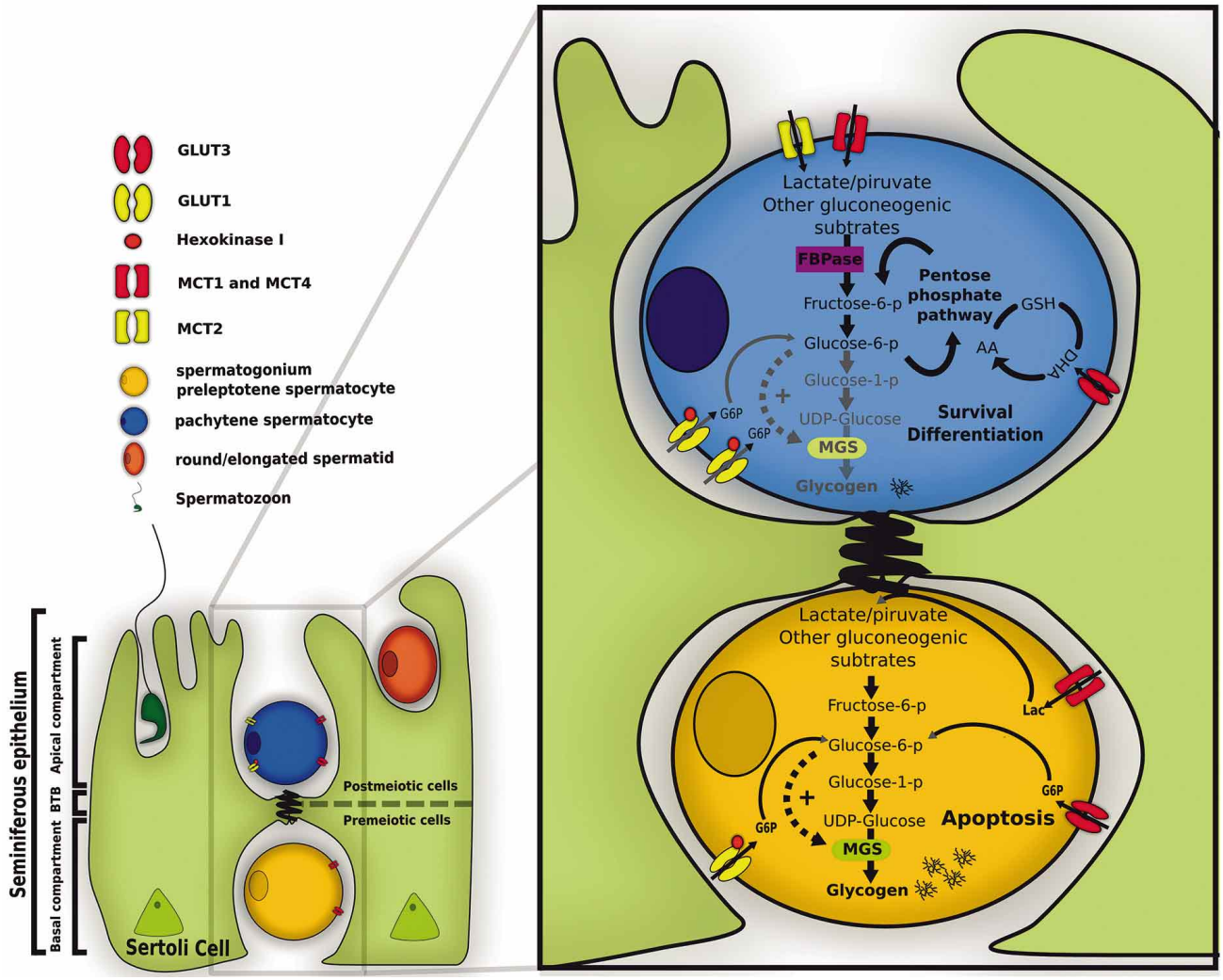

Fig. 1. Relationship between metabolic requirements during spermatogenic cell differentiation and proliferation. Premeiotic cells, before crossing the blood-testis barrier (BTB), are exposed to a glycolytic condition. GLUTs expression promotes mainly glucose uptake and glucose-6-phosphate accumulation could stimulate glycogen synthesis, while MCT-1 and -4 quickly support the ATP flux; in this scenario, glycogen accumulation could be a potential toxic metabolite and promote apoptosis. Furthermore, postmeiotic cells (at the adluminal compartment) have strong machinery for lactate utilization; in this case the FBPase expression indirectly promotes a strategy to remove the excess of lactate and pyruvate. The DHA uptake by GLUTs promotes an increase in redox power through ascorbic acid and glutathione recycling. In addition, the pentose phosphate pathway contributes to increase the redox power and clearance of gluconeogenic substrates promoting viability and differentiation of male germ cells. G6P, glucose-6-phosphate; Lac, lactate; AA, ascorbic acid; DHA, L-dehydroascorbic acid; GLUTs, glucose transporters; GSH, glutathione; MGS, muscle glycogen synthase; MCTs, monocarboxylate transporter.

Table I. Molecules expressed and synthesized in cells from the seminiferous tubules and its associated functions during testis development and spermatogenesis. This table is not meant to be exhaustive, and readers are encouraged to refer to relevant review articles cited throughout this review, in this table, and to references cited therein.

