

The Role of Glucose in Supporting Motility and Capacitation in Human Spermatozoa

ANDREW C. WILLIAMS AND W. CHRISTOPHER L. FORD

From the University Division of Obstetrics & Gynaecology, St Michael's Hospital, Bristol, United Kingdom.

ABSTRACT: Glucose has been reported to be beneficial to human sperm for optimal capacitation and fertilization, although it is unclear whether glucose is required for providing extra metabolic energy through glycolysis, or for generating some other metabolic product. In this study, the effects of sugars on human sperm capacitation, motility, and energy production were investigated. The glucose concentration that supported the greatest number of acrosome reactions was 5.56 mmol L⁻¹. Compared with incubations with no added sugar, this concentration of glucose, fructose, mannose, or galactose appeared to slightly increase the number of acrosome reactions occurring after 18 hours of capacitation, or following induction by 2 μmol A23187 + 3.6 mmol pentoxifylline L⁻¹, but only glucose had a statistically significant effect. Glucose supported increased penetration of zona-free hamster oocytes, but its advantage was not statistically significant. The addition of 5.56 mmol glucose or fructose L⁻¹ to sugar-free medium immediately increased the adenosine tri-

phosphate (ATP) concentration and motility of sperm. These parameters were then stable for 3 hours, but declined markedly after 18 hours. In the absence of a glycolysable sugar, motility began to decline in the first hour and only 2% or 3% of sperm remained motile after 18 hours. Glucose or fructose was required to support hyperactivated motility. 2-Deoxyglucose was detrimental to the ATP concentration and motility of sperm, and supported fewer spontaneous or progesterone-stimulated acrosome reactions than were observed in the absence of a sugar. We conclude that glycolytic ATP production is required for vigorous motility and hyperactivation in human sperm. Other products of glucose metabolism are not essential to support capacitation, but they may have a small, enhancing effect.

Key words: Acrosome reaction, ATP, hamster oocyte penetration test, glycolysis, hyperactivation.

J Androl 2001;22:680-695

Mammalian sperm require metabolic energy for a variety of functions, most notably to support motility. The principal sources of adenosine triphosphate (ATP) production are mitochondrial oxidative phosphorylation and glycolysis, but the balance between these 2 pathways varies greatly between sperm of different species. Human sperm obtain an unusually high proportion of their ATP from glycolysis (Ford and Rees, 1990) and respond to changes in energy demand by adjusting glycolytic flux rather than by mitochondrial respiration (Rees et al, 1990). Nevertheless, human sperm can remain motile in media lacking a glycolysable sugar (Suter et al, 1979; Ford and Harrison, 1981), although it is unclear whether such motility can be sustained for prolonged periods of time, or is sufficiently vigorous to achieve optimal fertilization.

The effect of glucose on capacitation also varies between species. Glucose is required for successful fertilization by epididymal mouse sperm (Hoppe, 1976; Fraser and Quinn, 1981; Urner and Sakkas, 1996a,b, 1999) but needs to be

present for only a short period of time at the end of capacitation, and the requirement is restricted to the later stages of fertilization. The specific steps for which it is necessary may include hyperactivated motility (Fraser and Quinn, 1981; Urner and Sakkas, 1996a) and sperm-oocyte fusion (Urner and Sakkas, 1996b), which is dependent upon the provision of NADPH by glucose metabolism via the pentose phosphate pathway (Urner and Sakkas, 1999). Glucose is also required for fertilization in the rat (Niwa and Iritani, 1978) and hamster (Dravland and Meizel, 1981), and is beneficial but not essential for the optimal *in vitro* capacitation of Macaque sperm (VandeVoort and Overstreet, 1995). By contrast, fertilization by guinea pig (Hyne and Edwards, 1985) and bovine sperm (Parrish et al, 1989) is inhibited by the presence of glucose.

A number of studies have suggested that human sperm may require glucose for optimal capacitation and fertilization. Following an initial preincubation period of at least 6 hours, glucose significantly enhanced the ability of human sperm to penetrate zona-free hamster oocytes, in comparison with the glycolysable sugars, fructose or mannose, or with the nonglycolysable sugar, galactose (Rogers and Perreault, 1990). By contrast, Hoshi et al (1982) reported that human sperm could penetrate zona-free hamster eggs in the absence of glucose but later observed that glucose was needed for optimal penetration of the zona pellucida, and suggested that glucose was required to support hyperactivated motility

This work was supported by a project grant from the Wellcome Trust and by the University of Bristol Centre for Reproductive Medicine.

Correspondence to: Christopher L. Ford, University Division of Obstetrics & Gynaecology, St Michael's Hospital, Southwell Street, Bristol BS2 8EG, United Kingdom (e-mail: chris.ford@bristol.ac.uk).

Received for publication November 28, 2000; accepted for publication February 16, 2001.

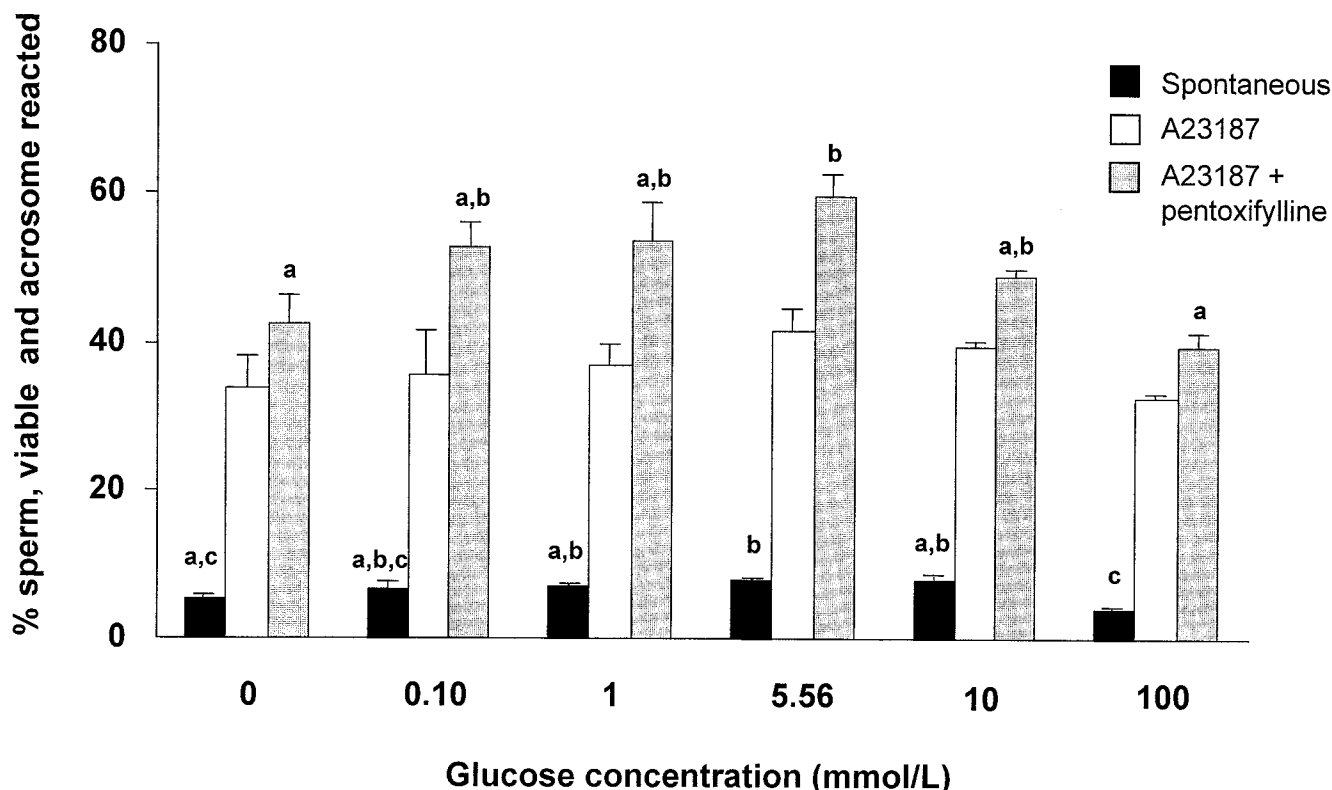


Figure 1. Effect of glucose concentration on the viable acrosome reaction rate in human sperm. Sperm were incubated overnight (18 hours) at 37°C under 95% air/5% CO₂ in BWW medium modified to contain glucose at various concentrations, and stimulated to acrosome react by further incubation for 1 hour with 2 μ mol A23187 L⁻¹ or 2 μ mol A23187 + 3.6 mmol pentoxifylline L⁻¹ or a buffer blank (spontaneous). Columns show means + SEM, n = 4. Significant differences ($P \leq .05$, one-way ANOVA with Scheffé post-hoc test) between glucose concentrations within each acrosome reaction stimulation regime are indicated by absence of a shared superscript.

(Hoshi et al, 1991). Finally, human in vitro fertilization rates were lower for sperm incubated in glucose-free media than for those incubated in media containing either 0.50 or 5.56 mmol glucose L⁻¹ (Mahadevan et al, 1997). A recent meta-analysis has recommended that glucose should be included in in vitro fertilization (IVF) media used for sperm culture and fertilization but omitted for the culture of the early embryo (Coates et al, 1999). However, it remains unclear whether the beneficial effects of glucose derive from the provision of extra metabolic energy from glycolysis or whether capacitation requires some other product of glucose metabolism. It has recently been proposed that glucose may be needed to produce NADPH, to allow a putative NADPH oxidase to generate superoxide, to promote protein tyrosine phosphorylation as part of the regulatory systems that control capacitation (Aitken et al, 1997, 1998; de Lamirande et al, 1997). Paradoxically, NADPH may also be required to protect sperm against oxidative stress (Ford et al, 1997; Storey, 1997).

We examined the effect of glucose on the acrosome reaction, hyperactivated motility, and the penetration of zona-free hamster oocytes by human sperm and its effect on their ATP concentration and motility in culture. Our aim was to

investigate the role of glucose in the optimal capacitation of human sperm and to determine to what extent its effects can be explained by the provision of ATP.

Materials and Methods

Sperm Preparation Procedure

Ejaculates were collected from donors whose semen exceeded World Health Organization (1992) criteria for normal semen variables, and who had previously participated in the University of Bristol's donor insemination program. Sperm were purified by centrifugation through 40%/80% discontinuous Percoll gradients (Ford et al, 1992). The resultant pellet was washed twice (350 g, 10 minutes) in modified Biggers, Whitten, and Whittingham (BWW) buffer (Aitken, 1983) containing 3 mg/mL bovine serum albumin (Fraction V, Sigma Chemical Co, Poole, United Kingdom) but lacking glucose. Sperm were then resuspended as appropriate for each experiment.

