High glucose concentrations *per se* do not adversely affect human sperm function *in vitro*

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Abstract

Diabetes mellitus (DM) represents one of the greatest concerns to global health and it is associated with diverse clinical complications, including reproductive dysfunction. Given the multifactorial nature of DM, the mechanisms that underlie reproductive dysfunction remain unclear. Considering that hyperglycemia has been described as a major effector of the disease pathophysiology, we used an *in vitro* approach to address the isolated effect of high glucose conditions on human sperm function, thus avoiding other *in vivo* confounding players. We performed a complete and integrated analysis by measuring a variety of important indicators of spermatozoa functionality (such as motility, viability, capacitation status, acrosomal integrity, mitochondrial superoxide production and membrane potential) in human sperm samples after incubation with D- and L-glucose (5, 25, or 50 mM) for 24 and 48 h. No direct effects promoted by 25 or 50 mM D-glucose were found for any of the parameters assessed ($P > 0.05$), except for the acrosome reaction, which was potentiated after 48 h of exposure to 50 mM D-glucose ($P < 0.05$). Interestingly, non-metabolizable L-glucose drastically increased superoxide production ($P < 0.05$) and suppressed sperm motility ($P < 0.05$) and capacitation ($P < 0.05$) after 24 h of treatment, whereas mitochondrial membrane potential ($P < 0.05$), acrosomal integrity ($P < 0.01$) and viability ($P < 0.05$) were later decreased. The overall results suggest that high glucose levels *per se* do not influence human sperm function *in vitro*, which stresses the importance of other factors involved in DM pathology. Nevertheless, the absence of metabolizable glucose contributes to a severe impairment of sperm function and thus compromises male fertility.

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Introduction

Diabetes mellitus (DM), which is clinically characterized by hyperglycemia, is a degenerative disease with serious and wide-range long-standing consequences in terms of human health (Marx 2002, ADA 2013). Worryingly, DM is progressing at epidemic rates, and the number of cases is predicted to increase about 40% by 2030 as compared to 2000 (Wild *et al.* 2004). This rising incidence will unavoidably result in an increased prevalence of DM in men before and during reproductive age (Agbaje *et al.* 2007). In fact, cumulative evidence suggests that DM may adversely affect male reproductive function on multiple levels (Miller *et al.* 1981, Lucas *et al.* 1989, Agbaje *et al.* 2007, Mulholland *et al.* 2011, La Vignera *et al.* 2012). Despite some conflicting results in the literature (La Vignera *et al.* 2012), standard semen analyses of diabetic individuals

in general revealed not only a decrease in sperm motility (Barták *et al.* 1975, Amaral *et al.* 2006, Delfino *et al.* 2007) and concentration (Handelsman *et al.* 1985, Amaral *et al.* 2006) but also the presence of a higher number of morphologically abnormal forms with defects in the acrosome, nucleus, mitochondria and plasma membrane (Barták *et al.* 1975, Vignon *et al.* 1991, Delfino *et al.* 2007) as well as generally increased semen plasma abnormalities (Agbaje *et al.* 2007, Tremellen 2008, Alves & Oliveira 2013). Interestingly, diabetic men who presented normal semen parameters showed significantly higher levels of damage in sperm nuclear and mitochondrial DNA, probably resulting from supra-physiological glucose levels and related oxidative stress (Agbaje *et al.* 2007). Additionally, advanced glycation end products that result from oxidative damage have been implicated in an increasing number of diabetic complications and have been detected at higher levels

in the reproductive tract, seminal plasma and sperm of type 1 and type 2 diabetic males (Wautier & Schmidt 2004, Mallidis *et al.* 2007, 2009), which suggests their involvement in reactive oxygen species (ROS)-initiated sperm DNA damage.

Although the existing literature suggests that diabetic conditions might compromise sperm quality and function, the mechanisms by which diabetes influences male fertility remain undefined. Considering that mitochondrial dysfunction has been associated with diabetes (Tremellen 2008, Sivitz & Yorek 2010) and taking into account the hub role of these organelles in reproduction (Ramalho-Santos *et al.* 2009), which has been shown by the association of several mitochondrial parameters with sperm quality and function (Ruiz-Pesini *et al.* 1998, Marchetti *et al.* 2002, 2012, Gallon *et al.* 2006, Nakada *et al.* 2006, Publicover *et al.* 2008, Sousa *et al.* 2011, Amaral *et al.* 2013a), it is possible that mitochondrial functionality might play a role in diabetes-induced alterations at the sperm level.

