Glycolysis and sperm motility: does a spoonful of sugar help the flagellum go round?

W.C.L.Ford

Department of Clinical Science at South Bristol, St Michael's Hospital, University of Bristol, Bristol, UK

To whom correspondence should be addressed at: Department of Clinical Science at South Bristol, St Michael's Hospital, Bristol, BS2 8EG, UK. E-mail: chris.ford@bristol.ac.uk

It is doubtful that diffusion can deliver sufficient ATP from the mitochondria to sustain activity at the distal end of the sperm flagellum. Glycolytic enzymes bound to the fibrous sheath could provide energy along the flagellum at the point it is required. An obligatory role for glycolysis is supported by the lack of progressive motility in sperm from mice where the gene for sperm-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDHs) had been 'knocked out'. Here, I review some evidence against this idea. First, pure diffusion from the mitochondrion is likely to be adequate in species with smaller sperm, and it is possible that rapid ATP delivery required in larger sperm could be achieved by an adenylate kinase shuttle. Second, experience with α-chlorohydrin demonstrates that sperm can remain motile with normal ATP concentrations despite inhibition of GAPDHs; adverse effects only occur if glucose is added and high levels of glycolytic intermediates accumulate. These observations undermine the GAPDHs knockout mouse as evidence for an essential role of local glycolysis. Third, sperm from many species can remain motile for long periods in sugar-free media and excepting dog sperm, evidence that gluconeogenesis is a possible explanation, is weak. In most species, it is unlikely that local glycolysis is the only way that ATP can be supplied to the distal flagellum.

Key words: adenylate kinase/energy metabolism/motility/spermatozoa/α-chorohydrin

Introduction

The sperm flagellum is long, about 50 μm in human, and thin, about 0.5 μm diameter. Many species have still longer flagella (Table I). Active bend propagation continues along the length of the flagellum and consequently ATP to drive active sliding must be available throughout the flagellum. The mitochondria, the site of oxidative phosphorylation, are located in the sperm midpiece at the extreme anterior end of the flagellum. There is a very legitimate question as to whether diffusion from the mitochondria can deliver ATP to the distal end of the flagellum rapidly enough to support active sliding there. Glycolytic enzymes are concentrated in the principal piece, and some are bound to the fibrous sheath of the flagellum (Storey and Kayne, 1975; Travis *et al*., 1998; Eddy *et al*., 2003) therefore glycolysis could produce ATP adjacent to the site it is required to support active sliding of the flagellar filaments. It has been suggested that glycolysis in the principal piece is critical for normal sperm function (Turner, 2003). A recent report that mice in which expression of the gene for the sperm-specific isoform of glyceraldehyde-3-phosphatedehydrogenase (GAPDHs) was knocked out have immotile sperm supports this idea (Miki *et al*., 2004).

This short article reviews the evidence that glycolysis is essential for local ATP production to support sperm motility, it will not deal with the equally interesting question of whether glucose is required for sperm capacitation or hyperactivation in some species (Fraser and Quinn, 1981; Urner and Sakkas, 1996; Urner *et al*., 2001; Williams and Ford, 2001).

Mammalian sperm share a common problem of ATP delivery along the flagellum but adopt a variety of metabolic strategies for ATP generation (Ford and Rees, 1990). This review will avoid detailed analysis of interspecies differences. It accepts that sperm of many species can support motility by glycolysis and by this means can remain motile under anaerobic conditions or when oxidative phosphorylation is blocked by inhibitors (Ford and Rees, 1990; Mukai and Okuno, 2004) but argues that multiple strands of evidence demonstrate that alternative mechanisms for ATP delivery to the flagellum must exist. The balance between different pathways is likely to vary between species.

Is diffusion really a problem?

Biophysicists have calculated the rate of diffusion of ATP along the sperm tail based on both a steady state (Nevo and Rikmenspoel, 1970) and a transient model taking into account the rate of power output along the flagellum (Adam and Wei, 1975). Both models suggested that diffusion was adequate to deliver ATP to the tail tip at least in bull and in sea urchin sperm. Whether this remains true in the larger sperm of rodents remains open to doubt (Turner, 2003), although a close linear relationship between mitochondrial volume and flagellar length supports a diffusion-based mechanism (Cardullo and Baltz, 1991).