Effects of Different Sugars on the Acrosome Reaction

Sperm prepared in sugar-free BWW were resuspended at a concentration of 10×10^6 /mL (motile concentration of approximately 8×10^6 /mL) in BWW containing 0–100 mmol glucose L⁻¹; 5.56

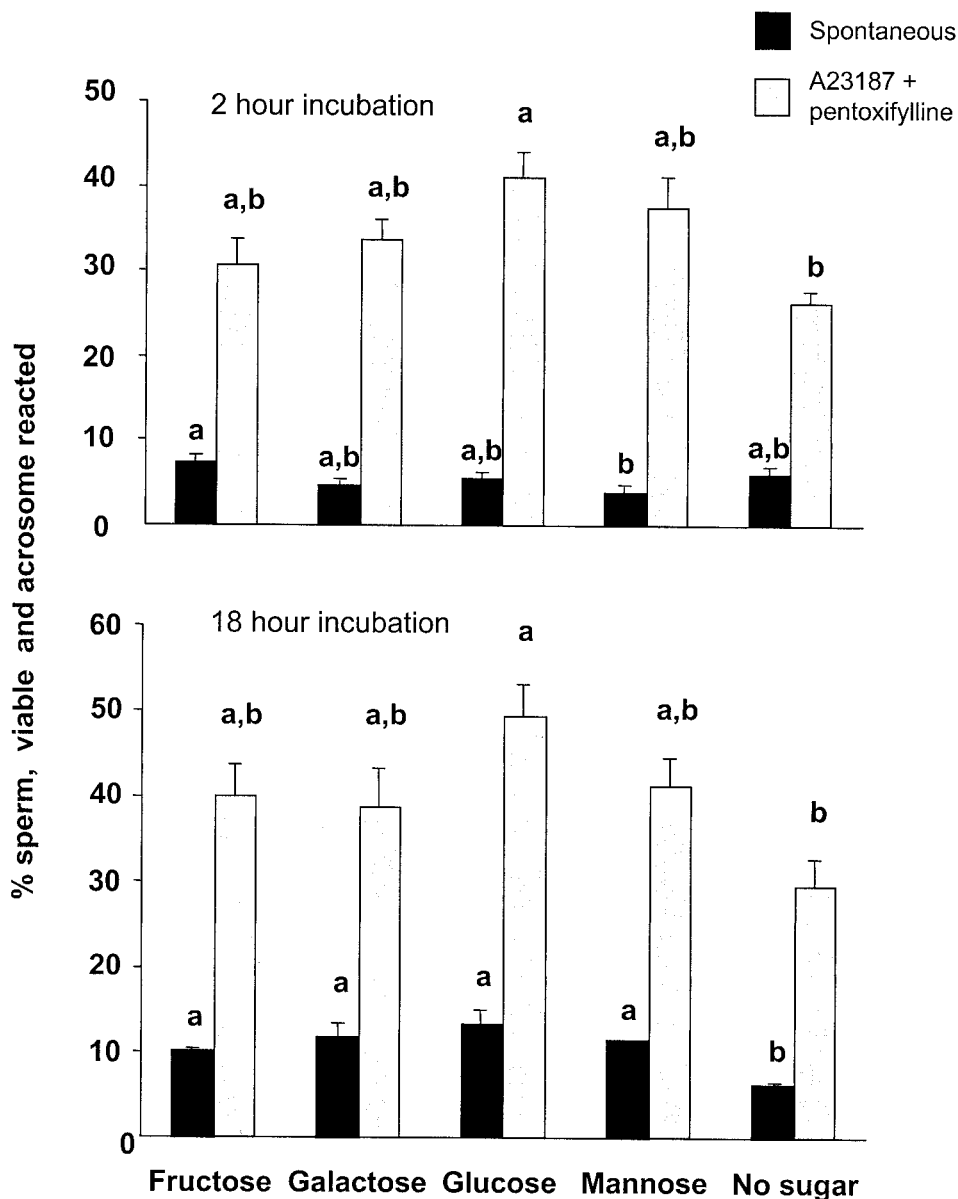


Figure 2. Effect of various sugars on the spontaneous and A23187 + pentoxifylline-stimulated acrosome reaction rate in human sperm, following incubation for 2 or 18 hours at 37°C under 95% air/5% CO₂. Sperm were incubated in BWB containing 5.56 mmol glucose, fructose, mannose, or galactose L⁻¹, or no sugar. At the end of the incubation period, acrosome reactions were induced by a further 1-hour incubation with 2 μmol A23187 + 3.6 mmol pentoxifylline L⁻¹ or with a buffer blank. Columns show means + SEM, n = 3. Significant differences ($P \leq .05$, one-way ANOVA with Scheffé post-hoc test) between sugars are represented by the absence of a shared superscript.

mmol fructose, 5.56 mmol galactose, 5.56 mmol glucose, 5.56 mmol mannose L⁻¹, or no sugar; or 5.56 mmol glucose, 5.56 mmol 2-deoxyglucose L⁻¹, 5.56 mmol glucose + 5.56 mmol 2-deoxyglucose L⁻¹, or no added sugar, as appropriate for each experiment. The samples were incubated for either 2 or 18 hours at 37°C under 95% air/5% CO₂. Following the initial incubation sperm were stimulated to acrosome react by a further 1 hour of incubation under 95% air/5% CO₂ at 37°C by the addition of 2 μmol A23187 + 3.6 mmol pentoxifylline L⁻¹, 3.18 μmol progesterone L⁻¹, or a buffer blank, as appropriate. A23187 and progesterone were prepared immediately prior to use from stock solutions in dimethyl sulfoxide (DMSO) that were stored at -20°C.

The proportion of viable acrosome-reacted sperm was measured by the combined use of the hypo-osmotic swelling test (HOST; Jeyendran et al, 1984) and the fluorescein isothiocyanate (FITC)-conjugated lectin agglutinin, *Pisum sativum* (PSA; Cross et al, 1986), as recently described by Whittington and Ford (1998). This technique differentiates, successfully, between physiological and degenerative acrosome reactions.

Effect of Different Sugars on the Penetration of Zona-Free Hamster Oocytes

Sperm were suspended at a concentration of 10 × 10⁶/mL (approximately 8 × 10⁶/mL motile) in BWB medium that contained

5.56 mmol fructose, 5.56 mmol galactose, 5.56 mmol glucose, or 5.56 mmol mannose L⁻¹; or in BWW that contained no added sugar. All incubations were carried out at 37°C under 95% air/5% CO₂. Three different capacitation protocols were used: 1) incubation in the appropriate buffer for 1 hour, 2) incubation in the appropriate buffer containing 2 μmol A23187 + 3.6 mmol pentoxifylline L⁻¹ for 1 hour, or 3) incubation of sperm in the appropriate buffer for 18 hours, followed by resuspension in fresh media. For capacitation protocol 3, 2 tubes containing sperm in sugar-free medium were prepared. After 18 hours, sperm in one of these tubes were resuspended in medium that contained 5.56 mmol glucose L⁻¹. Sperm in all the other tubes were resuspended in media containing the same sugar as before. Incubation was continued for a further 3 hours. At the end of the incubation, sperm were washed twice and resuspended in sugar-free BWW at a concentration of 10 × 10⁶/mL prior to the transfer of 100 μL of sperm to a plastic LP4 tube (Luckham, Burgess Hill, West Sussex, United Kingdom). The hamster oocyte penetration test (HOPT) was performed as described by Ford et al (1991) with 8–12 eggs being added to each tube.

Effects of Different Sugars on Motility and ATP Concentration in Sperm

Sperm were prepared in sugar-free BWW and resuspended at a concentration of between 5 and 10 × 10⁶/mL (approximately 4–8 × 10⁶/mL motile). All incubations were carried out at 37°C under 95% air/5% CO₂. Two experimental protocols were used. In the first, sperm were incubated for 18 hours with 5.56 mmol fructose, 5.56 mmol galactose, 5.56 mmol glucose, or 5.56 mmol mannose L⁻¹; or in medium containing no added sugar. Duplicate tubes were prepared containing no added sugar. After 18 hours, sperm in one of these were resuspended in fresh medium containing 5.56 mmol glucose L⁻¹, and at the same time, sperm in all other tubes were resuspended in fresh medium containing the same sugar as before. The sperm were then incubated for a further 30 minutes. Samples for motility assessment and the measurement of ATP and adenosine diphosphate (ADP) were taken after the initial resuspension in sugar-free BWW approximately 1 minute after the addition of sugar or buffer blank, after 1 and 18 hours incubation, and finally, 30 minutes after resuspension in fresh medium. In the second protocol, sperm were incubated for 18 hours with 5.56 mmol glucose, 5.56 mmol 2-deoxyglucose L⁻¹, or with no added sugar. Two tubes with no added sugar were set up as in protocol 1, and sperm in all tubes were resuspended after 18 hours in the same way as before. Sperm samples for motility assessment and the assay of ATP and ADP concentrations were removed at the following stages: after their initial resuspension in sugar-free BWW; 1 minute after the addition of sugar or buffer blank; after 1, 3, and 18 hours of incubation; and finally, 2 and 30 minutes after sperm resuspension in fresh medium. Sperm viability was monitored at each time point using the HOST technique.

Motility was assessed at 37°C by computer-aided sperm analysis (CASA) using a Hamilton Thorne sperm motility analyzer (version 7) as described previously (McLaughlin and Ford, 1992), except that measurements were made in 20-μm-deep “microcells” and the nonmotile head size was set at 8. We endeavored to analyze at least 200 motile sperm per sample, but occasionally, this was impossible in samples taken after over-

night incubation particularly in the sugar-free medium or in the presence of 2-deoxyglucose, and for this reason, values at these times are interpreted with caution.

Hyperactivated sperm were identified using the “sort fraction” facility of the Hamilton Thorne motility analyzer. The criteria upon which this was based were those of Burkman (1991); that is, linearity (LIN) ≤65%, curvilinear velocity (VCL) ≥100 μm/s, and amplitude of lateral head displacement (ALH) ≥7.5 μm.

To measure ATP and ADP, 200-μL samples of sperm suspension were added to 100 μL of ice-cold 1 mol perchloric acid L⁻¹ and mixed thoroughly. Denatured protein was removed by centrifugation and the supernatant was neutralized to pH 7.0–7.5 using 2.3 mol K₂CO₃ + 0.7 mol 2-[N-Morpholino]ethanesulphonic acid (MES) L⁻¹. Constant mixing avoided local alkalinization. Potassium perchlorate was removed by centrifugation (10 000 × g, 30 seconds) and the supernatant was stored at –20°C until analyzed. Both ATP and ADP were analyzed luminometrically as described previously (Rees et al, 1990).

