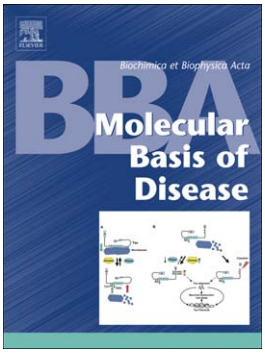

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Molecular mechanisms beyond glucose transport in Diabetes-related male infertility

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Abstract

Diabetes Mellitus (DM) is one of the greatest public health threats in modern societies. Although during a few years it was suggested that DM had no significant effect in male reproductive function, this view has been challenged in recent years. The increasing incidence of DM worldwide will inevitably result in a higher prevalence of this pathology in men of reproductive age and subfertility or infertility associated with DM is expected to dramatically rise in upcoming years. From a clinical perspective, the evaluation of semen parameters, as well as spermatozoa DNA integrity, are often studied due to their direct implications in natural and assisted conception. Nevertheless, recent studies based on the molecular mechanisms beyond glucose transport in testicular cells provide new insights in DM-induced alterations in male reproductive health. Testicular cells have their own glucose sensing machinery that react to hormonal fluctuations and have several mechanisms to counteract hyper- and hypoglycemic events. Moreover, the metabolic cooperation between testicular cells is crucial for normal spermatogenesis. Sertoli cells (SCs), which are the main components of blood-testis barrier, are not only responsible for the physical support of germ cells but also for lactate production that is then metabolized by the developing germ cells. Any alteration in this tied metabolic cooperation may have a dramatic consequence in male fertility potential. Therefore, we present an overview of the clinical significance of DM in the male reproductive health with emphasis on the molecular mechanisms beyond glucose fluctuation and transport in testicular cells.

1. Introduction

Diabetes Mellitus (DM) is one of the most prominent public health threats in modern societies and its prevalence is rapidly increasing. In 2000, the World Health Organization (WHO) reported that there were about 171 million people with DM, which represented a 60% increase relatively to 1995 [1]. A recent report by Wild and collaborators predicted that, if obesity rates are maintained constant, in 2030 there will be about 300 million individuals affected by DM, which could represent a 39% increase comparatively to 2000 [2]. However, the statistics that point to an overall 4.4% prevalence in the world population could be underestimated since the factors known to be responsible for the disease progression, such as obesity and lifestyle habits, may aggravate these numbers [2].

A closer look into fertility rates of modern societies reveals that the increased incidence of DM has been closely associated with falling birth rates and fertility [3, 4]. This is due to a disturbing increase of diabetic men in reproductive age. The great majority of patients with type 1 diabetes (T1D) are diagnosed before the age of 30 [5] and there is an alarming number of childhood and adolescent with T1D and type 2 diabetes (T2D) [6]. Moreover, western diets, lifestyle habits and obesity in young individuals strongly contribute for the increasing incidence of T2D in youth [7]. In Japan, there are already four times more adolescents with T2D than T1D [8], and, in USA, more than one third of the new DM diagnostics are in young adolescents [9]. Although there is no doubt that the disease is responsible for several pathological and biochemical alterations that reduce male fertility, the real impact of DM on male reproductive health remains obscure and is a matter of great debate. Sexual disorders, such as erectile dysfunction [10] or retrograde ejaculation [11, 12], are known to occur in diabetic individuals and usually end-up in a reduction of sexual appetite that is often attributed to lethargy and a certain degree of tiredness associated to the hyperglycemic state [13]. The decline in reproductive health of male diabetics can be apparently attenuated in a “well-

controlled" patient but the discussion on the molecular mechanisms behind such processes is scarce. Multiple molecular mechanisms and pathways are affected by DM with dramatic consequences to male reproductive function. The endocrine control of spermatozoa production event, spermatogenesis, is in fact severely altered in DM [14-16], and sperm quality and/or functioning of diabetic men can be compromised. This is a direct consequence of the unique characteristics of glucose metabolism that testicular cells present. Their glucose sensing machinery, hormonal control and specific mechanisms to counteract hyper- and hypoglycemia play a crucial role in the subfertility and/or infertility associated to DM.

Several clinical and animal studies have been focused on the molecular mechanisms responsible for the alterations induced by DM in male reproductive potential but much remains to be discussed. Important metabolic pathways, beyond glucose transport to the cells, remain undisclosed. Moreover, insulin (dys)function in male diabetic individuals is scarcely debated in the interpretation of the mechanisms responsible for male DM-induced subfertility/infertility. Therefore, we aim to present an up-to-date view on the male reproductive health of diabetic individuals and discuss the molecular mechanisms and pathways altered by DM beyond the expected increase in glucose transport to testicular cells. The role of insulin signaling will also be discussed. Finally, future directions in the study of the molecular mechanisms responsible for DM-induced male reproductive dysfunction will be presented.

2. Diabetes Mellitus at a glance

Diabetes is a chronic, metabolic disease characterized by hyperglycemia that can result from defects in insulin secretion and/or insulin action [17]. Moreover, there is a severe alteration in carbohydrate, lipid and protein metabolism [17] that causes several systemic complications and co-morbidities such as renal failure or hypertension [18, 19]. Therefore, the origin of the DM-induced dysfunctions is a result of multifactorial causes that cannot be disregarded and highly complicates the study of this disease.

The vast majority of the diagnosed DM cases are classified as T1D or T2D. In the first category, there is an absolute deficiency of insulin secretion, while in the second the cause can be a combination of insulin resistance and insufficient insulin secretion [17]. Noteworthy, in T2D the clinical symptoms are frequently detected only in an advanced phase of the disease, allowing the progression of functional changes in cells and tissues that may not be reverted even when a correct therapy is achieved [20]. One must also note that although the glycemic management in diabetic patients is crucial to reduce the development of several complications, it does not eliminate all the undesirable effects [21, 22]. Recent reports have questioned the safety of insulin [23] since some of the most important side-effects that diabetic patients suffer are related to the occurrence of hypoglycemic events, even if they occur transiently [24, 25]. Nevertheless, insulin remains the most powerful antihyperglycemic agent available although it is often combined with other antihyperglycemic agents to achieve euglycemia in diabetic patients. Noteworthy, recent studies reported that adults with DM can have a poor glycemic control due to intentionally missed insulin therapy [26] thus evidencing that clinical studies can, sometimes, be misled by this factor.