Table I. Sizes of spermatozoa from some domestic and laboratory animals

| Species	Length (μm)			
	Head	Midpiece	Principal piece	Total
Bull	6.8	9.8	36.9	53.5
Boar	8.5	10	36.1	54.6
Ram	8.2	14.0	43.0	65.2
Horse	7.0	9.8	43.8	60.6
Dog	5.9	10.6	45.8	62.7
Rat	12.1	67.0	110.0	190.1
Mouse	7.9	18.4	96.6	122.9
Hamster	15.2	50.5	126.3	186.8
Guinea pig	10.9	11.1	92.1	114.1
Honey possum	14.0	86.0	256.3	356.3
Man	4.5	4.0	48	56.5

Data from Cummins and Woodall (1985).

However, it is an over-simplification to consider ATP in isolation. ATP can only remain effective in transducing energy from metabolism to mechanical force if the reaction ATP↔ADP + inorganic phosphate (Pi) remains displaced from equilibrium. In the cytoplasm of a typical cell, the ATP/ADP ratio is about 10 000-fold greater than it would be at equilibrium (assuming a constant Pi of 10 mM) (Nicholls and Ferguson, 1992). In a diffusion model, in the more distal parts of the tail, the ATP concentration would be less and the concentration of ADP and Pi greater compared to the midpiece. Therefore, the ATPase reaction would come closer to equilibrium and the rate at which ATP could be hydrolysed to provide energy for sliding would be lower. It is unclear how great a problem this is; arguably less power is required for bending in the distal regions of the flagellum, because a lesser length needs to be deflected against viscous drag.

Enzymatic shuttles to facilitate ATP delivery

This problem of ATP delivery is not confined to spermatozoa, although it is particularly acute for them, and many cells notably muscle and nerve have to transfer ATP from its site of production in the mitochondrion to the site of energy dissipation and return the products through a highly structured cellular milieu. There are now very strong arguments that this is achieved by 'flux transfer chains'. These depend on the rapid transfer of the displacement from equilibrium of an enzyme reaction at one site to adjacent sites leading to the spatial transmission of a wave of disequilibrium. If product was added at one end and removed at the other then an efficient transport system would be established (Dzeja and Terzic, 2003). Creatine kinase, adenylate kinase and phosphoglycerate kinase shuttles have been proposed to transfer ATP away from the mitochondrion and to return ADP (Figure 1). Carbonic anhydrase and GAPDH shuttles (not shown Figure 1) are proposed to return hydrogen ions and Pi, respectively to the mitochondrion.