Statistical Analysis of Results

All analyses were conducted using personal computer-based Statistical Packages for the Social Sciences software, release 8. Data were tested for normality with the Kolmogorov-Smirnov test. Normal data were analyzed using one-way and two-way repeated measures analysis of variance (ANOVA) models, as appropriate. When significance was established (in the absence of a significant time × sugar interaction), one-way ANOVA models were used to examine the effect of individual sugars on motility and adenine nucleotides. Post hoc testing was carried out using the Scheffé test, with *P* ≤ .05, to reveal specific differences between sugars. Correlations between sperm motility and ATP concentration or the ATP:ADP ratio were made using Pearson’s product moment correlation. The HOPT data could not be transformed to conform to a normal distribution and were therefore analyzed using the nonparametric Kruskal-Wallis ANOVA on ranks and Friedman’s test. Despite these data being nonnormally distributed, they are presented as means (± range) because the number of observations was considered too small for medians and interquartile ranges to give a realistic impression of the data.

Results

Acrosome Reaction

First we determined the glucose concentration that supported the highest acrosome reaction rate following an overnight incubation. Both spontaneous and ionophore-stimulated rates were examined. The spontaneous acrosome reaction rate (mean ± SEM, *n* = 4) was low but increased significantly, albeit slightly, from 5% ± 0.4% in the absence of glucose to 8% ± 0.6% in the presence of 5.56 mmol glucose L⁻¹ (Figure 1). The rate then decreased at higher glucose concentrations. The acrosome reaction rate induced by 2 μmol A23187 L⁻¹

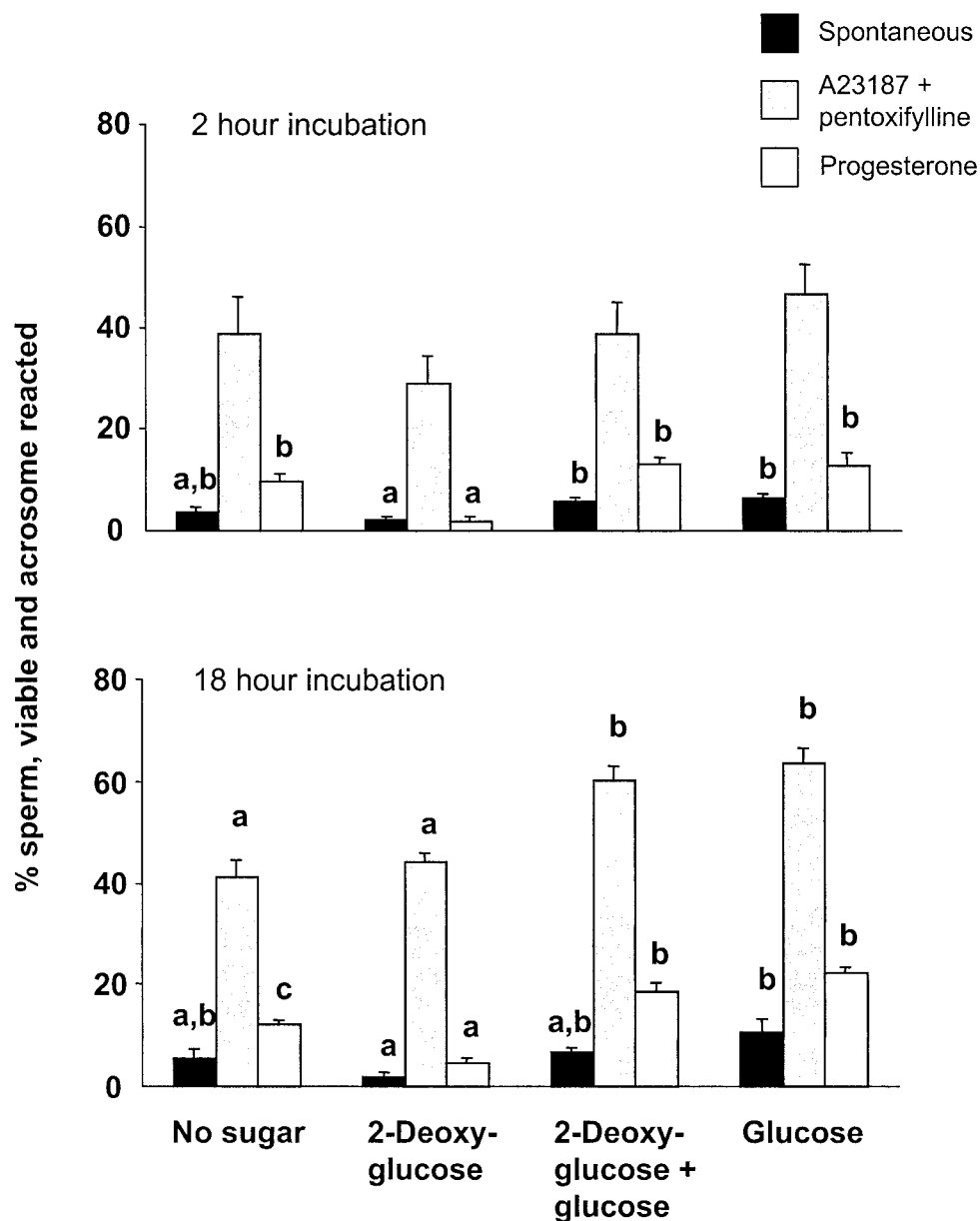


Figure 3. Effect of 2-deoxyglucose on the spontaneous and stimulated acrosome reaction rate in human sperm, following incubation for 2 or 18 hours at 37°C under 95% air/5% CO₂. Sperm were incubated in BWB containing 5.56 mmol glucose, 2-deoxyglucose, or glucose + 2-deoxyglucose L⁻¹, or no sugar. At the end of the incubation period, acrosome reactions were stimulated by further incubation with 2 μmol A23187 + 3.6 mmol pentoxifylline L⁻¹, 3.18 μmol progesterone L⁻¹, or with a buffer blank (spontaneous). Columns show means + SEM, n = 4. Significant differences ($P \leq .05$, one-way ANOVA with Scheffé post-hoc test) between sugars are indicated by the absence of a shared superscript.

was much higher than the spontaneous rate. Using this ionophore, there were no statistically significant effects of glucose concentration, although once again, the rate appeared maximal in the presence of 5.56 mmol glucose L⁻¹. Additional stimulation of the acrosome reaction with 3.6 mmol pentoxifylline in addition to 2 μmol A23187 L⁻¹ produced a further increase in the number of acrosome reactions detected. The proportion of sperm that exhibited viable acrosome reactions increased significantly from 43% ± 3.4% in the absence

of glucose to 60% ± 3.1% in the presence of 5.56 mmol glucose L⁻¹, declining to 40% ± 1.8% with 100 mmol glucose L⁻¹ (Figure 1). Glucose concentration had no statistically significant effect on sperm viability or the number of dead, acrosome-reacted sperm during the overnight incubation.

Next we compared the number of spontaneous and A23187 + pentoxifylline-stimulated acrosome reactions occurring after incubation for 2 or 18 hours in the presence of 5.56 mmol glucose L⁻¹, with the numbers

The effect of different sugars on the penetration of zona-free hamster oocytes by human sperm*

Sugar	Oocytes penetrated (%)			Decondensed sperm heads/oocyte		
	Capacitation period (hours)			Capacitation period (hours)		
	1	18 + 1		1	18 + 1	
	Stimulation regime			Stimulation regime		
	A23187		Spontaneous	A23187		Spontaneous
	Spontaneous	+ pentoxifylline		Spontaneous	+ pentoxifylline	
Fructose	10 (8–17)	80 (0–100)	25 (0–75)	0.14 (0.1–0.2)	4.5 (0–10)	0.38 (0–1.3)
Galactose	8 (0–17)	75 (0–85)	25 (0–63)	0.12 (0–0.3)	3.7 (0–9)	0.41 (0–0.7)
Glucose	12 (0–25)	91 (55–100)	50 (0–100)	0.13 (0–0.3)	5.6 (2–9)	0.80 (0–1.9)
Mannose	5 (0–17)	65 (0–100)	18 (0–72)	0.08 (0–0.3)	3.9 (0–10)	0.45 (0–1.9)
No sugar	10 (0–38)	60 (0–100)	15 (0–43)	0.12 (0–0.3)	3.5 (0–8)	0.48 (0–1.3)
No sugar + glucose at 18 h	—	—	20 (0–38)	—	—	0.47 (0–1.5)

* Sperm were resuspended in BWW medium containing the sugars indicated at a concentration of 5.56 mmol L⁻¹ or in medium containing no added sugar, and incubated for 1 hour. Acrosome reactions were spontaneous or were stimulated by the addition of 2 μmol A23187 + 3.6 mmol pentoxifylline L⁻¹ (A23187 + pentox) to the incubation medium. The penetration rate with spontaneous acrosome reactions was also measured in sperm that had been incubated overnight (18 hours) in similar medium before they were washed, resuspended in fresh, but otherwise identical, medium and incubated for a further 1 hour as above. Duplicate tubes containing no sugar were included in the overnight incubations, to which 5.56 mmol glucose L⁻¹ was added to just one for the final hour incubation. At the end of the incubations sperm were washed and resuspended in sugar-free medium and incubated with 8–12 zona-free hamster oocytes for a further 3 hours. Data are means (range). n = 5 for 1 hour incubations and n = 11 for 18 hour incubations.

occurring after incubation with the same concentration of other sugars. Under all conditions there were more acrosome reactions after 18 hours than after 2, and A23187 + pentoxifylline always produced a large increase in acrosome reactions above the spontaneous rate (Figure 2). After 18 hours all of the sugars supported a significantly higher spontaneous acrosome reaction rate than in the absence of an added sugar but there were no significant differences between the different sugars. After only 2 hours of incubation there was no significant difference between the number of spontaneous acrosome reactions occurring in the presence or absence of sugar, although fructose supported significantly more acrosome reactions than mannose did (Figure 2). After both 2 and 18 hours, the A23187 + pentoxifylline-stimulated acrosome reaction rate, supported by each of the sugars, appeared to be higher than in the absence of sugar, although at both time points this difference was only statistically significant in the case of glucose (Figure 2).