The complexity of DM diagnostic, especially in obese patients, led the researchers to establish an intermediate state, often called as pre-diabetes, where the patients present mild glycaemia that does not meet the established criteria for DM. Nevertheless pre-diabetic patients have important metabolic alterations that increase the risk for T2D development [27] and associated complications. Besides mild glycaemia, pre-diabetic individuals also present impaired glucose tolerance and insulin secretion as well as relative insulin insensitivity [27, 28]. Thus, special care must be taken in account when discussing the overall effects of DM in any relevant physiological condition because the spectrum of development stages of this disease is very complex [29]. It is also well known that DM alters the hypothalamic-pituitary-gonadal axis, which is responsible for some of the problems related to DM, such as impotence [16, 30]. Earlier studies reported elevated pituitary gonadotrophins in

diabetic rats [31] and altered levels of LH and FSH [32, 33]. Moreover, diabetic rats have abnormal sexual steroids feedback in the hypothalamic-pituitary axis described as a consequence of abnormal steroid transport or reduced pituitary sensitivity [15, 34]. Several reports also demonstrated that DM is associated with hormonal deregulation, particularly of sex steroids hormones [35-37].

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

3. Molecular basis of glucose metabolism in testis and sperm

Blood glucose concentrations depend on and alter the function of several organs and tissues. Liver and fat are usually known to suffer a tight control from glucose fluctuations, especially because they are known to play key roles in the use and storage of nutrients by hormonally regulated mechanisms. In testes, glucose metabolism is also a pivotal event. Moreover, spermatogenesis maintenance *in vivo* depends upon glucose metabolism [38] although there are low levels of this sugar in tubular fluid [39]. Therefore, blood-to-germ cells transport of glucose and other metabolic intermediates is highly controlled, particularly due to the presence of the blood-testis barrier (BTB). This barrier not only physically divides the seminiferous epithelium in two compartments but is also responsible for the maintenance of different levels of substances and metabolites between rete testis fluid and the lymph or plasma [40]. One of the most relevant testicular cells for all the functions of BTB is the Sertoli cell (SC). These cells have such an important role on male reproductive function that their number is usually associated with testis size [41] and they play a broad spectra of functions in the spermatogenic event. Besides being responsible for water transport from the interstitial space to the lumen [42], they also control the seminiferous fluid pH and ionic composition [43-45]. Furthermore, SCs are known as “nurse cells” because

they provide physical and nutritional support for the developing germ cells, which is pivotal for a normal spermatogenesis [46]. SCs are well known for their ability to produce lactate at a higher rate [39, 46-50] that is consumed, together with pyruvate, by pachytene spermatocytes and round spermatids [46, 47, 51] (Fig. 1).

Glucose passive transport across BTB, mediated by glucose transporters (GLUTs), is an important event to spermatogenesis. In SCs, the main components of BTB, GLUT1, GLUT3 and GLUT8 isoforms have been identified [52, 53]. GLUT1 and GLUT3 expression proved to be sensitive to several substances such as hormones, cytokines or growth factors, which are known to regulate glucose uptake by SCs [49, 50, 53, 54]. Recent *in vitro* studies reported that when glucose [55] or insulin [54] are removed from extracellular medium, SCs are capable to adapt their glucose transport machinery in order to ensure the appropriate lactate production.

In these processes, lactate dehydrogenase (LDH) is pivotal for the continued ATP production by glycolysis. Moreover, germ cells express a specific isozyme of LDH, known as LDH type C (LDHC) that is abundant in spermatids and spermatozoa [56, 57]. After glucose uptake by SCs and the conversion of pyruvate to lactate by LDH, it is imperative that this product of glycolysis becomes available for the developing germ cells. This event is mediated by active membrane monocarboxylate transporters (MCTs) that are responsible for lactate transport through the plasma membrane of SCs [49, 54, 55, 58] (Fig. 1). We [49, 50, 59] and others [53, 60, 61] reported that the testicular cells metabolic cooperation is under strict hormonal control leading us to conclude that the hormonal control of SCs metabolism is pivotal for spermatogenesis [62]. Therefore, understanding SCs energy metabolism may help to identify and promote new therapeutic approaches against subfertility or infertility caused by pathological conditions as spermatogenesis is in the basis of male fertility.

The molecular basis of glucose metabolism and the importance of its subproducts in testis are far beyond the simple maintenance of the germ cells nutritional status. In fact, a vast majority of patients suffering from subfertility and infertility have problems in

sperm function rather than lack of sperm [63]. The ATP levels in sperm are maintained by several glycolytic and non-glycolytic substrates since both glycolysis and mitochondrial respiration are active in mammalian sperm [64]. However, sperm capacitation is known to be stimulated by glucose [65], evidencing the regulatory role that this sugar can exert in the overall male reproductive function. Importantly, the loss of sperm functional competence is often associated to oxidative stress that arises from sperm metabolism [66], since sperm capacitation is achieved by biochemical and metabolic modifications.

4. Diabetes-related male subfertility/infertility

a) Clinical Data

Recently, a high prevalence (51%) of subfertility was reported among patients with DM [67]. This led to the perception that DM is responsible for inducing subtle but crucial changes in sperm quality and function [68]. It is well known that in men DM is responsible for important sexual disorders such as decreased libido and impotence [69]. Erectile dysfunction (ED) is also very prevalent in diabetic men [70], but it ultimately depends on the male age, the duration of the disease and the level of blood glucose levels control. In a population-based cohort study, there was an increase of ED in older men with DM [71] and it has been reported that the glycemic control is correlated with ED [72]. Ejaculation disturbance is another factor that has been long identified in men with DM, especially retrograde ejaculation [73, 74], a condition where semen passes backwards into the bladder.