It is also possible that the ATP consuming steps of glycolysis could occur on the mitochondrial surface whilst the ATP releasing steps occurred close to the site of ATP consumption. In a similar way, the metabolism of glycerol 1-phosphate arising from phospholipid hydrolysis could provide substrate for the latter half of
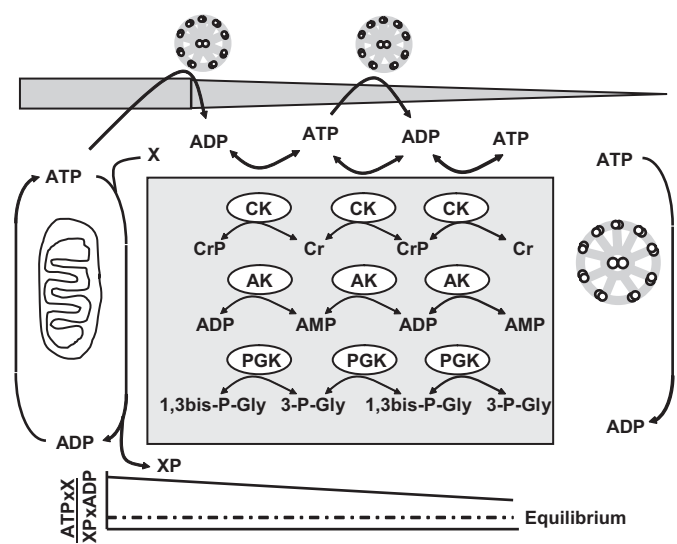
Figure 1. Potential ATP transfer shuttles in the sperm flagellum. Along the flagellum, ATP is hydrolysed to ADP to provide energy for active sliding of adjacent microtubules leading to propagation of flagellar bending. ADP is re-phosphorylated by phosphate transfer from creatine phosphate (CrP), ADP or 1,3 bisphosphoglycerate (1,3bis-P-Gly). These reactions are catalysed by creatine kinase, adenylate kinase or 3 phosphoglycerate (3-P-Gly) kinase, respectively. These enzymes operate close to equilibrium. In the midpiece, mitochondrial oxidative phosphorylation generates ATP that can support motility or the phosphorylation of creatine, AMP or 3-P-Gly together represented as X. The net effect is to establish a gradient of disequilibrium of the kinase reactions (XP + ADP↔ATP + X) along the flagellum so that their concerted action can sustain the ATP concentration in the distal regions of the flagellum without the need for diffusion over long distances. Based on Dzeja and Terzic (2003). Note that other shuttles may be required to transfer hydrogen ions and inorganic phosphate (Pi). The creatine phosphate (CrP) shuttle is important in invertebrate but not in mammalian sperm.

the glycolytic pathway. Mitochondrial glycerol-1-phosphate dehydrogenase could oxidize it to dihydroxyacetone 1-phosphate that could diffuse down the tail. Mitochondrial glycerol 1-phosphate dehydrogenase is present at high to moderate activity in ram, boar, bull, rat and mouse sperm but is present at much lesser activity in rabbit, human and monkey sperm (Storey and Kayne, 1980; Storey, 1980; Carey *et al.*, 1981; Ford, 1981). Glycerol-1-phosphate is an important substrate in the long-term survival of boar sperm in substrate-free media (Jones and Bubb, 2000).

The shuttle mechanisms adopted by sperm differ across the evolutionary spectrum. Invertebrate sperm usually contain relatively high amounts of creatine phosphate (CrP) and creatine phosphokinases or other phosphagens (Ellington and Kinsey, 1998; Ellington, 2001), these buffer the ATP/ADP ratio at the expense of CrP/creatine and provide a shuttle to facilitate diffusion of ATP along the flagellum (Figure 1). The CrP shuttle is particularly well characterized in sea urchin sperm (Tombes and Shapiro, 1985). By contrast, the CrP shuttle is likely to be absent or of only minor importance in mammalian sperm because they lack or contain only low concentrations of CrP or other phosphagens (Smith *et al.*, 1985; Robitaille *et al.*, 1987). Consistent with this view, mice in which the gene for the mitochondrial isotype of creatine kinase had been 'knocked out' were fertile, and their sperm had similar motility characteristics to the wild type (Steeghs *et al.*, 1995).

On the other hand, creatine phosphokinase activity has been detected in human sperm. This may be associated with cytoplasmic

retention during spermiation, because activity was higher in infertile men and was associated with abnormal head morphology and increased lipid peroxidation (Huszar and Vigue, 1993, 1994), The concentration of creatine phosphokinase (Huszar *et al.*, 1988) and the ratio between the CK-B and CK-M isoforms (Huszar *et al.*, 1992) have been proposed as clinical indices of male fertility, although the band identified as CK-M has turned out to be the heat shock protein HspA2 (Huszar *et al.*, 2000).

Evidence that creatine phosphokinase may have a role in energy provision in human sperm comes from observations that demembranated human sperm could be re-activated by CrP plus ADP and that in intact sperm the creatine phosphokinase inhibitor, dinitrofluorobenzene, impaired progressive motility when lactate was the only substrate provided (Yeung *et al.*, 1996). However, CrP activated motility less effectively than ATP, and formation of ATP + AMP from ADP via adenylate kinase activity could not be ruled out. Furthermore, interpretation of the effect on intact sperm depends critically on the assumption that the inhibitor acted specifically.