It is possible that the acrosome reaction could be supported by the metabolism of endogenous sugars and, therefore, that the previous experiments may have failed to reveal the full importance of glucose. For this reason, we examined the effect of 5.56 mmol 2-deoxyglucose L⁻¹ (a nonglycolysable glucose derivative that competitively inhibits both hexokinase and glucose 6-phosphate dehydrogenase) on the ability of sperm to acrosome react after capacitation for 2 or 18 hours, either spontaneously or after stimulation by 2 μmol A23187 + 3.6 mmol pentoxifylline L⁻¹. These experiments also provided an opportunity to examine the effect of glucose on the number of acrosome reactions

induced by a more physiological agonist, 3.18 μmol progesterone L⁻¹. After both 2 and 18 hours, 2-deoxyglucose appeared to decrease the number of spontaneous and progesterone-stimulated acrosome reactions below the rate observed with no added sugar, although only the difference in the progesterone-stimulated rate proved to be statistically significant. 2-Deoxyglucose had no significant effect on the acrosome reaction rate stimulated by 2 μmol A23187 + 3.6 mmol pentoxifylline L⁻¹. Glucose exerted similar effects to those seen in the previous experiments and seemed to support slightly higher spontaneous and A23187 + pentoxifylline-stimulated acrosome reaction rates than were seen in the absence of sugar, although this was statistically significant only for the stimulated rate after 18 hours. It also increased the number of acrosome reactions observed after progesterone stimulation, although again, statistically significantly only after an 18-hour incubation. The addition of 2-deoxyglucose together with glucose did not change significantly the responses seen with glucose alone (Figure 3).

Hamster Oocyte Penetration Test

The results of the HOPT varied greatly between sperm from different individuals. However, incubating sperm overnight consistently increased both the number of zona-free hamster oocytes penetrated as well as the number of decondensed sperm heads per egg in comparison with sperm that had been incubated for just 1 hour. Much larger increases were achieved by stimulating the acrosome reaction with A23187 + pentoxifylline (Table). Comparing the various sugars, glucose appeared to give the best performance overall, particularly after an overnight in-

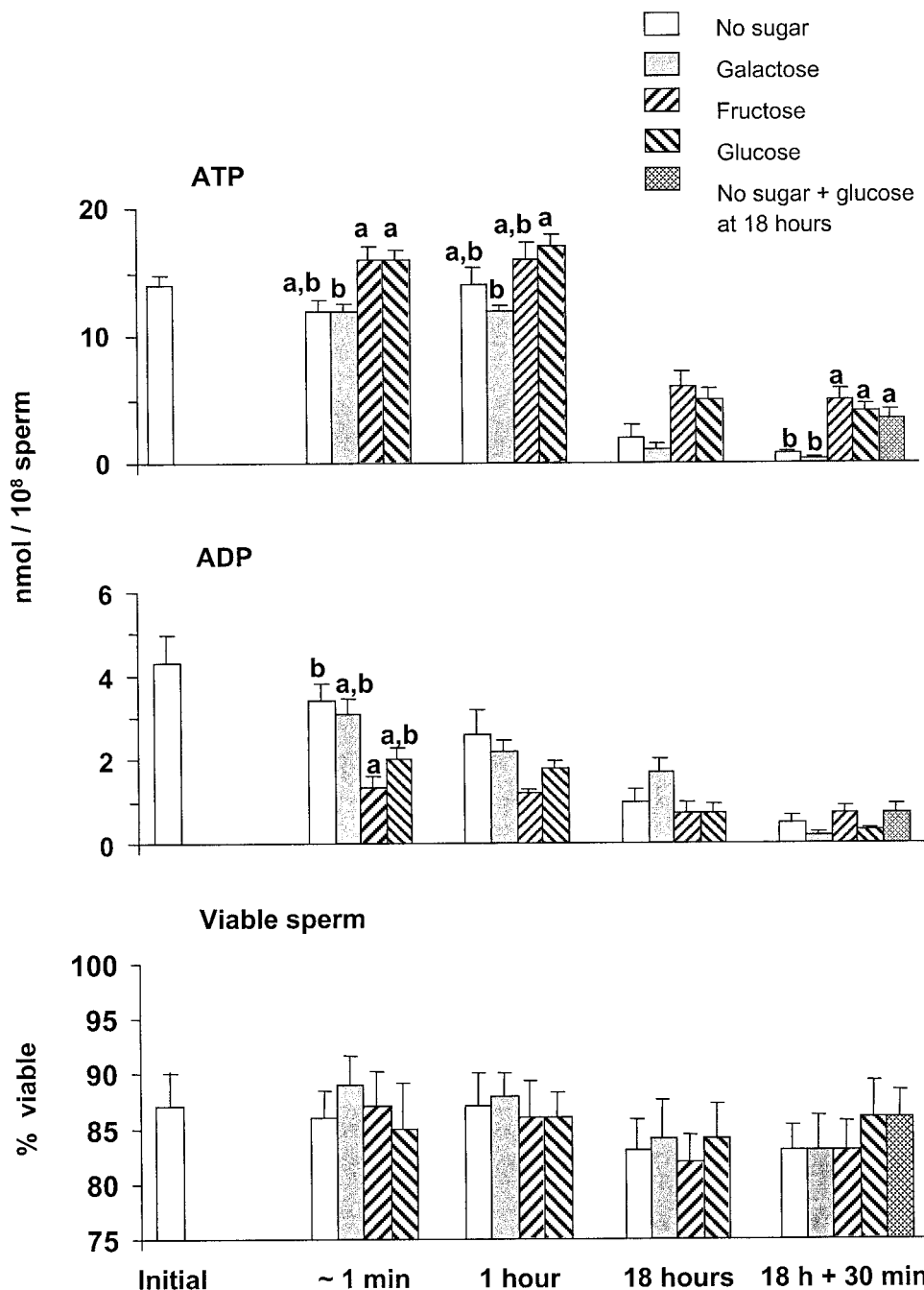


Figure 4. The effect of different sugars on the ATP and ADP concentrations, and viability, of human sperm during incubation in BWW medium at 37°C under 95% air/5% CO₂. Sperm were prepared in sugar-free BWW and a sample (Initial) for nucleotide analysis and viability assessment was taken immediately before the addition of 5.56 mmol fructose, galactose, or glucose L⁻¹ or a buffer blank (No sugar). After 18 hours, the sperm were resuspended in fresh medium and incubated for a further 30 minutes. Samples for analysis were taken at the times shown. Duplicate tubes containing no sugar were prepared, to which 5.56 mmol glucose L⁻¹ was added to just one tube after 18 hours. Sperm viability was assessed by HOST. Data are means ± SEM, n = 11. Significant differences between sugars at each time point ($P \leq .05$, one-way ANOVA with Scheffé post-hoc test) are signified by the absence of a common superscript.

cubation. However, there was no consistent pattern in the relative penetration rates supported by the different sugars and no significant differences were detected between the penetration rates supported by the individual sugars after

either capacitation time, whether spontaneous or after stimulation with A23187 + pentoxifylline (Table).

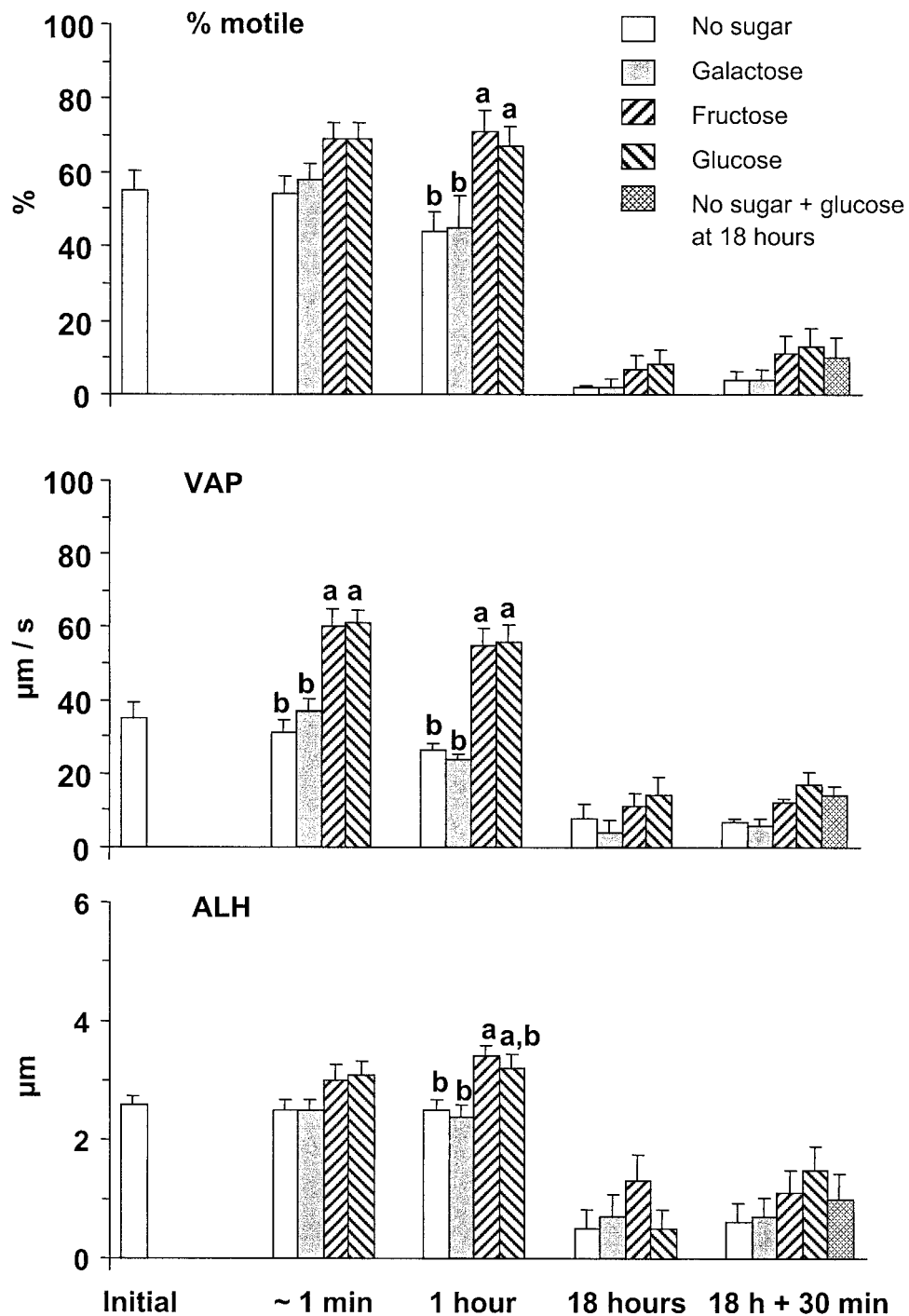


Figure 5. The effect of different sugars on the percentage of sperm motile (% Motile), VAP, and ALH during incubation in BWB medium at 37°C under 95% air/5% CO₂. Sperm were prepared in sugar-free BWB and a sample (Initial) for CASA was taken immediately before the addition of 5.56 mmol fructose, galactose, or glucose L⁻¹ or a buffer blank (No sugar). After 18 hours the sperm were resuspended in fresh medium and incubated for a further 30 minutes. Samples for CASA were taken at the times shown. Two tubes containing no sugar were prepared, one of which received 5.56 mmol glucose L⁻¹ after 18 hours. Data are means + SEM, n = 11. Significant differences between sugars at each time point (*P* ≤ .05, one-way ANOVA with Scheffé post-hoc test) are signified by the absence of a common superscript.