Diabetic male reproductive capacity is undoubtedly affect by ED and retrograde ejaculation, but when studying sperm parameters and sperm quality markers, the literature shows some conflicting results. In the 70's a study involving 25 diabetic individuals and 24 control individuals (16-22 years old), showed that T1D juveniles presented lower sperm values and significant differences in sperm motility and morphology [75]. A few years later, other study compared the ejaculated of 65 diabetic

and 77 control men and it was reported a negative effect of DM in the ejaculated. The parameters mostly affected were, in decreased order, sperm motility, morphology, volume and count [76]. Slightly different results were reported in a 1984 study, where T1D adolescents presented a minor, non-significant, decrease in sperm count relatively to control individuals [77]. The semen from these T1D adolescents had lower volume and motility, as well as altered morphology, and presented significantly higher fructose and glucose levels, evidencing that an ineffective metabolic control can be deleterious and/or responsible for the observed alterations in the semen [77]. Later, a study in testicular biopsies from impotent men with DM, concluded that the testes of impotent diabetic men presented ultrastructural lesions in apical SC cytoplasm and morphological changes in the interstitial compartment, thus suggesting spermatogenic disruption and subfertility or infertility problems [78]. Another work reported that T1D and T2D subjects had a significant increase in sperm count and concentration, and a significant decrease in sperm motility and semen volume [77]. Interestingly, the sperm morphology and the quality of sperm motility remained unaffected [79]. Spermatozoa motility of T1D patients was also evaluated by Niven and collaborators using a computerized image analysis system [80]. They reported no correlations between sperm motility and age, age of onset of DM and duration of DM. Nonetheless, when comparing the diabetic group with control subjects, they concluded that several parameters related to sperm motility, such as track speed, path velocity, progressive velocity, and lateral head displacement, were not altered. On the other hand, other sperm motility related parameters, such as linearity and linear index that analyze the straightness of swimming, were significantly greater in diabetic men [80]. The controversy about the real impact of DM in male reproductive health continued, and, in 2002, a sperm cryopreservation study of patients with several diseases, including DM, reported that only the sperm from diabetic men presented significant differences in sperm parameters, namely in sperm counts [81]. More recently, a study with both T1D and T2D patients reported that diabetic men may present normal semen parameters,

or only a significant decrease in semen volume, but they have a higher level of damage in sperm nuclear and mitochondrial DNA than control individuals [5].

There are apparently contradictory results concerning sperm motility and other male reproductive parameters when studying male diabetics. In fact, the conflicting nature of the existing data should be clarified. The fact that most of these studies are more focused on the clinical significance, rather than the molecular mechanisms behind DM-induced alterations, may explain at some extend the lack of consensus. For instance, the conventional semen analysis that was used in several of these studies, has been recognized by some authors, as very limited in the determination of the fertility status [82]. Moreover, there are several factors very difficult to control such as the duration of the disease, the glycemic control, the type of treatment as well as all the comorbidities associated to DM that mask the outcomes of the works related with DM and male fertility. Molecular studies with sperm from diabetics are still scarce and most of the mechanisms through which sperm manage to attain their energy metabolism in diabetic men remain to be disclosed. Therefore, there is an urgent need for more studies focused on the molecular mechanisms beyond glucose transport in sperm of diabetic men. However, there is a more profound lack of literature concerning the effect of DM in the functioning of testicular cells. It is expected that the metabolic cooperation between testicular cells may be compromised. To surpass these issues, as experimentation in humans has great restrictions and in most cases the tested hypotheses are impossible to assess in human material, the use of animal models that allow a tight control over experimental conditions are of extreme importance.

b) Animal Models data

Interestingly, data from animal models are successful in demonstrating that DM impairs male fertility and it is responsible for alterations in the reproductive health of individuals. As expected, there are more studies with animal models than with clinical data concerning DM-related male infertility. Since the 70's there are studies reporting a

reduced fertility in male diabetic rats [83] and in the early 80's several reports using BB Wistar rats, a strain that spontaneously develop DM, described gonadal dysfunction in these animals [84, 85]. A few years later the first study with a DM rat model induced by streptozotocin (STZ) and insulin therapy was done, reporting that STZ-treated rats presented decreased sperm counts and motility and that insulin treatment was able to restore these parameters [86]. This study evidenced that glycemic control may play a key role in reducing DM-related subfertility or infertility problems. Concomitant with that hypothesis, it was reported in the early 90's that long-term DM with sustained uncontrolled hyperglycemia is responsible for testicular dysfunction, resulting in decreased fertility potential [87]. Later it was described that STZ-induced DM male rats have altered sex behavior and diminished reproductive organ weight, testicular sperm content, epididymal sperm content, as well as sperm motility [88]. Studies on STZ-treated diabetic rats also showed that DM may cause regression of epididymis, leading to a decrease in caput weight, corpus, and caudal regions. Those studies also described atrophic changes in the caput, corpus, and caudal epididymis that resulted in voiding of spermatozoa from the epididymal lumen [89]. Insulin treatment was able to prevent some of these deleterious effects but only on certain epididymal regions [89]. Additionally, others studied the fertilization ability of spermatozoa from male STZ-induced diabetics and reported that these animals had a significant reproductive dysfunction that resulted from a decrease in the reproductive organ weights and in sperm counts, though not compromising sperm fertilizing ability using in utero insemination [90]. Also, a study comparing T1D and T2D using STZ-treated and Goto-Kakizaki (GK) rats showed that hyperglycemia had an adverse effect in sperm concentration and motility [91]. More recently, Kim and Moley [92] studied diabetic male mice sperm quality and fertilization capacity as well as subsequent embryo development. They concluded that DM decreased sperm concentration and motility and may cause male subfertility by altering steroidogenesis [92]. Others reported that the offspring of diabetic female rats, presented altered testicular parameters during

fetal life, which can affect reproductive health during post puberty [93]. The offspring males presented increased number of seminiferous tubules besides thickness of the testicular capsule and reduced number of Leydig, Sertoli and spermatogonia cells [93]. Although there are contradictory results concerning sperm motility and other male reproductive parameters in diabetic men, it seems more consensual that DM rat models present significant alterations that end-up in reduced male reproductive health (see table 1 for details). Nevertheless most of the works are not actually focused on the molecular mechanisms beyond glucose deregulation. The pathological alterations induced throughout the male reproductive tract of rodents are diverse in nature and although the histological changes are evident, most of the biochemical changes remain unknown. Thus, it is imperative to disclose the molecular mechanisms behind testicular cells metabolic dysfunction caused by DM to identify key points for possible therapeutic interventions.