Other reports suggest that although human sperm contain CK-B and the mitochondrial isoform CK-Mi, their activities have little predictive value for *in vitro* fertilization (Rolf *et al.*, 1998), Moreover, creatine kinase activity was not related to the ATP/ADP ratio in human spermatozoa (Vigue *et al.*, 1992). We have to conclude that on balance the CrP shuttle is unlikely to be of major importance in mammalian sperm.

This might be construed as circumstantial evidence in favour of mammalian sperm requiring local ATP production by glycolysis, but mammalian sperm contain high activities of adenylate kinase which catalyses the reaction 2ADP↔ATP + AMP (Schoff *et al.*, 1989) and glycolytic enzymes including 3-phosphoglycerate kinase. Therefore, they could rely on adenylate kinase and 3-phosphoglcerate kinase shuttles for ATP transfer along the flagellum. Consistent with this, ADP could maintain the motility of permiabilized bull sperm (Schoff *et al.*, 1989).

On the other hand, mice in which the gene for adenylate kinase 1 had been deleted exhibited no overt physiological abnormalities (Janssen *et al.*, 2000) implying that they were normally fertile. However, it remains possible that adenylate kinases 2 and 3, normally restricted to the mitochondrion, have a different distribution in sperm or that sperm contain a novel isotype of adenylate kinase. Novel adenylate kinase isoforms characterized by an N terminal extension targeting them to the flagellum have been demonstrated in *Trypanosoma brucei*, and closely related gene sequences were detected in the human genome (Pullen *et al.*, 2004). A novel AK anchored to the outer dynein arm protein Oda5p has been reported in *Chlamydomonas* (Wirschell *et al.*, 2004).

Further research is required to confirm that these shuttles operate and that they are capable of sustaining flagellar activity driven by mitochondrial oxidative phosphorylation.

Evidence from the sperm-specific GAPDHs knockout mouse that glycolysis is required for mammalian sperm motility

Sperm from mice in which the gene for the testis-/sperm-specific isoenzyme of GAPDHs had been 'knocked out' were not progressively motile and contained a very low ATP concentration, although mitochondrial respiration, assessed by the rate of oxygen
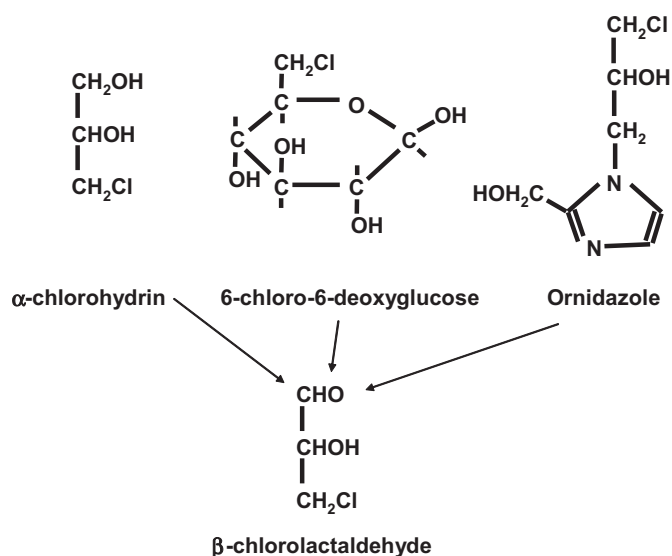

Figure 2. The chemical structure of α-chlorohydrin and related male contraceptive compounds and the putative common active intermediate β-chlorolactaldehyde.

uptake, was unimpaired. These results were interpreted to imply that the energy required for sperm motility is generated by glycolysis (Miki *et al.*, 2004). This conclusion fails to take account of experience with the putative male contraceptive α-chlorohydrin (1-chloropropan-2,3–diol; Figure 2). α-Chlorohydrin has a rapid and effective oral male contraceptive action in many species. It appears to act by inhibiting GAPDHs and to a lesser extent triose phosphate isomerase and exerts similar biochemical effects in sperm exposed to it *in vitro*. α-Chlorohydrin itself does not inhibit GAPDHs but has first to be metabolized to an active intermediate probably β-chlorolactaldehyde (Jones, 1983; Jones and Cooper, 1999). Other related compounds, 6-chloro-6-deoxysugars (Ford and Harrison, 1981; Ford *et al.*, 1981) and ornidazole (Bone *et al.*, 2000) have a similar contraceptive effect probably because they can generate the same active intermediate *in vivo* (Figure 2).