Adenine Nucleotide Concentration and Sperm Motility

The addition of either of the glycolysable sugars, glucose or fructose, to human sperm suspended in sugar-free BWW medium produced a rapid (<1 minute) increase in ATP concentration and a decrease in ADP, whereas addition of the nonglycolysable sugar, galactose, had no effect. Whatever sugar was present, the nucleotide concentrations remained stable for 1 hour, but after 18 hours the concentration of ATP had fallen dramatically, as too had the concentration of ADP, although to a much lesser extent. Sperm incubated with glucose or fructose appeared to maintain higher ATP and lower ADP concentrations than sperm incubated without a glycolysable sugar, but this effect was not statistically significant. Only slight changes occurred after the sperm had been resuspended in fresh medium and incubated for a further 30 minutes, but at this time the higher ATP concentration in the presence of glucose or fructose achieved statistical significance. The addition of glucose, at 18 hours, to sperm previously incubated without sugar increased their ATP concentration to a value similar to that observed in sperm incubated continuously with glucose (Figure 4).

The effects of the different sugars on motility (Figure 5) reflected their effects on ATP. The addition of a glycolysable sugar produced an immediate, albeit nonsignificant, increase in the percentage of sperm that were motile with significant improvements in path velocity (VAP), the percentage of sperm rapidly motile (ie, with a VAP >25 $\mu\text{m/s}$) and in progressive velocity (not shown) as well as a smaller increase in lateral head displacement. Motility was sustained for an hour but decreased dramatically after incubation overnight. At this time, most motility parameters appeared somewhat higher in the presence of a glycolysable sugar but the effect was not statistically significant (Figure 5). Resuspending the sperm in fresh medium had little effect on motility but the addition of glucose to sperm previously incubated with no sugar appeared to increase their motility to similar values to those incubated in glucose all along (Figure 5).

Sperm viability remained largely unchanged throughout the time course of the experiments, with no significant differences seen between sperm incubated with any of the sugars (Figure 4).

A second experiment was carried out to examine the effect of 2-deoxyglucose on sperm motility and adenine nucleotides. Extra time points were included to study ATP and ADP concentrations and motility 3 hours after the start of the experiment and immediately (<2 minutes) after resuspension following an overnight incubation (Figures 6 and 7). Glucose produced a rapid increase in ATP concentration together with an accompanying decrease in ADP. These adenine nucleotide concentrations were maintained at a stable level for a further 3 hours. However,

following an overnight incubation the concentration of ATP fell, although to a lesser extent than was observed in the absence of added sugar. The addition of glucose to sperm previously incubated overnight without sugar increased the ATP concentration. In the absence of sugar ATP decreased in the first hour, whereas ADP increased very slightly, with both observations becoming more marked after 3 hours. The concentration of both ATP and ADP decreased to very low values after the sperm had been incubated overnight. In the presence of 2-deoxyglucose the worsening of the sperms' energy status was markedly accelerated, with the ATP concentration reaching a very low value after just 3 hours (Figure 6).

Consistent with its effect upon the energy status of sperm, glucose maintained a high level of motility for 3 hours and although all the motility parameters decreased after an overnight incubation they remained higher than those observed in the absence of sugar. In the absence of sugar the percentage of sperm that were motile or swimming rapidly began to decline during the first hour, had decreased further by 3 hours, and by 18 hours had reached very low levels. By contrast, VAP and ALH changed relatively little during the first 3 hours of incubation, but had declined markedly after 18 hours. In the presence of 2-deoxyglucose, motility declined steeply after the first hour, with only 14% of sperm remaining motile after 3 hours and very few motile following an overnight incubation (Figure 7).

As was the case in the first experiment, viability remained unaffected by time, or by the type of sugar present during the incubation (Figure 6).

Hyperactivated motility was also monitored during these experiments and the results are illustrated in Figure 8. In the presence of glucose or fructose approximately 8% of sperm exhibited hyperactivated motility 10 minutes to 3 hours after the addition of the sugar, but this proportion fell to <2% after 18 hours (not shown). In the presence of galactose or with no sugar, the mean proportion of sperm hyperactivated never exceeded 2%, and no hyperactivated sperm were detected after 18 hours (Figure 8a). In the second series of experiments the proportion of sperm that were hyperactivated in the presence of glucose was slightly higher than in the first (~12% vs ~8%, respectively). Once again this prevalence of hyperactivation was maintained for only the first 3 hours; by 18 hours <2% of sperm were hyperactivated (not shown). Very few sperm (<3%) incubated in either 2-deoxyglucose or no sugar exhibited hyperactivation (Figure 8b).

Discussion

Glucose, or another glycolysable sugar, was required to sustain an optimal ATP concentration in human sperm and to support optimum motility, even when sperm were

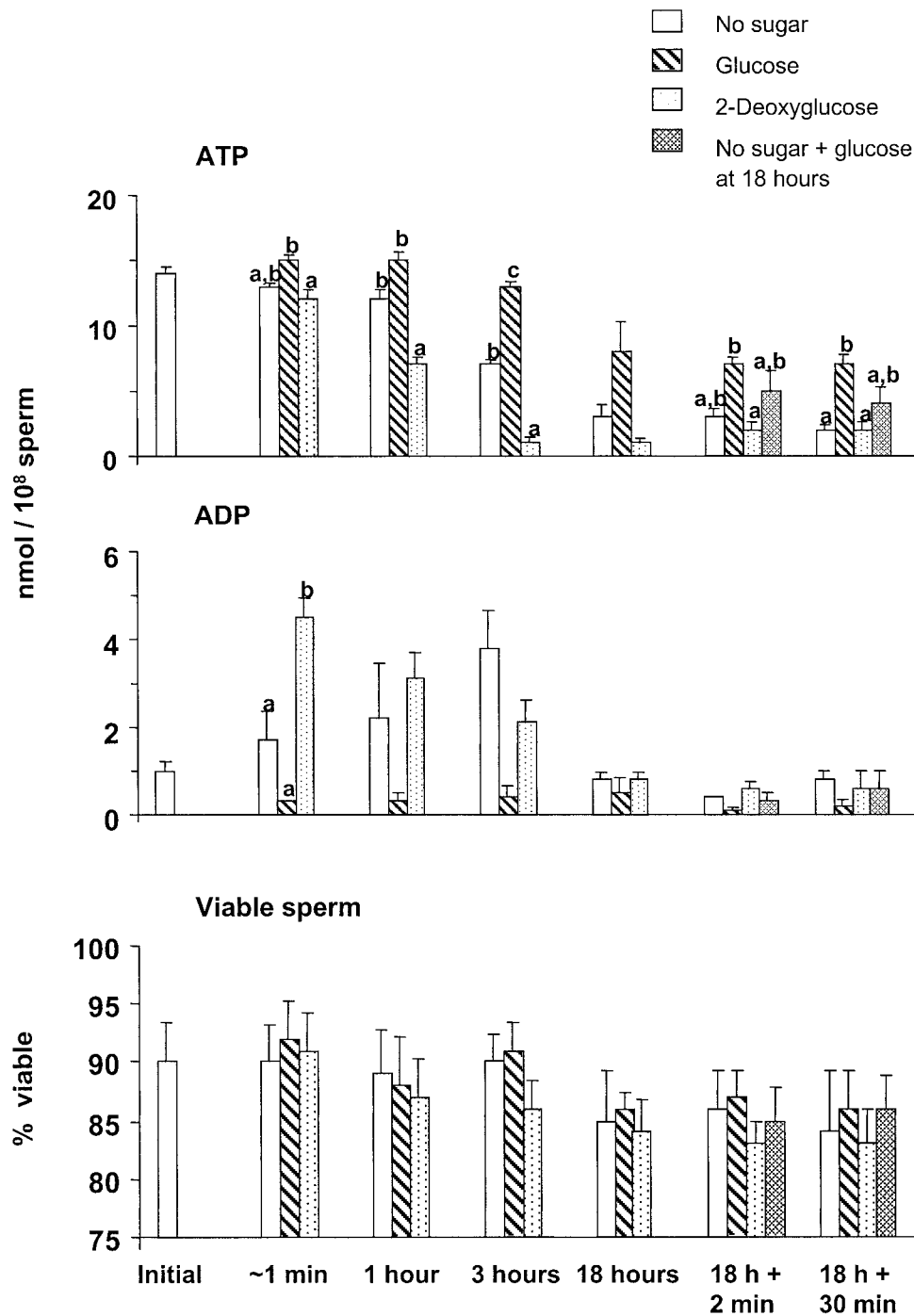


Figure 6. The effect of 2-deoxyglucose on the ATP and ADP concentration, and viability, of human sperm during incubation in BWB medium at 37°C under 95% air/5% CO₂. Sperm were prepared in sugar-free BWB and a sample (Initial) for nucleotide analysis and viability assessment was taken immediately before the addition of 5.56 mmol 2-deoxyglucose, or glucose L⁻¹, or a buffer blank (No sugar). After 18 hours, the sperm were resuspended in fresh medium and incubated for a further 30 minutes. Samples for analysis were taken at the times shown. Two tubes containing no sugar were prepared, to which 5.56 mmol glucose L⁻¹ was added to just one tube after 18 hours. Sperm viability was assessed by HOST. Data are means + SEM, n = 4. Significant differences between sugars at each time point ($P \leq .05$, one-way ANOVA with Scheffé post-hoc test) are signified by the absence of a common superscript.