Specie	Cell kind	Molecule	Function	Reference
Human, mouse, and rat.	Spermatocytes, spermatids, sperm, sustentocytes, interstitial endocrine cells	GLUT1 and GLUT3	Glucose and DHA uptake	Kayano *et al.* (1988); Haber *et al.* (1993); Heijnen *et al.* (1997); Angulo *et al.* (1998); Wilson (2002), Wilson (2005); Rauch *et al.* (2006); Angulo *et al.* (2011)
Human, mouse, and rat.	Spermatids and sperm	GLUT5 and GLUT8	Fructose uptake	Angulo *et al.* (1998); Ibberson *et al.* (2002); Angulo *et al.* (2011)
Human, mouse, and rat.	Spermatocytes, spermatids, sperm, sustentocytes (primary culture and 42GPA9 cell line)	SVCT1 and SVCT2	Ascorbic acid uptake	Angulo *et al.* (1998); Wilson (2002); Wilson (2005); Angulo *et al.* (2008); Angulo *et al.* (2011)
Human, mouse, rat, boar and dog.	Spermatids and sperm	Liver FBPase	Gluconeogenesis and indirect glycogen synthesis	Yañez *et al.* (2003); Albarracín *et al.* (2004); Yañez *et al.* (2007); Galardo *et al.* (2014)
Human, mouse, rat, boar, stallion, and dog.	Spermatocytes (and GC1 cell line), spermatids, sustentocytes (primary culture and 42GPA9 cell line), and sperm (and whole testis)	Muscle glycogen synthase	Glycogen synthesis	Arzac (1950); Fabbrini *et al.* (1969); Fouquet & Guha (1969); Villarroel-Espíndola *et al.* (2013)

uptake by these cells might play a role during cell differentiation. In both cases, the physical orientation of meiotic and post-meiotic cells in seminiferous tubules determines nutrient access and oxygen availability to maintain glycolytic or oxidative metabolism. Antioxidant molecules, such as ascorbic acid and glutathione produce favorable conditions when the metabolic status is high, and pro-oxidant molecules are being accumulated. The fructose 1,6-bisphosphatase enzyme may also contribute by diminishing non-hexose substrates such as lactate/ pyruvate when mitochondria activity is low or oxygen access is limited. All of these situations may represent a concert of mechanisms that allow cell proliferation and differentiation in germinal epithelium.

Under conditions of limited nutrient access, testicular tissue may metabolize glycogen as a source of glucose. The presence of three isoforms of glycogen phosphorylase enzyme in this tissue suggests a new supply strategy for energetic precursors according to demand, especially when cell metabolism is strongly glycolytic. However, when glycogen is non-metabolizable and accumulates, because of some abnormal structure or a high rate of synthesis, the glycogen molecule may then have a negative effect, for example, as a pro-apoptotic agent in spermatogenic cells. Glycogen would thus seem to have a dual character, dependent on its structure and quantity and probably also dependent on cell characteristics.

Until now, metabolism of the male germ cells and its regulation are unclear. Many authors have focused their studies on somatic cells (sustentocytes and interstitial endocrine cells) or on the global changes during spermatogenesis. However, the single changes that occur during male germ cell differentiation could explain some controversial hypothesis about male infertility and other pathologies. In synthesis, male germ cells during differentiation can be considered as specialized individual cells with specific metabolic requirements and a particular sensitivity to their environment (Table I).

Depending on their differentiation state, these cells could be studied as a single cell, considering both its external and internal characteristics, such as morphological changes or gene expression, but also the metabolic flux, metabolites synthesized or accumulated and the impact of these on viability and spermatogenesis progression.

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ABREVIATIONS

AA, ascorbic acid; BTB, blood-testis barrier; DHA, L-dehydroascorbic acid; GDE, glycogen debranching enzyme; GLUTs, glucose transporters; SVCTs, sodium/vitamin C co-transporters; FBPase, fructose-1,6-biphosphatase; MGS, muscle glycogen synthase isoform; PFK, phosphofructose-1 kinase; TCA, tricarboxylic acid; MCT, monocarboxylate transporter; STZ, streptozotocin; PPP, pentose phosphate pathway; GSH, glutathione; G6PD, glucose 6-phosphate dehydrogenase.

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RESUMEN: La espermatogénesis es un proceso fisiológico complejo que implica la proliferación celular, la división meiótica y la diferenciación celular final de células post-meióticas en espermatozoides. Durante este proceso las células germinales masculinas también se someten a un proceso de diferenciación metabólico, en el que las células espermatogénicas post-meióticas (espermátidas) y las células espermatogénica no meióticas (espermatocitos) responden diferencialmente al metabolismo de la D-glucosa, la distribución de los transportadores de glucosa (GLUTs) y el uso de sustratos no-hexosa, tales como el lactato/piruvato o la dihidroxiacetona. Estas diferencias podrían explicarse por la necesidad de un proceso metabólico específico para apoyar la diferenciación de células o en algunos casos, la viabilidad celular. Además, aunque se considera que el glucógeno es la principal fuente glucosa, en las células germinales masculinas este polímero puede desempeñar un nuevo papel en la proliferación celular, actuando como un nuevo marcador para los eventos apoptóticos en el tejido testicular a través de un mecanismo aún desconocido. En este artículo, resumimos los principales cambios metabólicos que se producen durante la diferenciación germinal masculina, con un enfoque específico en las fuentes metabólicas durante la transición de espermatocito a espermátida. Este último, considerando que estas células proceden de la misma estirpe celular como células especializadas, pero no se aíslan desde su entorno, describiendo sus roles desde moléculas individuales a polímeros que actúan sobre la viabilidad de las células germinales masculinas.

PALABRAS CLAVE: Testículo; Carbohidratos; GLUTs; Glucógeno; Glucosa; Gluconeogénesis; Espermatogénesis.

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