Therefore, using a previously established *in vitro* approach (Tavares *et al.* 2013, 2015, Sousa *et al.* 2014), we intended to address whether some aspects of sperm function are affected by exposure to high glucose levels, which is the foremost recognized signature of this pathology and one of the major factors responsible for diabetes-associated complications.

Materials and methods

Chemicals

Unless stated otherwise, all chemicals and reagents were provided by Sigma–Aldrich.

Human biological material

Human semen samples were obtained at the Human Reproduction Service of the University Hospitals of Coimbra (Coimbra, Portugal) from 48 healthy, non-diabetic men who were undergoing routine semen analysis for fertility treatment in which male factor was excluded.

All the donors signed informed consents and the biological material was used in accordance with the proper ethical and Internal Review Board (IRB) guidelines provided by the University Hospitals of Coimbra. The present study was approved by the IRB.

Semen samples were obtained by masturbation after 3–5 days of sexual abstinence. Seminal analysis was performed according to the World Health Organization (WHO) guidelines (WHO 2010) and only normozoospermic samples with no detectable leukocytes or other round cells were used.

Experimental design

After liquefaction, sperm cells were isolated by density-gradient centrifugation (SupraSperm; Medicult-Origio, Jyllinge,

Denmark) and allowed to capacitate in sperm preparation medium (SPM; Medicult-Origio) for at least 3 h at 37 °C and 5% CO₂ before initial experiments were performed, except for the capacitation status assay in which spermatozoa were allowed to capacitate under the different glucose conditions for the entire incubation period. Sperm samples, with an adjusted concentration of 10×10^6 spermatozoa/ml, were then incubated at 37 °C and 5% CO₂ in PBS supplemented with 0.9 mM CaCl₂, 0.5 mM MgCl₂, 0.3% (w/v) BSA, 1 mM sodium pyruvate, 10 mM sodium lactate, and 1% (v/v) penicillin/streptomycin, pH=7.4 (Tavares *et al.* 2013, 2015, Sousa *et al.* 2014), as well as D-glucose at either: i) 5 mM, the optimal concentration for sperm preparation *in vitro* (Mahadevan *et al.* 1997, Amaral *et al.* 2011); ii) 25 mM to mimic the diabetic condition; or iii) 50 mM to evaluate cell response to massive doses of glucose. To control for the possible effects of the increased solution osmolarity being driven by the elevated concentrations of D-glucose, equal concentrations of L-glucose, a non-metabolizable and non-physiologic form of glucose, was used as a control. Media were daily renewed and incubations were maintained over a maximal period of 2 days.

The influence of glucose on sperm was assessed daily by measuring a variety of important indicators of spermatozoa functionality.

Motility and viability evaluation

Sperm motility and viability, which were assessed using 0.5% (w/v) Eosin Y, were determined according to WHO (2010) recommendations and monitored by phase-contrast optical microscopy (Nikon Eclipse E200, Nikon Instruments, Inc., Melville, NY, USA) at 200× and 400× magnification respectively. For each condition a total of 200 spermatozoa were evaluated in different fields.

Sperm motility is expressed as the percentage of total motility (i.e., progressive motility + *in situ* motility) and viability was expressed as the percentage of live spermatozoa.

Capacitation status: assessment of tyrosine phosphorylation

To further address whether D-glucose has an effect on the sperm capacitation process, spermatozoa were allowed to capacitate under glucose exposure in the PBS-based medium described in the experimental design section, supplemented with 25 mM NaHCO₃. Capacitation status was assessed through the detection of phosphorylated tyrosines using a rabbit anti-phosphotyrosine polyclonal antibody (1:10; Zymed, San Francisco, CA, USA) as described previously (Ramalho-Santos *et al.* 2007). Samples were mounted on glass slides with Vectashield Mounting Media containing 4',6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA, USA) to counterstain spermatozoa nuclei. At least 200 spermatozoa were observed in different fields by means of a Leica fluorescence microscope (Leica DM4000B, Wetzlar, Germany) at 1000× magnification. Whereas capacitated spermatozoa exhibited green fluorescence in both the flagellum and midpiece, non-capacitated spermatozoa only displayed the

blue nuclear DAPI counterstain. Results are presented as the percentage of capacitated sperm.