Contraceptive doses of α-chlorohydrin or 6-chloro-6–deoxyglucose (Brown-Woodman *et al.*, 1978; Ford *et al.*, 1981) did not decrease mitochondrial respiration, and sperm from rats made infertile with 6-chloro-6-deoxyglucose remained motile with a normal ATP concentration when incubated with pyruvate plus lactate (Ford and Harrison, 1981).

With this in mind, in the course of research into the mode of action of α-chlorohydrin, we investigated why sperm treated with α-chlorohydrin could not obtain energy to support motility and fertility from mitochondrial oxidative phosphorylation.

Ram epididymal or boar-ejaculated sperm were incubated in media containing no substrate, 5 mM D-glucose, 10 mM L-lactate plus 1 mM pyruvate or 5 mM-D-glucose plus 10 mM-L-lactate plus 1 mM pyruvate, each in the presence or absence of 1 mM RS-α-chlorohydrin. The principal conclusion was that over a 1 h incubation, α-chlorohydrin had no significant effect on motility or ATP concentration when glucose was absent, but when glucose was present both motility and ATP concentration decreased rapidly to very low values (Figure 3). The principal difference between ram and boar sperm was that in the latter lactate plus pyruvate provided
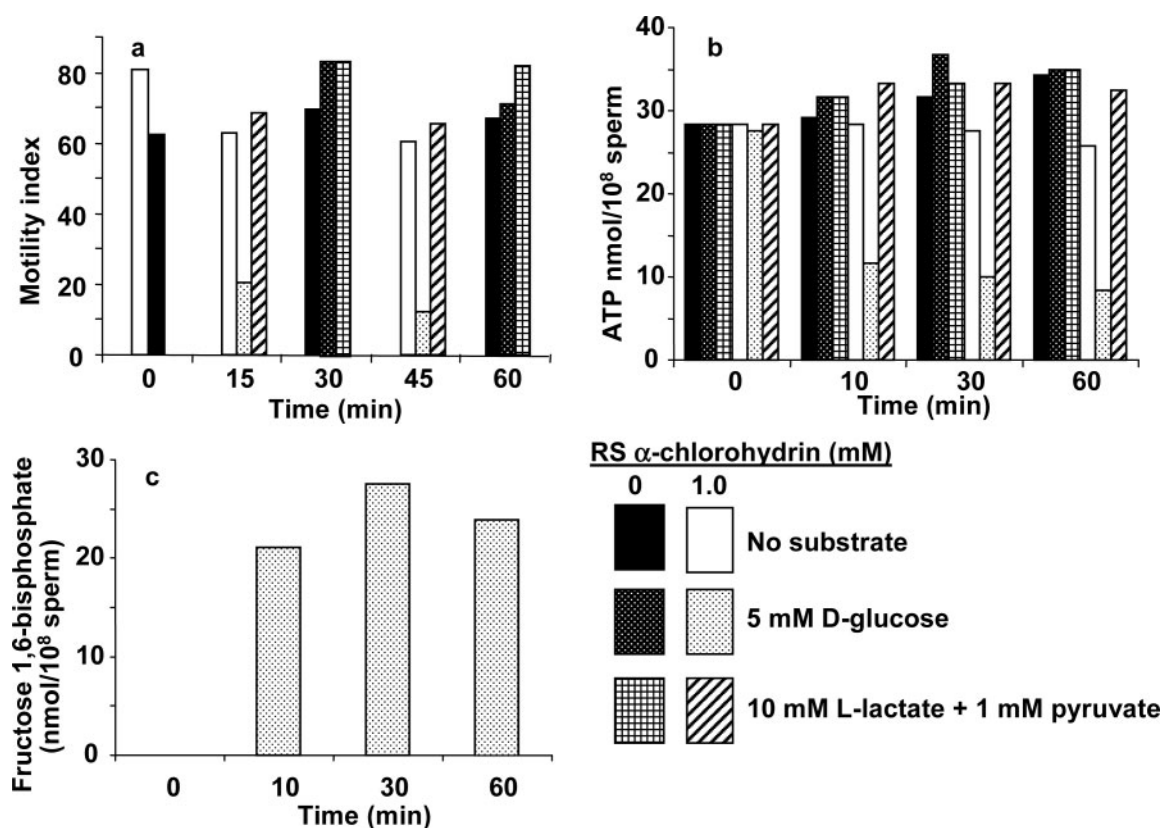
Figure 3. Effects of α-chlorohydrin and glucose on ram spermatozoa (**a**) motility, (**b**) ATP concentration (**c**) fructose 1,6-bisphosphate concentration. (**a** and **b**) Ram epididymal spermatozoa were incubated with 1 mM –RS-α-chlorohydrin or a buffer blank (control) for 10 min before the addition of 5 mM–D-glucose, 10 mM L-lactate plus 1 mM–pyruvate or a buffer blank (no substrate). Samples for motility measurements were taken from α-chlorohydrin flasks 0, 15 and 45 min, and from control flasks 0, 30 and 60 min after addition of substrates. Samples for ATP assay were taken from all flasks at 0, 10, 30 and 60 min. (**c**) Experimental conditions were the same except that 1 mM glucose was added. The concentration of fructose 1,6-bisphosphate remained <1 nmol/10^8 sperm except when both α-chlorohydrin and glucose were present. Replotted from previously published data (Ford and Harrison, 1985, 1986).