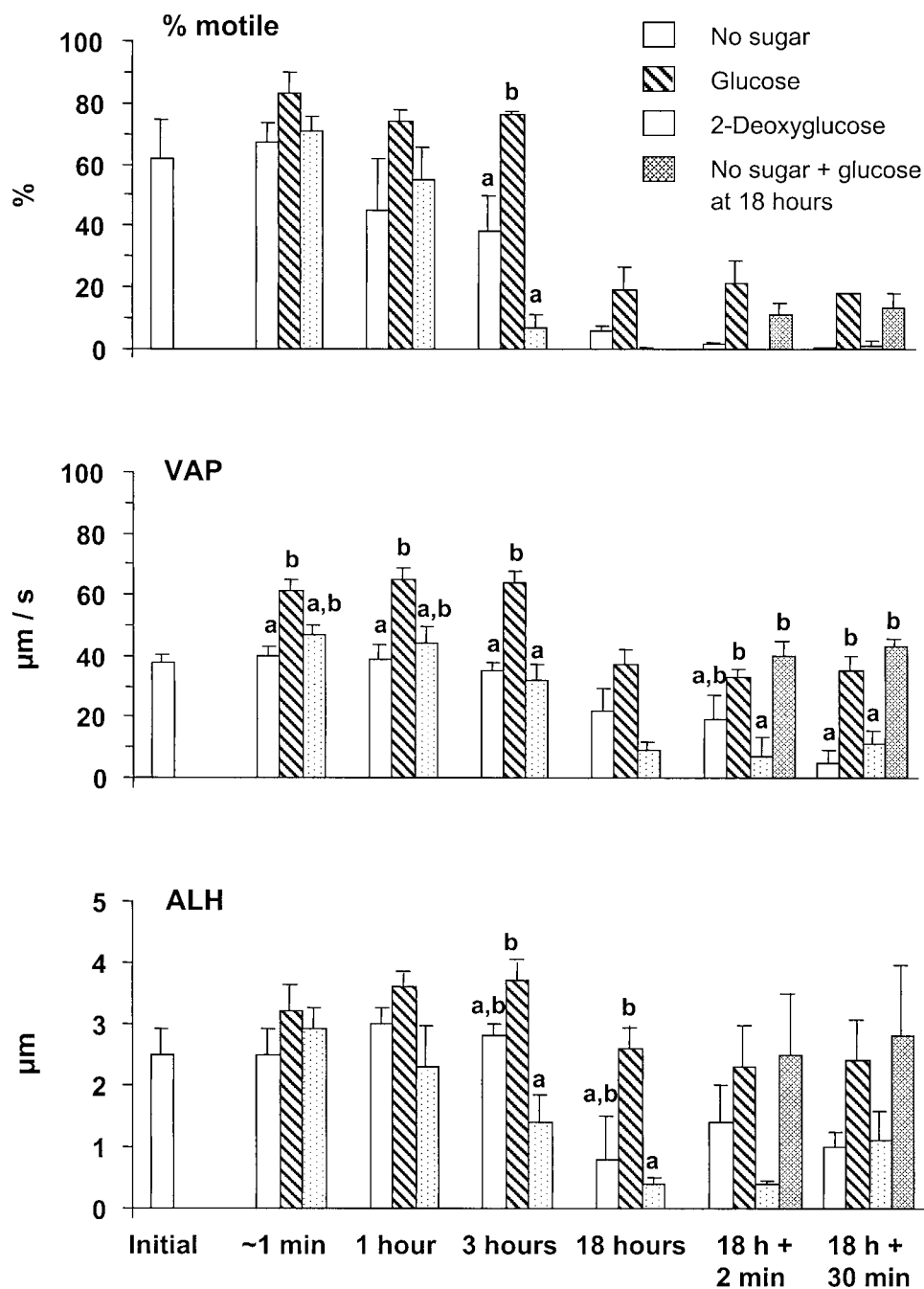


Figure 7. The effect of 2-deoxyglucose on the percentage of sperm motile (% Motile), VAP, and ALH during incubation in BWB medium at 37°C under 95% air/5% CO₂. Sperm were prepared in sugar-free BWB and a sample (Initial) for CASA was taken immediately before the addition of 5.56 mmol 2-deoxyglucose, or glucose L⁻¹, or a buffer blank (No sugar). After 18 hours the sperm were resuspended in fresh medium and incubated for a further 30 minutes. Samples for CASA were taken at the times shown. Two tubes containing no sugar were prepared, one of which received 5.56 mmol glucose L⁻¹ after 18 hours. Data are means + SEM, n = 4. Significant differences between sugars at each time point ($P \leq .05$, one-way ANOVA with Scheffé post-hoc test) are signified by the absence of a common superscript.

supplied with adequate lactate and pyruvate to support mitochondrial respiration. By contrast, the ability of the sperm to acrosome react or to penetrate zona-free hamster oocytes was affected only slightly by the presence of glucose, if at all. Glucose was required to support significant

levels of hyperactivated motility, but could be substituted by fructose, and the extent of hyperactivation did not increase with time as might have been expected had the degree of capacitation increased during the incubation. These data emphasize the importance of glycolysis for

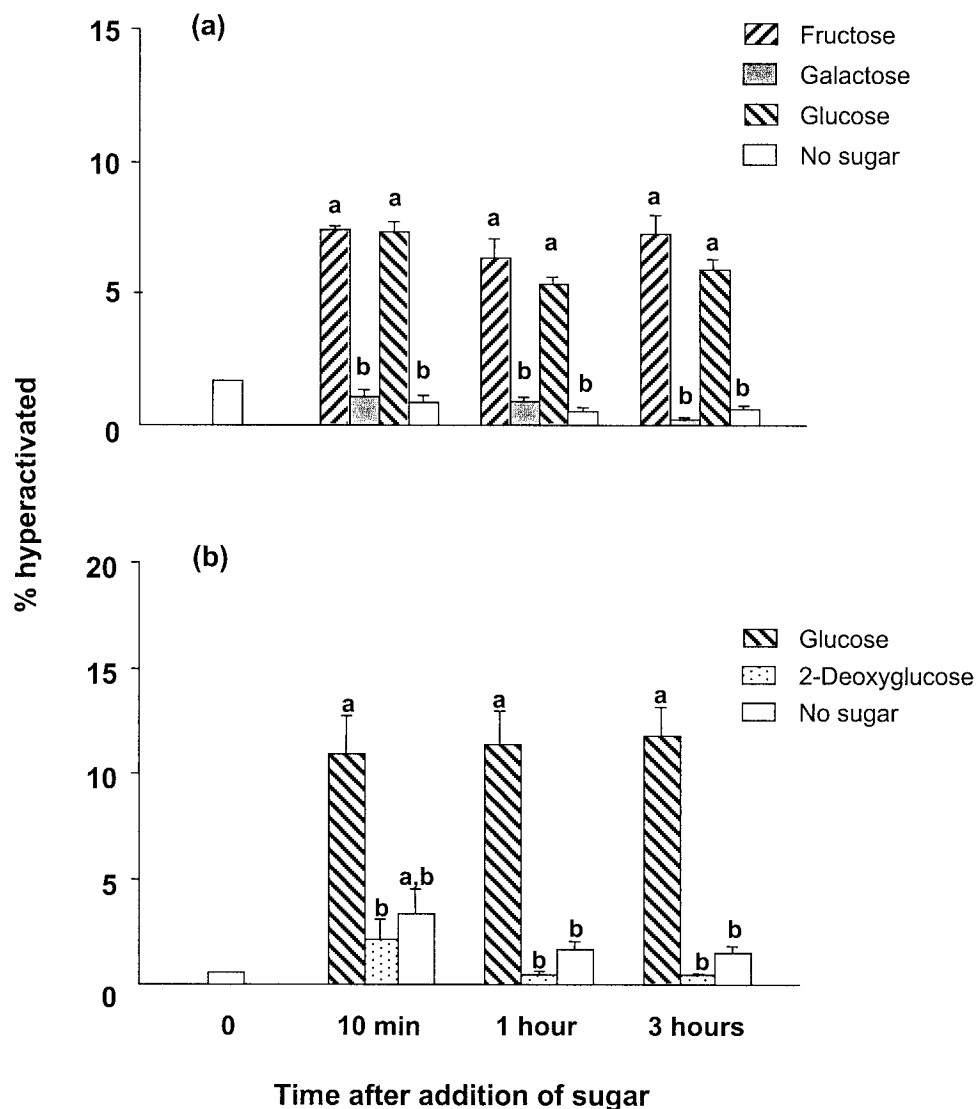


Figure 8. Effect of (a) various sugars or (b) 2-deoxyglucose on the percentage of human sperm exhibiting hyperactivated motility during incubation in BWW medium at 37°C under 95% air/5% CO₂. Sperm were prepared in sugar-free BWW, and 5.56 mmol glucose, fructose, galactose, or 2-deoxyglucose L⁻¹ or a buffer blank was added at time zero. Samples for CASA were taken at the times indicated and hyperactivation was defined according to the method of Burkman (1991). Columns show means + SEM, n = 11 (a) or n = 4 (b). Significant differences ($P \leq .05$, one-way ANOVA with Scheffé post-hoc test) between sugars are indicated by the absence of a shared superscript.

energy production in human sperm (Ford and Rees, 1990) but cast doubt on glucose having any significant role in supporting human sperm capacitation, aside from the provision of ATP. If such a role exists, it must be a subtle one or one that can easily be substituted by other mechanisms.

Effect of Glucose on ATP Concentration and Motility

As discussed above, in comparison to sperm from other species, human sperm obtain an unusually high proportion of their energy from the glycolysis of glucose to lactate, and the effect of glucose or fructose on motility can be explained by their effect on ATP concentration. Accordingly, there is a highly significant correlation between

the percentage of sperm that were motile ($r = .84$, $P < .001$) or path velocity (VAP; $r = .65$, $P < .001$) and their ATP concentration (Figure 9a and b). Sperm in these experiments sustained vigorous motility for 3 hours but motility declined markedly after incubation overnight. The stress of prolonged incubation was accompanied by a decrease in the concentration of both ATP and ADP. Because sperm viability remained high throughout the experiments this was not due to leakage of adenine nucleotides from dead and dying cells. The loss of ATP and ADP is likely due to the conversion of ADP to ATP and adenosine monophosphate (AMP) by adenylate kinase to maintain the reaction, $ATP \leftrightarrow ADP + P_i$, away from equilibrium and the subsequent removal of AMP by de-