Acrosomal integrity evaluation

Acrosomal integrity was evaluated using the acrosomal content marker *Pisum sativum* agglutinin conjugated with FITC (PSA-FITC) as described previously (Mota *et al.* 2012). Briefly, blocked sperm samples were incubated with PSA-FITC (1:200) for 1 h at 37 °C and mounted with Vectashield Mounting Media containing DAPI after being washed. At least 200 spermatozoa were examined in different fields by means of a Leica fluorescence microscope (Leica DM4000B) at 1000× magnification. Whereas intact acrosomes exhibited bright green homogeneous fluorescent staining, acrosome-reacted spermatozoa showed either no fluorescence signal in the acrosomal region, heterogeneous spots of fluorescence, or only a fluorescing band at the equatorial segment of the sperm head (Ramalho-Santos *et al.* 2007). Results are presented as the percentage of spermatozoa with intact acrosome.

Flow cytometric analysis: mitochondrial membrane potential and superoxide production

Flow cytometric analysis was performed on human spermatozoa following incubation with specific fluorescent probes, as described in the following sections. Sample analysis was performed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with an argon laser that operated with an excitation wavelength of 488 nm and coupled with the following emission filters: 530/30 band pass (FL-1/green), 585/42 band pass (FL-2/red), and >620 nm long pass (FL-3/far red).

Based on the distinct light scatter characteristics of the spermatozoa (forward and side scatter), non-sperm specific events were gated out of the analysis (Amaral *et al.* 2013a). A total of 20 000 sperm-specific events per condition were recorded, and data acquisition and analysis were performed using the BD Cell Quest Pro Acquisition program (BD Biosciences).

Evaluation of sperm mitochondrial superoxide production

Sperm mitochondrial superoxide generation was determined using the fluorescence probe MitoSOX-Red (Molecular Probes, Eugene, OR, USA) as reported previously (Amaral *et al.* 2013a). Briefly, sperm suspensions (2.5×10^6 spermatozoa/ml) were incubated with 3 μ M of MitoSOX-Red for 15 min at 37 °C in the dark. For each sample an appropriate control was performed by simultaneous incubation with 80 μ M of antimycin A – a mitochondrial complex III inhibitor that is known to produce a burst of mitochondrial superoxide (Amaral *et al.* 2013a). Results are expressed as the percentage of MitoSOX-positive cells.

Evaluation of sperm mitochondrial membrane potential

Sperm mitochondrial membrane potential (MMP) was assessed using the fluorescent probe JC-1 (Molecular Probes) which differentially labels mitochondria according to their membrane potential by reversibly shifting the fluorescent emission from

green (low MMP) to red (high MMP) (Amaral & Ramalho-Santos 2010, Amaral *et al.* 2013a, Sousa *et al.* 2014).

Sperm suspensions (2.5×10^6 spermatozoa/ml) were stained with 2 μ M of JC-1 for 20 min at 37 °C in the dark. Additionally, for each sample an appropriate control was prepared by simultaneous incubation with 50 μ M of *p*-trifluoromethoxy carbonyl cyanide phenyl hydrazine (FCCP), an uncoupler that collapses MMP (Amaral *et al.* 2013a, Sousa *et al.* 2014).

Results are presented as the percentage of sperm-specific events with high MMP following exposure to different concentrations of glucose.

Statistical analysis

Statistical analysis was performed using the SPSS Software for Windows version 20.0 (SPSS, Inc.). All the variables were evaluated for normal distribution by Shapiro–Wilk test and the homogeneity of variances was assessed by Levene's test.