some protection against the concerted effect of α-chlorohydrin and glucose (Ford and Harrison, 1985). Similarly, in mouse sperm incubated with 1 or 10 mM α-chlorohydrin, motility was partially (1 mM) or completely (10 mM) inhibited if 10 mM glucose was present but was normal if glucose was replaced by 10 mM β-hydroxybutyrate (Tanaka *et al*., 2004). The decline in ATP and motility was always accompanied by a marked increase in the concentrations of glycolytic intermediates upstream of GAPDHs, notably fructose 1,6 bisphosphate and the triose phosphates (Figure 3c) (Ford and Harrison, 1986). Because of their neurotoxic side effects (Jacobs and Ford, 1981; Ford and Waites, 1982), interest in the α-chlorohydrin family of male contraceptives waned, and their mode of action was never fully resolved. However, we were able to establish that the increased concentrations of glycolytic intermediates did not increase futile substrate cycling sufficiently to explain the decline in ATP concentration, and the most likely explanation is that they sequestered so much of the phosphate present in the sperm that none remained available for oxidative phosphorylation (Ford and Harrison, 1987).

These results demonstrate that sperm in which GAPDHs has been inhibited by α-chlorohydrin can function well for at least 1 h when no external glucose is present and the metabolism of any internal carbohydrate store is blocked. This confirms that glycolysis is not required to support normal motility. Adverse effects on ATP

and motility ensue if sufficient glucose is present to cause the accumulation of glycolytic intermediates which impair oxidative phosphorylation in some way. Consequently, the absence of motility in sperm from GAPDHs knockout mice cannot be taken as proof that glycolysis is essential to maintain motility, because the sperm were incubated in medium that contained glucose and they accumulated 4× normal concentrations of glyceraldehyde 3-phosphate (Miki *et al*., 2004).

If glycolysis is required for energy transfer, how can sperm be motile in glucose-free media?

Sperm from many species including human can remain motile in glucose-free media. Bull sperm provide a striking example, because glucose impedes capacitation in this species. The sperm remain motile in sugar-free media *in vitro* and bovine oviductal fluid contains only 50–100 μM glucose yet supports capacitation and fertility (Galantino-Homer *et al*., 2004).

If glycolysis is required to deliver ATP to the distal regions of the epididymis, where does the substrate come from? A number of recent papers support the presence of glycogen stores in mammalian sperm and even suggest that they might be capable of gluconeogenesis.