5. Role of insulin dysfunction in male infertility

Euglycemia is very difficult to achieve in diabetic individuals although glycemic control is crucial to reduce DM-related complications [22]. In fact, hypoglycemia and the correlated hyperinsulinemia are common events in diabetic individuals. Glucose and insulin fluctuations lead to important molecular alterations that may result in detrimental effects to the reproductive health of the diabetic men. The undesirable effects of DM can be controlled depending on the DM type and the stage of the disease progression but hyperinsulinemia and hypoglycemia are common in both T1D and T2D [94, 95]. Moreover, throughout the diabetic individual's life, especially T2D subjects, become progressively more insulin deficient and, consequently, more vulnerable to the undesirable effects of a poor glycemic control. Furthermore, contrarily to non-diabetic individuals that are very sensitive to small glucose or insulin variations in plasma, diabetic individuals gradually lose their sensitiveness to glucose and insulin [96, 97]. This is very important because, as discussed earlier, the number of diabetic

adolescents is increasing and their reproductive health may be compromised at a very early age. Noteworthy, although the use of insulin analogues and/or insulin is essential they do not guaranty total effectiveness. In addition, insulin therapy can be a double-edged sword and its safety has been questioned [23-25]. Thus, it is imperative to discuss the molecular mechanisms altered in testicular cells when insulin deregulation occurs.

Few studies have been focused on the molecular mechanisms that are altered in testicular cells by insulin deregulation. In the early 80's the first studies concerning the effect of insulin in SCs glucose metabolism were published. Initially, it was reported that insulin acted synergistically with other hormones but its importance to male reproduction remained unknown [98]. Others reported that insulin stimulated the uptake of total nucleotide pool and in ATP, GTP, and UTP pools [99] as well as transferrin secretion by SCs [100]. Then, it was reported that insulin at micromolar concentrations induced stimulatory effects not only in DNA and protein synthesis but also in SCs lactate production [101]. Later, it was reported that after 3 hours of insulin addition to SCs, they showed a marked stimulation of lactate production [102] (Fig. 2). This insulin effect on cultured immature rat SCs was described as being mediated by insulin receptors [102] (Fig. 2), however it was only a few years later that the authors reported not only the presence of insulin receptors but also suggested that insulin and insulin-like growth factor-I (IGF-I) had precise functions responsible for the regulation of specific SCs activities during spermatogenesis initiation and maintenance [103, 104]. Interestingly, Leydig cells cultured in a Sertoli cell-conditioned medium had also their functions controlled by the presence or absence of insulin [105]. In the 90's it was reported that insulin could not only regulate glycine metabolism but also lipid metabolism in cultured SCs [106]. Later, a study in SCs and Leydig cells of STZ-treated rats, evidenced that the biosynthesis of arachidonic acid, a polyunsaturated fatty acid essential for several cellular functions, was under insulin regulation [107] (Fig. 2). Importantly, it was reported that insulin could promote the differentiation of

spermatogonia into primary spermatocytes by binding to the IGF-I receptor [108]. More recently, it was suggested that insulin restores the reproductive health in diabetic males by the normalization of the hypothalamic-pituitary-gonadal axis, and thus via the LH and testosterone levels, rather than having a direct interaction in the testis [109]. However, it was also recently reported that insulin deprivation induced several important metabolic alterations in cultured SCs [54, 59]. Insulin-deprived cultured SCs presented lower glucose consumption that was followed by a lower lactate production, down-regulation of metabolism-associated genes of lactate production and export and also an intriguing modulation of GLUT1 and GLUT3 [54] (Fig. 2). Noteworthy, acetate secretion, which is also reported to be one of the major SCs functions, was completely suppressed by SCs cultured under insulin deprivation conditions [59]. Although the exact meaning of this mechanism to spermatogenesis was not completely disclosed, the reduction of acetate production, necessary for the continuous membrane remodeling that occurs in the developing germ cells, may compromise the spermatogenic event.

Insulin role on the male reproductive tract goes far beyond testicular cells. In the 70's the first studies pointed to a stimulation of hexoses metabolism by sperm under insulin action [110]. However, the discussion continued whether insulin could modulate sperm metabolism or not with a study reporting that spermatozoa glucose oxidation could be independent from extracellular glucose concentration and insulin was not able to alter neither glucose metabolism nor spermatozoa motility [111]. A few years later, it was reported that both plasma membrane and the acrosome of spermatozoa are targets for insulin action and thus are under insulin hormonal control [112]. Also, intratesticular injection of insulin resulted in a decrease in spermatozoa motility in vas deferens and an increase in motile spermatozoa percentage on incubation medium after removal [113]. Moreover, defects in insulin secretion may change testicular and sexual glands function [114]. Interestingly, a study about the incidence of insulin resistance in men with ED, concluded that these men have a high incidence of insulin resistance thus

evidencing a possible role for insulin in the reproductive health of men [115]. Others also reported that in men with increased insulin resistance there is a decrease in Leydig cell testosterone secretion and, thus, severe male reproductive dysfunction [116]. Importantly, a recent study from Aquila and collaborators [117] proved that not only human ejaculated spermatozoa secrete insulin, as there is a physiological role for this insulin in the autocrine glucose metabolism regulation. This finding opened a new possible role for insulin in spermatozoa capacitation by controlling their glucose metabolic pathways. Others have also reported that insulin treatment in washed human spermatozoa from normozoospermic donors significantly increased total and progressive motility and acrosome reaction, as well as nitric oxide (NO) production, thus evidencing that insulin can enhance human spermatozoa fertilization capacity [118]. Moreover, a mitochondrial citrate carrier that contributes to the acquisition of sperm fertilizing ability through insulin secretion control has been identified [65], showing that insulin can act in sperm metabolism in a more complex way than the first proposed mechanisms.