Statistical comparisons between the control and the different glucose concentrations were performed using a *t*-test or a related Mann–Whitney *U* test, and Wilcoxon tests were used for non-normal variables. Values of $P \leq 0.05$ were considered significant. Results are presented as means $\pm$ S.E.M. and the number of experiments is indicated.

Results

Motility and viability

No significant differences, either in motility or in viability, were detected between samples incubated with glucose at 5 mM and higher concentrations (25 or 50 mM) on any day of incubation ($P > 0.05$, Fig. 1A and B). Nevertheless, statistically significant differences were found when comparing D-glucose and L-glucose. In fact at day 1, L-glucose-incubated spermatozoa showed a significantly lower motility ($P < 0.05$, Fig. 1A), although the viability was not affected ($P > 0.05$, Fig. 1B). The percentage of viable cells decreased only after 2 days of incubation with the non-metabolizable substrate ($P < 0.05$, Fig. 1B).

Capacitation and acrosomal status

Capacitation and the subsequent acrosome reaction are essential processes, as only capacitated sperm with an intact acrosome have the ability to fertilize (Ecroyd *et al.* 2003, Ramalho-Santos *et al.* 2007). Therefore these processes are considered important indicators of sperm functionality.

As detected by protein tyrosine phosphorylation (Fig. 2A), capacitation was not influenced by the high D-glucose conditions ($P > 0.05$, Fig. 2B). Nonetheless, glucose availability also seemed to be important for sperm capacitation. This started to be particularly evident on day 1, when the percentage of capacitated spermatozoa incubated in 50 mM L-glucose-containing media was significantly lower than that obtained when

Figure 1 Spermatozoa motility and viability after exposure to different glucose levels. (A) Percentage of total motility. (B) Percentage of live spermatozoa. Samples were incubated for 2 days in PBS-based medium with D-glucose (D-glu), whereas osmotic controls were incubated with L-glucose (L-glu), as described in the Materials and methods section. Results are presented as means $\pm$ S.E.M. and the number of experiments is specified. * $P < 0.05$, ** $P < 0.01$.

exposed to 50 mM D-glucose media (12 ± 3.77 and $43 \pm 10.62\%$ respectively, $P < 0.05$, Fig. 2B) and became more pronounced on day 2, when the differences between D- and L-glucose were significant for all of the concentrations ($P < 0.001$, Fig. 2B).

The percentage of intact acrosomes (Fig. 3A) was not statistically different between the spermatozoa incubated with 5 mM D-glucose and those incubated with high glucose levels on day 1 ($P > 0.05$, Fig. 3B). However, after 2 days of incubation, the higher level of D-glucose (50 mM) had significantly affected acrosomal integrity ($P < 0.05$, Fig. 3B). Furthermore, the presence of a metabolizable substrate was essential for the maintenance of acrosomal integrity following 2 days of incubation, as was evidenced by the results obtained with L-glucose ($P < 0.01$, Fig. 3B).

Spermatozoa mitochondrial function

Mitochondria play a central role in sperm function (Marchetti *et al.* 2002, Gallon *et al.* 2006, Sousa *et al.* 2011). Moreover, mitochondrial impairment is also an important event in DM (Sivitz & Yorek 2010). We

therefore monitored two aspects of mitochondrial function: superoxide production and MMP.

No differences were observed in the mitochondrial superoxide generation of samples incubated in the different conditions ($P > 0.05$, Fig. 4A). However, a significant increase in superoxide production was observed on both days 1 and 2 in L-glucose-incubated spermatozoa ($P < 0.05$, Fig. 4A).

Similarly, no significant glucose-induced effects on MMP were found during any time of incubation ($P > 0.05$), yet substrate availability was shown to be important for mitochondrial activity, as was proven by the decrease in MMP in spermatozoa that were incubated in L-glucose media for 2 days ($P < 0.05$, Fig. 4B).