The traditional view is that glycogen is lost during the spermatocyte stage of spermatogenesis and that mature mammalian sperm do

not contain glycogen (Mann, 1964). Based on respiratory quotients and other evidence, their primary endogenous substrate is thought to be phospholipid (Ford and Rees, 1990). However, recent evidence demonstrates that dog, ram, boar and horse sperm do contain glycogen together with measurable activities of glycogen synthetase and glycogen phosphorylase. In dog sperm, glycogen was depleted in sperm incubated in substrate-free medium but accumulated in media containing millimolar concentrations of glucose or fructose with the site of deposition in the cell varying according to the hexose provided (Ballester *et al.*, 2000; Palomo *et al.*, 2003).

Subsequently, it was reported that dog sperm incubated in medium containing 21.5 mM lactate + 0.25 mM pyruvate but no hexose accumulated glycogen in the first 2 h of the incubation, and radioactivity from [U-^{14}C] lactate was incorporated into it. The pyruvate carboxylase inhibitor phenylacetic acid prevented glycogen synthesis and inhibited motility without a significant effect on viability. Immunocytochemical evidence indicated presence of the key gluconeogenic enzymes fructose-1,6 bisphosphatase and aldolase B (Albarracin *et al.*, 2004). Therefore, there is strong if not conclusive evidence that dog sperm are capable of gluconeogenesis, and this may be important to maintain motility and to allow them to capacitate in glucose-free media.

Evidence for gluconeogenesis in other species is less convincing. 2-Deoxyglucose, which can be phosphorylated by hexokinase but not further metabolized was used to block glycolysis in mouse sperm (Mukai and Okuno, 2004). Although only a weak competitor with glucose, it decreased the motility of sperm incubated with pyruvate (but not glucose), although it had no effect on mitochondrial membrane potential. On this basis, the authors postulated that mitochondrial energy was used to drive gluconeogenesis and so provide glucose for glycolytic energy production in the flagellum. This interpretation is open to challenge, because metabolism of 2-deoxyglucose leads to the accumulation of high concentrations of 2-deoxyglucose 6-phosphate. As described above, for the increase in glycolytic intermediates induced by α-chlorohydrin, this could bind most of the phosphate in the sperm making it unavailable for oxidative phosphorylation. By preventing the discharge of the proton motive force, this situation would increase rather than decreasing mitochondrial membrane potential. The toxicity of 2-deoxglucose is illustrated by its detrimental effect on the ATP/ADP ratio of human sperm metabolizing lactate plus pyruvate (Williams and Ford, 2001). Secondly, no evidence was presented that gluconeogenesis could be detected. Analysis of the metabolome of boar sperm suggested that gluconeogenesis did not occur in that species (Marin *et al.*, 2003).

Conclusions

Local glycolysis can deliver energy to the distal flagellum but evidence that it is required to achieve this is weak. Firstly, it is likely that diffusion reinforced by adenylate kinase and other shuttles is sufficient to exchange ATP, ADP and Pi between the flagellum and the mitochondria in the midpiece at the rate required to sustain motility. Secondly, experience with the GAPDHs inhibitor α-chlorohydrin indicates that sperm can maintain motility for long periods when glycolysis is blocked, so long as high concentrations of glycolytic intermediates do not accumulate. This needs to be taken into account in the interpretation of results from GAPDHs knock-out mice. Thirdly, sperm of most species can remain fully motile in sugar-free media especially if mitochondrial substrates are provided

and with the exception of the dog evidence for gluconeogenesis to provide substrate for the distal flagellum is lacking or weak.

To understand ATP delivery to the mammalian sperm flagellum better, further research is needed to confirm the role of adenylate kinase and other shuttles in energy distribution along the mammalian sperm flagellum and to reveal the reasons why sperm from GAPDHs knock-out mice are immotile. Are demembranated or permiabilized sperm from these mice motile if supplied with ATP, and are intact sperm motile if protected from contact with glycolysable sugars as experience with α-chlorohydrin suggests?

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