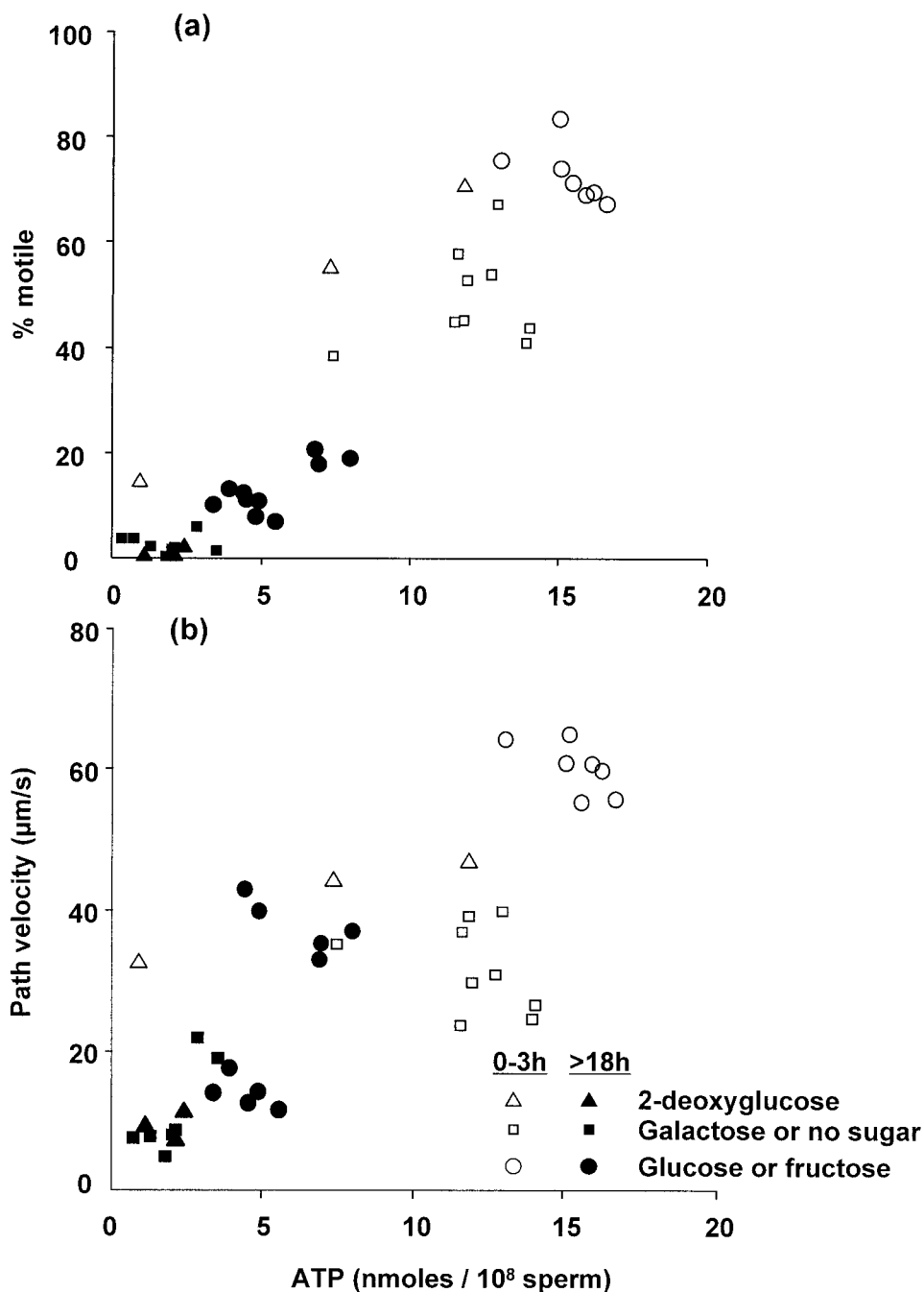


Figure 9. The relationship between ATP concentration in sperm incubated for 0–3 or >18 hours in the presence of 5.56 mmol glucose, fructose, galactose, or 2-deoxyglucose L⁻¹ or no added sugar, and (a) the percentage motile, and (b) their mean VAP.

amination or dephosphorylation to allow the process to continue (Chulavatnatol and Harsungcharern, 1977). In these circumstances, given that we were unable to measure Pi or AMP, the ATP:ADP ratio is possibly the best available index of the cell's energy status. There was a quantitatively different relationship between the ATP:ADP ratio and motility after short- and long-term incubations. At both times, there was a hyperbolic relationship

between ATP:ADP and motility. However, for a given ATP:ADP ratio the percentage of sperm motile or their mean path velocity was considerably less after an overnight incubation (Figure 10a and b). This could be a consequence of the decreased concentration of adenine nucleotides, but alternatively, it might reflect changes in protein phosphorylation within the axoneme. The latter proposition would be consistent with the view that motility is

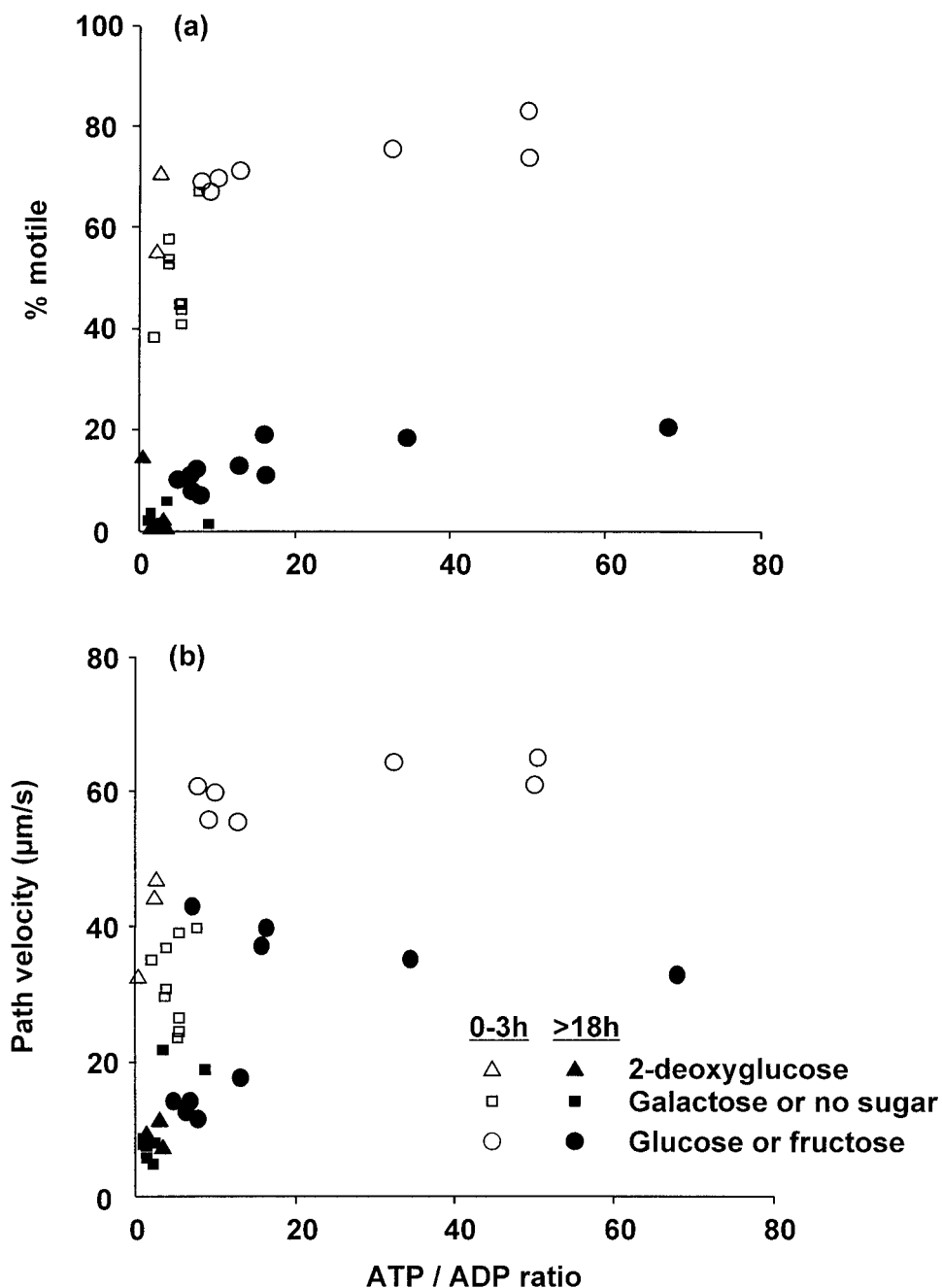


Figure 10. The relationship between the ATP:ADP ratio in sperm incubated for 0–3 or >18 hours in the presence of 5.56 mmol glucose, fructose, galactose, or 2-deoxyglucose L^{-1} or no added sugar, and (a) the percentage motile, and (b) their mean VAP.

not limited by the availability of metabolic energy unless ATP synthesis is constrained by a lack of substrate or the presence of a metabolic inhibitor (Ford and Rees, 1990). Depriving sperm of a glycolysable sugar did not permanently damage the energy status of the sperm because the subsequent addition of glucose after 18 hours restored ATP and motility to values similar to those expected had glucose been present all along.

2-Deoxyglucose had an extremely adverse effect on the

energy status of sperm. This is probably due to 2 factors. First, the consumption of ATP during the phosphorylation of 2-deoxyglucose to 2-deoxyglucose 6-phosphate; and second, the sequestration of the sperm's limited pool of inorganic phosphate, thus limiting the amount of phosphate available to rephosphorylate ADP or AMP. Consistent with its effect on ATP concentration, 2-deoxyglucose substantially decreased the motility of human sperm after just 3 hours. In view of its marked effect on the energy

status of sperm, 2-deoxyglucose is not a suitable inhibitor with which to investigate the role of other products of glucose metabolism in capacitation, such as NADPH (Aitken et al, 1998).

Effects of Glucose on Capacitation

Our data demonstrate that there is no absolute requirement for glucose for human sperm to acrosome react or to fuse with zona-free hamster oocytes. A similar conclusion was reached by Hoshi et al (1982) who also found that human sperm incubated in glucose-free medium could penetrate zona-free hamster oocytes. The slight enhancement by glucose of the acrosome reaction rate and penetration of zona-free hamster oocytes, observed by us, often lacked statistical significance. By contrast, Rogers and Perreault (1990) reported much higher penetration rates in the presence of glucose and found that glucose could only be partially substituted by mannose or fructose. It is, however, important to note that these authors performed both the fertilization and capacitation incubations in the presence of sugars, whereas the fertilization stage, in our experiments, was conducted in the same common sugar-free medium, whatever sugar was present during capacitation. If glucose or other sugars are present during the fertilization step it is possible that they may affect the eggs rather than the sperm or, alternatively, that enhanced sperm motility in the presence of sugars may promote a higher penetration rate through a higher collision frequency. A second difference from our data is that Rogers and Perreault (1990) observed that glucose supported better progressive motility than fructose, whereas we found both sugars to be equally effective. Rogers and Perreault assessed motility subjectively and were unable to relate their observations to the adenine nucleotide concentrations in the sperm.