Discussion

Effects of glucose on spermatozoa functionality

Although it is somewhat contradictory, the literature suggests that DM might alter sperm function and semen quality, even though the mechanisms involved are poorly understood. In fact, current knowledge has mostly relied on the assessment of conventional sperm parameters that have been shown to be of limited predictive value in the determination of fertility status

Figure 2 Influence of the different glucose concentrations on protein tyrosine phosphorylation. (A) Immunocytochemical identification of a capacitated spermatozoon on the left (green fluorescence) and a non-capacitated cell on the right (magnification 1000 $\times$). (B) Percentage of capacitated spermatozoa. Samples were incubated for 2 days in PBS-based medium with D-glucose (D-glu), whereas osmotic controls were incubated with L-glucose (L-glu), as described in the Materials and methods section. Results are presented as means $\pm$ S.E.M. and the number of experiments is specified. * $P < 0.05$, *** $P < 0.001$. \$Represents statistical trends.

Figure 3 Influence of different glucose concentrations on acrosomal integrity. (A) Spermatozoa with intact acrosomes exhibit homogeneous green fluorescence on the anterior region of the head, whereas acrosome-reacted sperm show only blue nuclear counterstain (magnification 1000 $\times$). (B) Percentage of spermatozoa with intact acrosomes. Samples were incubated for 2 days in PBS-based medium with D-glucose (D-glu), whereas osmotic controls were incubated with L-glucose (L-glu), as described in the Materials and methods section. Results are presented as means $\pm$ S.E.M. and the number of experiments is specified. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

(Jequier 2005), which stresses the importance of evaluating more accurate functional sperm parameters that are often ignored in both *in vivo* and *in vitro* studies.

Given the extension and multifactorial nature of diabetes-induced physiological changes, there are many mechanisms that might be responsible for the associated reproductive dysfunction. Therefore, the present study strictly addressed the isolated and direct effect of high glucose levels in human spermatozoa during a 2-day incubation period by means of an *in vitro* experimental system in which male gametes were kept for extended periods of time in a PBS-based medium supplemented with a variety of frequently renewed substances that closely mimic *in vivo* conditions (Tavares *et al.* 2013, 2015, Sousa *et al.* 2014).

Although basic sperm parameters, such as motility and viability, were unaffected by high glucose levels, subtle aspects of sperm function/status were further analyzed.

To become functionally competent, spermatozoa must undergo capacitation within the female tract (Ecroyd *et al.* 2003) and even though glucose has been reported to be beneficial for human sperm capacitation (Mahadevan *et al.* 1997, Williams & Ford 2001), the present results suggest that high glucose levels do not interfere with this process. To our knowledge the present

report is the first to address the effects of high glucose levels on capacitation.

On the other hand, 50 mM D-glucose significantly reduced acrosomal integrity on day 2, and this effect was not justified by alterations in sperm viability, that together with the lack of noticeable changes in capacitation, suggests that the acrosome is more prone to alterations that can possibly interfere with the fertilization process. However, this glucose concentration is not physiologically relevant; it served only as a putative positive control in the present study.

Considering the impact of mitochondrial impairment on both diabetes (Ceriello 2003) and reproductive function (Ramalho-Santos *et al.* 2009, Amaral *et al.* 2013b, Ramalho-Santos & Amaral 2013), we further investigated the possible effect of glucose on sperm mitochondria, particularly on membrane potential, which is essential to the oxidative phosphorylation process, and on ROS production (Ramalho-Santos *et al.* 2007). To our knowledge, the present study is the first to address such effect on human sperm mitochondria

Figure 4 Influence of high glucose levels on sperm mitochondrial superoxide production (A) and mitochondrial membrane potential (MMP) (B). Samples were incubated for 2 days in PBS-based medium with D-glucose (D-glu), whereas osmotic controls were incubated with L-glucose (L-glu), as described in the Materials and methods section. Results indicate the percentage of MitoSOX-Red-positive cells and the percentage of cells with high MMP, respectively. Data is presented as means $\pm$ S.E.M. and the number of experiments is specified. * $P < 0.05$, ** $P < 0.01$. \$Represents statistical trends.

functionality. However, the absence of changes at this level might suggest that the alterations previously reported in literature on diabetic sperm function are mitochondria-independent, which is in accordance with reports on rat testicular mitochondria (Amaral *et al.* 2009).