In sum, from the literature overview we conclude that insulin dysfunction has a crucial role in male infertility and/or subfertility related to DM and it is vital to study how insulin and insulin analogues therapy is administered and used to maintain the glycemic control. Moreover, insulin-induced hypoglycemia, a phenomenon that is frequent and in some rare cases fatal, has unpredictable effects in the overall metabolism of testicular cells. In fact, neither the magnitude of insulin dysfunction nor the mechanisms by which insulin controls testicular cells and sperm metabolism are fully disclosed. This issue should deserve more attention in the future.

6. Molecular mechanisms of testicular glucose metabolism in diabetic conditions

There is no doubt that spermatogenesis is a metabolically active process that depends upon strict metabolic cooperation between the several testicular cell types. During spermatogenesis, spermatozoa are produced within the seminiferous tubule in a

process that takes several days and is under endocrine and paracrine control through the SCs [10]. In addition, SCs are responsible for the conversion of glucose, a non-metabolized substrate by developing germ cells, in lactate which is the preferential substrate for those cells. The molecular mechanisms of testicular glucose metabolism in diabetic conditions are far from being disclosed. Nevertheless, *in vitro* and morphological studies of human biopsies from diabetic men allowed the collection of small but vital information concerning those molecular mechanisms. Human biopsies from diabetic men showed that there are morphological changes in testicular cells, namely in SCs, which presented extensive vacuolization and had a high degree of degeneration [78]. Moreover, germ cells exhibited a normal morphology, but the seminiferous tubules were depleted, and the number of Leydig cells was also very variable, with these cells presenting lipid droplets and variable number of vacuoles [78]. All these changes certainly have dramatic consequences to testicular cells glucose metabolism and to the overall metabolic cooperation between SCs and developing germ cells. In fact, impairment of glucose metabolism is often related with increased fatty acid metabolism. Early studies reported that DM caused an increased endogenous oxygen uptake and reduced lactate production by testicular cells [119]. The same study also reported that DM increased cholesterol, non-esterified fatty acids, triglycerides and phospholipids in rat testis tissues [119]. In the 80's, Hutson [120] studied the biochemical responses of rat SCs and peritubular cells cultured under simulated diabetic conditions and reported that the *in vitro* metabolic functioning of these testicular cells was very sensitive to glucose concentrations. In fact, SCs cultured with high glucose concentration increased lactate secretion. At that time, it had already been reported that lactate enhanced respiration rates and protein and RNA synthesis in isolated pachytene spermatocytes and round spermatids [47], by interacting in other metabolic pathways and producing adenosine triphosphate (ATP) [121]. Besides, lactate was also described as a modulator of nicotinamide adenine dinucleotide phosphate-oxidase (NADH) oxidation and the pentose phosphate pathway in those

cells [122]. In the 90's, new functions for the lactate produced in the testis were described. Intratesticular lactate infusion was reported to improve the spermatogenesis in adult cryptorchid rat testis [123] and later, it was described that germ cell death is inhibited in a dose-dependent way by lactate [124]. Therefore, any condition that promotes an alteration in testicular lactate levels or lactate production by SCs may compromise germ cell development and this can occur through several distinct mechanisms.

Recently, *in vitro* works focused on the molecular mechanisms of glucose or insulin deprivation in SCs. It was reported that decreased glucose levels in SCs culture medium increases glucose uptake to maintain lactate production, which was only slight decreased [55]. This was achieved by modulating GLUT1 and GLUT3 expression, through activation of AMP-activated protein kinase (AMPK), phosphatidylinositol 3-kinase (PI3K)/PKB, and p38 MAPK-dependent pathways [55]. The authors do not infer about possible alterations in the key steps of glycolysis. One of those steps would be the conversion of fructose 6-phosphate to fructose 1,6-biphosphate by phosphofructokinase (PFK) as well as LDH action, which is responsible for the interconversion of lactate into pyruvate. Finally, the effect on the mechanisms of lactate export by MCTs remained to be elucidated. Nevertheless, that work was pioneer in evidencing that SCs can adapt their glucose metabolism by modulating GLUTs expression. Interestingly, as discussed earlier, the same behavior in GLUTs expression was recently described in human SCs cultured in insulin deprivation conditions [54]. That work was one of the first attempts to disclose the mechanisms by which insulin deprivation or insulin resistance, that are common DM features, alter carbohydrate metabolism, namely glucose metabolism. In insulin-deprived cells, total glucose uptake was not altered, mainly due to the discussed GLUTs adaptation, but pyruvate consumption was compromised. The insulin-deprived cells not only stopped to consume pyruvate but started to produce this substrate that was then exported into the extracellular medium [54]. Consequently, lactate production was compromised and two

mechanisms of lactate metabolic control were found to be altered: LDH and MCT4. Moreover, it was later proposed that SCs could produce and export acetate to the developing germ cells and that this acetate production could also be regulated by external insulin [59]. In fact, it was reported that cultured human SCs secreted high amounts of acetate that was suppressed by insulin deprivation, through a mechanism mediated by the modulation of Acetyl-CoA synthase and Acetyl-CoA hydrolase expression. Therefore, at least *in vitro*, the molecular mechanisms of testicular glucose metabolism are severely altered by glucose and insulin fluctuations in ways that can compromise spermatogenesis.

Although the exact mechanisms by which DM alters the glucose metabolism in testicular cells are not easy to follow *in vivo*, *in vitro* evidence cannot be disregarded even those dispersed and indirect. As discussed above, it is well known that DM is a metabolic disease that induces crucial alterations in sex hormones levels [125]. Recent work in both, rat and human SCs, showed that their energy metabolism is influenced by sex steroid hormones. Cells treated with 5α-Dihydrotestosterone (DHT) consumed less glucose and thus, produced less lactate. Both, 17β-estradiol (E2) and DHT were able to alter lactate metabolism-associated gene transcript levels such as MCT4, LDH and also GLUTs [49, 50]. Moreover, sex steroids are also known to regulate apoptotic signaling pathways in SCs thus deregulated levels of these hormones may lead to apoptosis and necrosis [126, 127]. Nonetheless, when analyzing all these molecular mechanisms that are certainly related to DM pathological conditions, one cannot neglect the possible contribution of glycogen. It has been reported that SCs possess glycogen and glycogen phosphorylase activity [128, 129] but these reports from the 80's were not consolidated and therefore should deserve special attention in a near future to understand the adaptive mechanisms of SCs in pathological conditions. The presence of glycogen and glycogen metabolism related machinery can be a valuable mechanism to explain some of the assumptions concerning DM-related effects in glucose testicular cells.