Glucose increases the number of sperm that bind to the egg and the proportion of eggs fertilized in human in vitro fertilization (Hoshi et al, 1991; Mahadevan et al, 1997) but we are not aware of any attempts to compare it with other sugars in this system. The effect of glucose on IVF was ascribed to the sperm having more vigorous motility and to a greater proportion of the sperm exhibiting hyperactivated motility. This is consistent with our observation that hyperactivated motility was the only capacitation-related phenomenon that clearly depended on the presence of a glycolysable sugar. Our data are consistent with a requirement for glycolytic ATP production to supply sufficient metabolic energy to support the very vigorous motility needed to achieve the high VCL and ALH inherent to hyperactivation. It was surprising that the proportion of hyperactivated sperm failed to increase over time as more capacitated. We think this is unlikely to be due to our definition of hyperactivation because although the Burkman (1991) criteria gave a lower estimate of per-

centage activation than some other definitions, the results were significantly correlated (Sukcharoen et al, 1995).

Sperm were able to acrosome react and fuse with zona-free hamster oocytes after an overnight incubation or in the presence of 2-deoxyglucose, despite a significant decline in their ATP concentration. It is unlikely that a significant number of the acrosome reactions were degenerative because the viability of the sperm was confirmed using the HOST test. Although many sperm became immotile, few died. This implies that the acrosome reaction and subsequent fusion with the zona-free oocyte require very little metabolic energy, or that a separate pool of ATP exists in the acrosome or elsewhere in the sperm head, to support it.

The small beneficial effect of glucose on the acrosome reaction may simply be due to the increased production of ATP through glycolysis. As well as supporting more vigorous motility this can have more subtle effects. The addition of glucose or fructose but, interestingly, not 2-deoxyglucose, to human sperm suspended in sugar-free medium caused the transient hyperpolarization of the plasma membrane, as a result of the augmented ATP available to the Na⁺, K⁺-ATPase (Guzmán-Grenfell et al, 2000). It has been proposed that hyperpolarization is a key element in capacitation (Florman et al, 1998), and that enhancement of it could explain the small stimulatory effects of the glycolysable sugars on the acrosome reaction. It is also possible that the higher ATP concentrations found in the presence of glucose might favor protein phosphorylation. Capacitation is characterized by the tyrosine phosphorylation of key proteins subsequent to an activation of protein kinase A (Visconti and Kopf, 1998). Although the ATP turnover involved in protein phosphorylation is trivial compared with the amounts used to support motility, it is possible that a restricted supply of ATP could retard it. Alternatively, there could be some regulatory feedback from the energy status of the sperm to the protein kinase/phosphatase system.

To conclude, these data demonstrate that the presence of glucose, or another glycolysable sugar, enhances ATP production and motility in human sperm. This is adequate to explain why a glycolysable sugar was required for substantial numbers of sperm to exhibit hyperactivated motility. We found no evidence of an essential requirement for any other product of glucose metabolism, such as NADPH, to support capacitation or the acrosome reaction.

Acknowledgment

We thank the late Professor M.G.R. Hull and other staff in the University of Bristol Centre for Reproductive Medicine for their help and support.

References

- Aitken RJ. The zona-free hamster egg penetration test. In: Hargreaves TB, ed. *Male Infertility*. Berlin: Springer Verlag; 1983:75–86.
- Aitken RJ, Fisher HM, Fulton N, Gomez E, Knox W, Lewis B, Irvine S. Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by the flavoprotein inhibitors diphenylene iodonium and quinacrine. *Mol Reprod Dev*. 1997;47:468–482.
- Aitken RJ, Harkiss D, Knox W, Paterson M, Irvine DS. A novel transduction cascade in capacitating human spermatozoa characterised by a redox-regulated, cAMP mediated induction of tyrosine phosphorylation. *J Cell Sci*. 1998;111:645–656.
- Burkman LJ. Discrimination between non-hyperactivated and classical hyperactivated motility patterns in human spermatozoa using computerised analysis. *Fertil Steril*. 1991;55:363–371.
- Chulavatnatol M, Harsungcharern A. Stabilization of adenylate energy charge and its relation to human sperm motility. *J Biol Chem*. 1977;252:8088–8091.
- Coates A, Rutherford AJ, Hunter H, Leese HJ. Glucose-free medium in human in vitro fertilization and embryo transfer: a large-scale, prospective, randomized clinical trial. *Fertil Steril*. 1999;72:229–232.
- Cross NL, Morales P, Overstreet JW, Hanson FW. Two simple methods for detecting acrosome-reacted human sperm. *Gamete Res*. 1986;15:213–226.
- de Lamirande E, Jiang H, Zini A, Kodama H, Gagnon C. Reactive oxygen species and sperm physiology. *Rev Reprod*. 1997;2:48–54.
- Dravland E, Meizel S. Stimulation of hamster sperm capacitation and acrosome reaction in vitro by glucose and lactate and inhibition by the glycolytic inhibitor alpha-chlorohydrin. *Gamete Res*. 1981;4:515–523.
- Florman HM, Arnoult C, Kazam IG, Li CQ, O'Toole CMB. A perspective on the control of mammalian fertilization by egg-activated ion channels in sperm: a tale of two channels. *Biol Reprod*. 1998;59:12–16.
- Ford WCL, Harrison A. The role of oxidative phosphorylation in the generation of ATP in human spermatozoa. *J Reprod Fertil*. 1981;63:271–278.
- Ford WCL, McLaughlin EA, Prior SM, Rees JM, Wardle PG, Hull MGR. The yield, motility and performance in the hamster egg test of human spermatozoa prepared from cryopreserved semen by 4 different methods. *Hum Reprod*. 1992;7:654–659.
- Ford WCL, Rees JM. The bioenergetics of mammalian sperm motility. In: Gagnon C, ed. *Controls of Sperm Motility: Biological and Clinical Aspects*. Boca Raton, Fla: CRC Press; 1990:175–202.
- Ford WCL, Rees JM, McLaughlin EA, Goddard RJ, Hull MGR. The effect of A23187 concentration and exposure time on the outcome of the hamster egg penetration test. *Int J Androl*. 1991;14:127–139.
- Ford WCL, Whittington K, Williams AC. Reactive oxygen species in human sperm suspensions: production by leukocytes and the generation of NADPH to protect sperm against their effects. *Int J Androl*. 1997;20(suppl 3):44–49.
- Fraser LR, Quinn PJ. A glycolytic product is obligatory for initiation of the sperm acrosome reaction and whiplash motility required for fertilization in the mouse. *J Reprod Fertil*. 1981;61:25–35.
- Guzmán-Grenfell AM, Bonilla-Hernández MA, González-Martínez MT. Glucose induces a Na⁺, K⁺-ATPase-dependent transient hyperpolarization in human sperm. I. Induction of changes in plasma membrane potential by the proton ionophore CCCP. *Biochim Biophys Acta*. 2000;1464:188–198.
- Hoppe PC. Glucose requirement for mouse sperm capacitation. *Biol Reprod*. 1976;15:39–45.
- Hoshi K, Saito A, Suzuki M, Hayashi K, Yanagimachi R. Effects of substrates on the penetration of human spermatozoa into the zona pellucida of human eggs and the zona free hamster eggs. *Jpn J Fertil Steril*. 1982;27:439–444.
- Hoshi K, Tsukikawa S, Sato A. Importance of Ca²⁺, K⁺ and glucose in the medium for sperm penetration through the zona pellucida. *Tohoku J Exp Med*. 1991;165:99–104.
- Hyne RV, Edwards KP. Influence of 2-deoxy-D-glucose and energy substrates on guinea-pig sperm capacitation and acrosome reaction. *J Reprod Fertil*. 1985;73:59–69.
- Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJD. Development of an assay to assess the functional integrity of the human sperm plasma membrane and its relationships to other semen characteristics. *J Reprod Fertil*. 1984;70:219–228.
- Mahadevan MM, Miller MM, Moutos DM. Absence of glucose decreases human fertilization and sperm movement characteristics in vitro. *Hum Reprod*. 1997;12:119–123.
- McLaughlin EA, Ford WCL. The contribution of the toxicity of a glycerol-egg yolk-citrate cryopreservative to the decline in human sperm motility during cryopreservation. *J Reprod Fertil*. 1992;95:749–754.
- Niwa K, Iritani A. Effect of various hexoses on sperm capacitation and penetration of rat eggs in vitro. *J Reprod Fertil*. 1978;53:267–271.
- Parrish JJ, Susko-Parrish JL, First NI. Capacitation of bovine sperm by heparin: inhibitory effect of glucose and role of intracellular pH. *Biol Reprod*. 1989;41:683–699.
- Rees JM, Ford WCL, Hull MGR. Effect of caffeine and of pentoxifylline on the motility and metabolism of human spermatozoa. *J Reprod Fertil*. 1990;90:147–156.
- Rogers BJ, Perreault SD. Importance of glycolysable substrates for in vitro capacitation of human spermatozoa. *Biol Reprod*. 1990;43:1064–1069.
- Storey BT. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. *Mol Hum Reprod*. 1997;3:203–213.
- Sukcharoen N, Keith J, Irvine DS, Aitken RJ. Definition of the optimal criteria for identifying hyperactivated human spermatozoa at 25 Hz using in-vitro fertilization as a functional end-point. *Hum Reprod*. 1995;10:2928–2937.
- Suter D, Chow PYW, Martin ICA. Maintenance of motility in human sperm by energy derived through oxidative phosphorylation and addition of albumin. *Biol Reprod*. 1979;20:505–510.
- Urner F, Sakkas D. Glucose is not essential for the occurrence of sperm binding and zona-pellucida-induced acrosome reaction in the mouse. *Int J Androl*. 1996a;19:91–96.
- Urner F, Sakkas D. Glucose participates in sperm-oocyte fusion in the mouse. *Biol Reprod*. 1996b;55:917–922.
- Urner F, Sakkas D. A possible role for the pentose phosphate pathway of spermatozoa in gamete fusion in the mouse. *Biol Reprod*. 1999;60:733–739.
- VandeVoort CA, Overstreet JW. Effects of glucose and other energy substrates on the hyperactivated motility of macaque sperm and the zona-pellucida-induced acrosome reaction. *J Androl*. 1995;16:327–333.
- Visconti PE, Kopf GS. Regulation of protein phosphorylation during sperm capacitation. *Biol Reprod*. 1998;59:1–6.
- Whittington K, Ford WCL. The effect of incubation periods under 95% oxygen on the stimulated acrosome reaction and motility of human spermatozoa. *Mol Hum Reprod*. 1998;4:1053–1057.
- World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction*. 3rd ed. Cambridge, United Kingdom: Cambridge University Press; 1992.