In summary, the effect of DM on human sperm that has been reported in other *in vivo* studies may arise from alterations at the spermatogenesis level and/or from factors other than glucose or, alternatively, from a combined effect of glucose and other factors. In fact, it is known that there are many biochemical alterations (e.g., altered insulin levels, increased inflammatory factors, and oxidative status) in diabetic patients, and if those alterations are reflected at the testicular level, along the reproductive tract, or in seminal plasma, their influence on sperm function could be cause for concern.

Effects of substrate availability on spermatozoa functionality

During all of the experiments osmotic controls were performed using a non-metabolizable glucose (L-glucose). Interestingly, this condition also allowed us to mimic what happens in a diabetic environment, where glucose is present at high levels but is not efficiently used by the cells (Muio & Newgard 2008).

L-glucose-incubated spermatozoa presented a significantly lower motility and viability than spermatozoa incubated with a metabolizable glucose (D-glucose). Motility was affected on day 1 but did not interfere with cell viability, so we hypothesized that this effect is a result of a lack of substrate and not of cell death-related processes, although the same cannot be assumed for day 2. The present results indicate that spermatozoa require glucose to support optimal motility, even when media is supplied with lactate and pyruvate that support mitochondrial respiration. In fact, mammalian sperm require metabolic energy for a variety of functions (Williams & Ford 2001, Ferramosca & Zara 2014), and it is known that human spermatozoa obtain a significant proportion of their energy from glycolysis (Williams & Ford 2001). Nevertheless, the origin of ATP for sperm motility is still being intensely discussed (Ford & Harrison 1981, Williams & Ford 2001, Ruiz-Pesini *et al.* 2007, Bucci *et al.* 2011, Piomboni *et al.* 2012).

Additionally, the present results showed that glucose availability is also important for sperm capacitation and acrosomal integrity; this was particularly noticeable on the second day of incubation. In accordance, a number of studies have already demonstrated that the capacitation of human spermatozoa substantially relies on glucose, which suggests a key role for glucose in sustaining hyperactivation (Williams & Ford 2001), in tyrosine phosphorylation (Travis *et al.* 2001), and in ensuring IVF (Rogers & Perreault 1990, Mahadevan *et al.* 1997). However, it is still unclear whether the beneficial

effects of glucose derive from the provision of extra metabolic energy through glycolysis or from the generation of other metabolic products (Aitken *et al.* 1997, De Lamirande *et al.* 1997, Williams & Ford 2001). The higher acrosomal loss observed in L-glucose-incubated spermatozoa may be explained by an early triggered acrosome reaction, possibly because of a continuous lack of metabolizable glucose.

Moreover, in L-glucose-containing media, spermatozoa produced higher amounts of mitochondrial superoxide that may contribute to the decline of several aspects of sperm function (Koppers *et al.* 2008, Tremellen 2008, Amaral *et al.* 2013a). Interestingly, we observed that the increased production of ROS occurred before any alteration in MMP became apparent, an effect that has been previously described in human spermatozoa (Koppers *et al.* 2008, Aitken *et al.* 2012, Amaral *et al.* 2013a, Sousa *et al.* 2014). This points to a deleterious effect of mitochondrial superoxide on mitochondrial functionality which creates a panorama of general mitochondrial dysfunction (Amaral *et al.* 2013a).

The lower functionality of L-glucose-incubated spermatozoa might be explained by mitochondrial impairment which might also be related to the observed significant decrease in capacitation. In accordance, several studies have demonstrated that concomitant with capacitation, there is an abrupt increase in mitochondrial activity both in mice (Boell 1985, Fraser & Lane 1987) and human sperm (Hicks *et al.* 1972, Stendardi *et al.* 2011). Therefore, if mitochondrial functionality is altered, it can be expected that capacitation might be affected to some extent, as was observed in the present study. However, we cannot exclude that other factors might also be involved.

Overall, glucose availability is essential for the long-term *in vitro* maintenance of spermatozoa function. Additionally, the present results lead us to suggest that the adverse effects of DM at the sperm level could be explained by a cellular glucose hypometabolism, resultant from the dysfunctional action of insulin in diabetic conditions. Under these conditions, cells have an impaired accessibility to glucose which may lead to cell injury and further reproductive impairment, as has already been reported in the diabetic brain (Liu *et al.* 2009, Roberts *et al.* 2014).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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