Exogenous pyruvate accelerates glycolysis and promotes capacitation in human spermatozoa

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BACKGROUND: There has been an ongoing debate in the reproductive field about whether mammalian spermatozoa rely on glycolysis, oxidative phosphorylation or both for their energy production. Recent studies have proposed that human spermatozoa depend mainly on glucose for motility and fertilization but the mechanism behind an efficient glycolysis in human spermatozoa is not well understood. Here, we demonstrate how human spermatozoa utilize exogenous pyruvate to enhance glycolytic ATP production, motility, hyperactivation and capacitation, events that are crucial for male fertility.

METHODS: Purified human spermatozoa from healthy donors were incubated under capacitating conditions (including albumin, bicarbonate and glucose) and tested for changes in ATP levels, motility, hyperactivation and tyrosine phosphorylation after treatment with pyruvate. The experiments were repeated in the presence of sodium cyanide in order to assess the contribution from mitochondrial respiration. The metabolism of 13C labeled glucose and pyruvate was traced by a combination of liquid chromatography and mass spectrometry.

RESULTS: The treatment of human spermatozoa with exogenous pyruvate increased intracellular ATP levels, progressive motility and hyperactivation by 56, 21 and 130%, respectively. In addition, added pyruvate induced a significant increase in tyrosine phosphorylation levels. Blocking of the electron transport chain did not markedly affect the results, indicating that the mechanism is independent of oxidative phosphorylation. However, the observed effects could be counteracted by oxamate, an inhibitor of lactate dehydrogenase (LDH). Metabolic tracing experiments revealed that the observed rise in ATP concentration resulted from an enhanced glycolytic flux, which was increased by more than 50% in the presence of exogenous pyruvate. Moreover, all consumed 13C labeled pyruvate added was converted to lactate rather than oxidized in the tricarboxylic acid cycle.

CONCLUSIONS: Human spermatozoa seem to rely mainly, if not entirely, on glycolysis as the source of ATP fueling the energy-demanding processes of motility and capacitation. The efficient glycolysis is dependent on exogenous pyruvate, which indirectly feeds the accelerated glycolysis with NAD⁺ through the LDH-mediated conversion of pyruvate to lactate. Pyruvate is present in the human female reproductive tract at concentrations in accordance with our results. As seen in other mammals, the motility and fertility of human spermatozoa seem to be dictated by the available energy substrates present in the conspecific female.

Key words: capacitation / glycolysis / human spermatozoa / pyruvate / sperm metabolism

Introduction

Mammalian spermatozoa are dependent on an efficient generation of ATP to fuel the progressive and hyperactive motility crucial for capacitation and fertilization (Ho et al., 2002). While progressive motility is essential for spermatozoa to travel through the cervix to the oviduct, the vigorous and asymmetrical swimming pattern of hyperactivation aids the release of spermatozoa from the oviductal epithelium (Demott and Suarez, 1992) and subsequent penetration of the oocyte cell membrane (Stauss et al., 1995). Hyperactive motility and tyrosine phosphorylation represent two hallmarks of capacitation, a process required to prepare spermatozoa for fertilization.
The main question has been how spermatozoa provide the vast amount of ATP needed for these energy-demanding processes. It is known that mammalian spermatozoa solve their energy requirements by switching between different metabolic pathways depending on oxygen availability and the composition of metabolic substrates in their environment. Glucose, pyruvate and lactate are present at high concentrations in oviductal fluid (Ruiz-Pesini et al., 2007) and hence they are commonly utilized as energy substrates by mammalian spermatozoa (Hoshi et al., 1991; Williams and Ford, 2001; Mukai and Okuno, 2004). In addition, other carbon sources such as sorbitol (Cao et al., 2009), glycerol (Jones et al., 1992) and fructose (Murdoch and White, 1968; Rigau et al., 2001) can be utilized. The use of metabolic substrates for ATP production has been shown further to vary between species (Storey, 2008). Bull spermatozoa rely primarily on oxidative phosphorylation to support capacitation from oxidizable substrates (Hutson et al., 1977; Van et al., 1977), whereas human spermatozoa rely substantially on glucose-derived ATP under capacitation to support hyperactivation (Hoshi et al., 1991; Williams and Ford, 2001), tyrosine phosphorylation (Travis et al., 2001) and fertilization in vitro (Mahadevan et al., 1997). Despite these findings, it has been unclear and debated in the field whether glycolysis or oxidative phosphorylation is the major contributor of the ATP needed for fertilization in humans (Ford, 2006; Ruiz-Pesini et al., 2007). In spermatozoa, these two metabolic pathways are sub-cellularly separated. The mitochondria are concentrated exclusively in the midpiece, whereas the glycolytic enzymes are tightly anchored to the fibrous sheath (FS) in the flagellum. As mitochondria are rather large organelles, this particular organization may have evolved to allow for the propeller motion of the tail. Because of the long distance between the mitochondria and the distal end of the speed-generating flagellum (35–256 μm) (Cummins and Woodall, 1985), it has been questioned if ATP diffusion- and carrier systems are able to supply the tail with mitochondrial ATP supporting motility and hyperactivation (Nevo and Rikmenspoel, 1970; Adam and Wei, 1975; Turner, 2003; Kim et al., 2007). In addition, the ability of glycolysis to provide sufficient ATP for sperm motility and capacitation is disputed, since one molecule of glucose yields only two molecules of ATP if incompletely metabolized by glycolysis and lactic acid fermentation. By contrast, complete aerobic combustion of one molecule of glucose theoretically generates more than 30 molecules of ATP.