7. Role of alternative fuels and oxidative stress in testis from male diabetics

As discussed earlier, euglycemia is very difficult to achieve and maintain in diabetic individuals that often face hyper- and hypoglycemia. Those fluctuations in insulin and glucose concentrations lead to several problems. Under glucose deprivation and/or insulin deregulation, testicular cells are expected to suffer metabolic adaptations and use alternative substrates. In fact, the role of these alternative substrates is underestimated when analyzing some of the available data. SCs have an enormous metabolic plasticity and can use several metabolic substrates such as palmitate and ketone bodies as well as glutamine, alanine, leucine, glycine and valine [130, 131]. Besides, these cells can produce ATP via the β-oxidation of free fatty acids or even through recycled lipids from apoptotic spermatogenic cells and residual bodies [132]. DM is known to induce severe alterations in lipid metabolism and studies in mice with inactivated genes of lipid metabolism proved that lipid metabolism are essential for a normal spermatogenesis [133]. Therefore, although there is a lack of literature regarding the use of these alternative substrates in testicular cells of diabetic individuals, we cannot disregard their contribution to the overall metabolic dysfunction that occurs in the testis of diabetic individuals.

There are also growing evidence that DM increases cellular oxidative stress due to the overproduction of reactive oxygen species (ROS) and decreased antioxidant efficiency [134]. This is a direct consequence of increased glycolytic capacity induced by higher glucose availability. Therefore, in testicular cells such as SCs where glucose metabolism is very active to produce lactate, it is expected increased levels of oxidative stress. This is an issue that deserves special attention in the near future because ROS generation has a real potential toxic effect in sperm quality and function. High levels of ROS are reported to be detrimental to fertility potential in natural and assisted conception [135]. Moreover, ROS production stimulated by hyperglycemia is recognized as a major cause for the clinical complications associated with DM and

obesity [136]. Hyperglycemia-induced superoxide overproduction inhibits glucose-6-phosphate dehydrogenase [137] thus decreasing the antioxidant defenses and ROS overproduction and is also intimately related with mitochondrial dysfunction. It has been reported that mitochondrial fission/fusion machinery is sensitive to the increased ROS production after hyperglycemia stimulation [138] and mitochondrial testicular antioxidant capacity is also known to be modulated by DM as an adaptive response to the increased oxidative stress [139]. In fact, hyperglycemia was reported to induce important alterations in sperm concentration and motility by altering energy production and free radical management [91], which is associated with the fact that human spermatozoa are known to be highly sensitive to oxidative stress. Sperm plasma membrane and nuclear or mitochondrial DNA fragmentation also occur in response to ROS, compromising the fertility potential of the individuals [140]. Increased sperm DNA damage was reported in diabetic men in reproductive age and associated to high oxidative stress resulting from sperm overexposure to glucose [5]. These mechanisms of sperm nuclear DNA damage in diabetic men promoted by ROS are suggested to be mediated through advanced glycation end products (AGEs) [141] and these AGEs accumulate in the reproductive tract of diabetic men [142]. Moreover, increased oxidative stress and higher DNA fragmentation is interconnected with apoptosis [143, 144], which has been reported to be increased in sperm from T1D and T2D men and suggested as a possible mechanism to explain subfertility in these individuals [145]. Recently, a possible central role for ROS in the pathogenesis of insulin resistance has also been highlighted [146], thus evidencing that ROS production stimulated by DM may have roles that go far beyond the glucose transport and metabolism.

8. Conclusion

It is obvious that not all diabetic men are infertile and there are conflicting results concerning the effect of DM in sperm parameters but, even when conventional sperm parameters do not differ, the glucose transport mechanisms are expected to be altered.

Glucose availability and/or insulin dysfunction induced by DM are expected to induce important metabolic adaptations in testicular cells, besides changes in oxidative stress that are often reflected in mitochondrial and nuclear DNA fragmentation. Moreover, glucose metabolism is as important for testicular cells as for mature sperm. In fact, sperm hyperactivation and/or capacitation depend of this substrate, although it is not clear if glucose is necessary for direct energy production or as a precursor for other metabolic products [147]. Besides, it remains to be disclosed the mechanisms responsible for the DM-induced alterations in these processes. Although we have to admit that from a clinical perspective the knowledge of the molecular mechanisms beyond glucose transport are not as appellative as DNA integrity and oxidative stress, which seem to have a more direct effect in natural and assisted conception, disclosing the pathways by which the chronic metabolic changes, beyond glucose transport, are altered by DM in testicular cells, will open new possibilities for pharmacological intervention. The presence of several DM-related comorbidities also complicates the study of DM-induced alterations in testicular cells and sperm. Most of the time the patients are treated in a polypharmacy regimen and the inappropriate use of these agents are associated with significant morbidity and mortality [148]. This is a crucial point that, in some cases, is not correctly weighed in clinical studies. The possible subtle molecular alterations in the male reproductive health promoted by pharmacological agents currently used in DM treatment should also deserve special attention in the near future.

In conclusion, the effects of DM on the molecular mechanisms responsible for the maintenance of the male reproductive health have been somewhat neglected, apart from the concerns about impotence, semen quality and, at some extend, oxidative stress and DNA fragmentation. Nevertheless, studies on the molecular mechanisms beyond glucose transport in DM-related male infertility will be on the spotlight in the next few years to uncover the metabolic plasticity in sperm and testicular cells of

diabetic individuals. This will certainly be a step forward for a possible metabolic therapeutic intervention.