In order to rely on glycolysis-derived ATP for motility and capacitation, spermatozoa are dependent on the sperm-specific variant of lactate dehydrogenase (LDH-C) (Blanco and Zinkham, 1963; Goldberg, 1963). LDH-C accounts for 80–100% of the LDH activity in human spermatozoa (Zinkham et al., 1964; Clausen and Ovlesen, 1965), catalyzing the reversible conversion of pyruvate to lactate with the concomitant conversion of nicotinamide adenine dinucleotide (NADH) to NAD⁺ during lactic acid fermentation. LDH-C has been shown to be essential for sperm motility (Rodriguez-Paez et al., 2002) and spermatozoa from mice carrying a targeted disruption of the LDH-C gene did not show tyrosine phosphorylation and hyperactive motility, resulting in impaired fertility (Odet et al., 2008).

In the present study, we investigated the effect of exogenous pyruvate on sperm ATP generation, motility and capacitation. The molecular mechanism was elucidated by metabolic tracing and liquid chromatography and mass spectrometry (LC-MS) experiments.

Materials and Methods

Media and reagents

Experiments were performed in sperm cell medium containing 138 mM NaCl, 5.3 mM KCl, 1.3 mM CaCl₂, 0.5 mM MgCl₂, 0.4 mM MgSO₄, 0.4 mM KH₂PO₄, 0.3 mM Na₂HPO₄, and 4.2 mM NaHCO₃, pH 7.4. For establishing capacitating conditions, spermatozoa were incubated in sperm cell medium supplemented with 1% human serum albumin (HSA) (Octapharma, Lachen, Switzerland) and 22 mM NaHCO₃ (Sigma-Aldrich, St. Louis, USA) in a 5% CO₂ atmosphere at 37°C (Burkman, 1991; Sukcharoen et al., 1995). Lysis buffer contained 1% sodium dodecyl sulfate (SDS) in phosphate-buffered saline (Sigma-Aldrich). Sodium cyanide (NaCN) was dissolved in distilled water (dH₂O) to 1 M stocks, rotenone was dissolved in dimethylsulphoxide to 25 mM stocks, antimycin A was dissolved in ethanol to 1 mM stocks and aliquots were stored at −20°C (all from Sigma-Aldrich). Sodium oxamate (Sigma-Aldrich) was diluted in dH₂O to 430 mM and stored at −20°C in aliquots. D-glucose, L-lactate and pyruvate (all obtained from Sigma-Aldrich) were stored as 1 M stocks at 4°C. 13C₆ glucose and 13C₁ pyruvate (both from Larodan, Malmö, Sweden) were diluted in dH₂O to 1 M and stored at 4°C. Methylene blue solution was obtained from Sigma-Aldrich.

Sperm samples and preparation

Human spermatozoa were obtained from freshly ejaculated semen from healthy donors after 3 days of abstinence. Normal morphology and motility (WHO standards) was assessed in a computer-assisted sperm analyzer (CASA) (HTM-IVOS system, version 12, Hamilton-Thorne Research, Beverly, USA). Semen samples were liquefied at 37°C in a shaker for 30 min. Spermatozoa were purified by discontinuous percoll density centrifugation. One part 1.54 M NaCl and nine parts percoll (Sigma-Aldrich) were mixed to make a 90% percoll solution. This solution was diluted again in Hanks Balanced Salt Solution (HBSS) (catalogue/product number 24020 Invitrogen) to make 80 and 40% percoll working solutions. The semen samples were added on top of two cushions of 80 and 40% percoll and centrifuged at 1250g for 20 min. Motile cells were collected from the lower 80% percoll layer. Spermatozoa were then washed once in HBSS and kept in sperm cell medium supplemented with 5 mM glucose at room temperature. Immediately before the experiments, cells were washed twice in glucose-free sperm cell medium.

ATP measurements

Endogenous ATP concentrations were measured in a luciferase-based kit (ATP-lite) from Perkin Elmer (Boston, USA). Human spermatozoa were diluted to 2 × 10⁶/ml in sperm cell medium and incubated under capacitation conditions in a 96-well white microtiter plate (Nunc, Roskilde, Denmark). Luminescence was measured by a Gemini EM microplate spectrophuorometer (Molecular Devices, Sunnyvale, USA) and mol ATP was determined according to a standard curve. The effect of pyruvate and oxamate on endogenous ATP concentrations was studied by incubation of spermatozoa in the presence of 28 nM–5 mM and 3.6 μM–15 mM pyruvate and oxamate, respectively for 30 and/or 120 min. In the mitochondrial respiration inhibition experiments, purified spermatozoa were incubated for 120 min with increasing concentrations of NaCN (24 μM–25 mM), rotenone (128 pM–50 μM) and antimycin A (5 nM–2 μM) in the absence or presence of different metabolic substrates as described in the figure legends. The effect of methylene blue on ATP levels was investigated by incubation of spermatozoa with 10 mM NaCN, 10 μM rotenone or 1 μM antimycin A in the presence of 5 mM glucose and either 5 mM pyruvate or 50 μM methylene blue for
120 min. The concentration of methylene blue used was determined by a dose–response experiment.

**Motility experiments**

A HTM-IVOS system (Hamilton-Thorne Research) was used for motility analysis with the following settings: Slow spermatozoa were counted as static. Progressive cells were defined as average path velocity > 25 μm/s and straightness > 80%. Number of frames: 30, frame rate: 30 Hz. Parameters measured included curvilinear velocity (VCL, μm/s) which is defined as the time-average velocity of a sperm head along its actual curvilinear trajectory and amplitude of lateral head displacement (ALH, μm) which describes the magnitude of lateral displacement of a sperm head about its spatial average trajectory. Hyperactive spermatozoa were defined by Burkman (1991) and set to linearity (LIN, %) < 65, ALH (μm) > 7.5 and VCL (μm/s) > 100. All motility experiments were performed under capacitating conditions. Cells were diluted to 2 × 10^7/ml and incubated in 48 wells cell culture plates (Corning, Schiphol-Rijk, The Netherlands) or eppendorf tubes. An aliquot of 5 μl of sperm solution was added to a 20 μl, two chambers slide (Leja, Nieuw-Vennep, The Netherlands) and a total of 20 fields were counted per sample. The effect of pyruvate and oxamate on sperm motility was studied by incubation of spermatozoa in the presence of 64 mM–5 mM and 39 μM–40 mM pyruvate and oxamate, respectively for 30 and/or 120 min. To study the effect of an inhibited mitochondrial respiration on sperm motility, spermatozoa were treated with 10 mM NaCN in the presence or absence of 5 mM glucose, pyruvate or a combination of the metabolites and incubated for 120 min. Regeneration of motility by methylene blue was investigated by incubation of spermatozoa with 10 mM NaCN, 10 μM rotenone or 1 μM antimycin A in the presence of 5 mM glucose and either 5 mM pyruvate or 50 μM methylene blue for 120 min. To confirm the viability of spermatozoa treated with NaCN, cells were incubated with 5 mM glucose and 10 mM NaCN under capacitating conditions for 120 min and motility was measured by CASA. Five mM of pyruvate were then added and reactivation was tested in CASA at time 180 min.

**Immunoblotting**

Purified spermatozoa were diluted in sperm cell medium to 5 × 10^6/ml and incubated in 48 well plates. The non-capacitated control sample was diluted in sperm cell medium and placed on ice directly after purification. The rest of the samples were treated with or without 10 mM NaCN, 5 mM glucose and 5 mM pyruvate under capacitating conditions for 30 and 120 min and put straight on ice after incubation. The samples were centrifuged at 4 °C, the supernatant was removed and the pellet resuspended in lysis buffer with 4% SDS–PAGE gel (105 cells/lane) and the proteins were transferred heavily vortexed for 30 s. The samples were loaded on a 10% tris–glycine SDS–PAGE gel (10^5 cells/lane) and the proteins were transferred onto a poly(vinylidene) difluoride membrane (Millipore, Billerica, USA). The membrane was blocked with anti-phosphotyrosine (pY) antibodies (catalogue/product number 9411, Cell Signaling Technologies, Danvers, USA) in tris-buffered saline (TBS) supplied with 0.1% tween 20 (Sigma-Aldrich) and 4% skimmed milk. As loading control, antibodies against α-tubulin (catalogue/product number T9026, Sigma-Aldrich) were used. All blots were treated with anti-mouse-horse-radish peroxidase as the secondary antibody (catalogue/product number 55563, MP Biomedicals, Aurora, USA) and developed by the Supersignal West Dura Extended Duration Substrate from Pierce (Rockford, USA).