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Figure Legends

Figure 1. Schematic illustration of the metabolic cooperation between Sertoli and germ cells. The glucose from the interstitial fluid enters the Sertoli cells through glucose transporters, mainly GLUT1 and GLUT3, and is converted into glucose-6-phosphate that is then converted into pyruvate by phosphofructokinase (PFK). The pyruvate can then be: a) transported into the mitochondrial matrix to form Acetyl-CoA; b) converted into lactate by lactate dehydrogenase (LDH); or c) converted into alanine by alanine aminotransferase (ALT). The lactate produced by Sertoli cells is exported to the intratubular fluid by proton-linked plasma membrane transporters (MCTs), mainly MCT4. The germ cells uptake the lactate produced by Sertoli cells through MCTs, which is then directed for ATP production.

Figure 2. Schematic illustration of the insulin action in Sertoli cells metabolic pathways. The insulin effects, mediated by its interaction with insulin receptors, can induce several effects in metabolism-associated transporters. Some of those effects can be stimulatory, such as in monocarboxylate transporter 4 (MCT4), glucose transporter 3 (GLUT3), lactate dehydrogenase (LDH), acetyl-CoA synthase and acetyl-CoA hydrolase; while other effects can be inhibitory such as in glucose transporter 1 (GLUT1) and arachidonic acid synthesis. ⊕ - Stimulatory effect; ⊖- inhibitory effect.

Tables

Table 1. Summary of the main studies reporting diabetes-related reproductive effects.

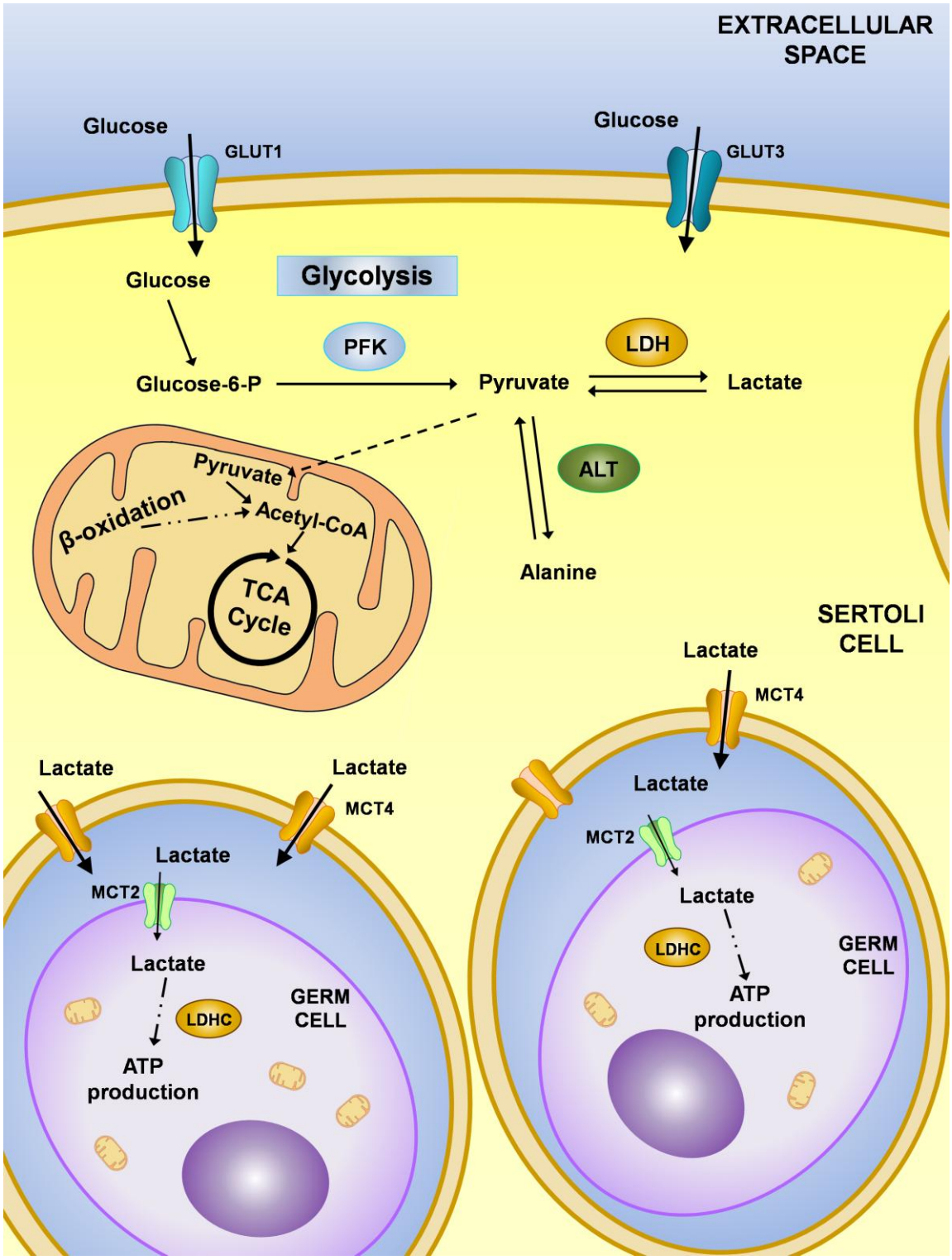
EXTRACELLULAR SPACE
Glucose
GLUT1
Glucose
GLUT3
Glucose
Glycolysis
PFK
LDH
Glucose-6-P
Pyruvate
Lactate
Pyruvate
β-oxidation
Acetyl-CoA
TCA Cycle
ALT
Alanine
SERTOLI CELL
Lactate
MCT4
Lactate
Lactate
MCT4
Lactate
MCT2
Lactate
MCT2
Lactate
LDHC
GERM CELL
ATP production
Lactate
LDHC
GERM CELL
ATP production

Figure 1

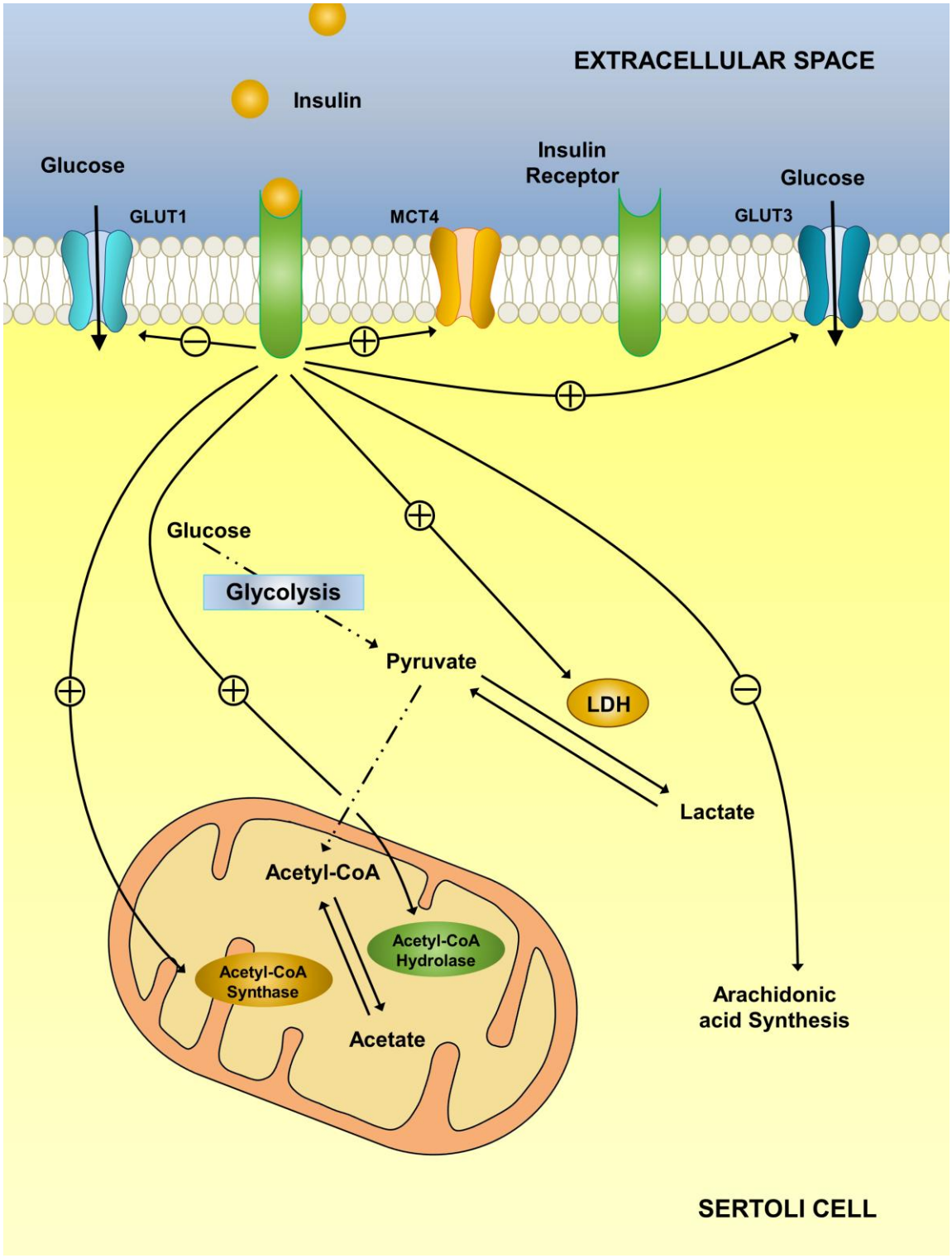

Figure 2

Table 1. Summary of the main studies reporting diabetes-related reproductive effects.

Model			Type of Diabetes	Reproductive Effects
Animal Studies	*Genetic Models*	BB Rat	T1D	↓ Testis weight [87]
				Severe germ-cell depletion [85]
				↓ Serum testosterone [85,87]
				Disruption of seminiferous tubular morphology [85, 87]
				Sertoli-cell vacuolization [85]
				↓ Sperm production [87]
				↓ Fertility [84,87]
		GK Rat	T2D	Decreased sperm production [91]
	Chemically-Induced Models	STZ Rat	T1D	↓ Testis weight [86,90]
				Disruption of epididymis morphology and density [89]
				↓ LH, FSH and testosterone serum levels [86, 88, 90]
				↓ Sperm production [88, 89]
				↓ Sperm counts and motility [86, 88, 90, 91, 92]
				Erectile dysfunction [70]
				Ejaculation dysfunction [88, 90]
				↓ Mating behaviour [88, 90]
				↓ Fertility [90, 92]
		ALX Rat	T2D	↓ LH, FSH and testosterone serum levels [93]
				Disruption of seminiferous tubular morphology [93]
				↓ Number of Leydig and Sertoli cells [93]
				↓ Number of spermatogonia [93]
Clinical Studies			T1D	Disruption of seminiferous tubular morphology [78]
				Germ-cell depletion and Sertoli-cell vacuolization [78]
				Disruption of Blood-Testis Barrier [78]
				Erectile dysfunction [70, 76]
				Ejaculation dysfunction [73, 74]
				↓ Semen volume [76, 77]
				↓ Sperm counts, motility and morphology [75, 76, 77]
			T2D	Erectile dysfunction [69]
				↓ Semen volume [5, 79]
				↓ Sperm motility [79]
				↑ Sperm DNA fragmentation [79]

Legend: BB Rat – BioBreeding genetic rodent model; GK Rats – Goto-Kakizaki genetic rodent model; STZ rat – Streptozotocin-induced rodent model; ALX rat – Alloxan-induced rodent model. T1D – type 1 diabetes; T2D – type 2 diabetes; ↑ - increase; ↓ - decrease; superscript numbers are references as indicated in references section.

Highlights

- The increasing incidence of Diabetes (DM) is affecting men in reproductive age.
- DM associated male subfertility/infertility will dramatically rise.
- DM may compromise the metabolic cooperation between testicular cells.
- Insulin (de)regulation is a major player in male reproductive health.
- Effects of DM on testicular metabolic pathways have been somewhat neglected.