**Cellular respiration assay**

Oxygen consumption of capacitating spermatozoa at 37 °C was determined by high-resolution respirometry (Oroboros Oxgraph-2K, Innsbruck, Austria). The integrated software (DATLAB 4.2) presents respiration as oxygen flux; pmol O_2/10^6 cells/s. The stirrer speed was set to 750 r.p.m. Spermatozoa (1.5 × 10^7/ml) were incubated under capacitating conditions for 120 min and transferred to the chambers maintained at 37 °C. Basal respiration was measured for 15 min followed by stepwise mitochondrial uncoupling by carbonyl cyanide-P-trifluoromethoxy-phenylhydrazide (FCCP) at step titration of 0.5 μM until maximal respiration was obtained. This respiration rate corresponds to respiration capacity of the spermatozoa under the given condition. NaCN was subsequently added at 1 mM aliquots up to 10 mM. Respiration rates for the individual batches of spermatozoa were presented relative to respiration capacity of spermatozoa supplemented with glucose and pyruvate in combination.

**Metabolic tracing**

2 × 10^7/ml purified spermatozoa (100 μl) were incubated in eppendorf tubes for 0 and 120 min under capacitating conditions in the presence of 1 mM 13C6 glucose and a combination of 1 mM 13C6 glucose and 1 mM 13C2 pyruvate. The experiment was performed both in the absence and in the presence of 10 mM NaCN. The samples were deproteinized with 300 μl methanol immediately after incubation, centrifuged at 16 000g and supernatant was collected for LC-MS. The quantitative LC-MS measurements of metabolites in the metabolic tracing experiments were performed on a Perkin Elmer series 200 HPLC system interfaced with an API 2000TM triple quadrupole mass spectrometer (Applied Biosystems/MD Sciex, Canada) equipped with a TurboIonSprayTM electrospray. Chromatographic separation of the analytes was achieved by isocratic elution on a 2.1 × 150 mm SunfireTM 5 μm particles C18 column (Waters, Milford, USA) operated at ambient temperature. The mobile phase (50% methanol in water) was delivered at 150 μL/min and 30 μL samples were injected. All samples were run in duplicate. For calculation of metabolite concentrations, calibration curves were created and half-maximal effective concentration (EC50) values calculated from the titration experiments by pyruvate and oxamate. Potencies were defined as percentage maximal increase/decrease compared with untreated controls.

**Statistics and presentation**

All experiments were repeated between three and five times (see figure legends) with new donor materials. Data sets are presented as mean ± SEM and P-values were calculated from a Student’s paired t-test. Dose–response curves were created and half-maximal effective concentration (EC50) values calculated from the titration experiments by pyruvate and oxamate. Potencies were defined as percentage maximal increase/decrease compared with untreated controls.

**Results**

Exogenous pyruvate enhances motility and hyperactivation of human spermatozoa

Previous studies on sperm pyruvate metabolism have focused on the role of pyruvate as an oxidizable substrate when present as sole donor material and in combination with other substrates. In the present study, we focused on the role of pyruvate in the capacitation process of human spermatozoa. We found that exogenous pyruvate enhances motility and hyperactivation of human spermatozoa.
metabolite. Our goal was to investigate the effect of pyruvate on motility and hyperactivation of human spermatozoa in combination with glucose. Purified spermatozoa were incubated under capacitating conditions (see Materials and Methods) in the presence of 5 mM glucose and incremental concentrations (64 nM–5 mM) of pyruvate (●). Effects of exogenous pyruvate on sperm motility were determined by CASA as (A) motility (%), (B) progressive motility (%), (C) VCL (μm/s) and (D) hyperactive motility (%). All data sets are presented as mean ± SEM from four separate experiments (n = 8).

Table I Effects of exogenous pyruvate on motility and intracellular ATP concentration in human spermatozoa incubated under capacitating conditions.

| Sperm parameter       | EC50 (μM)a 30 min | Maximal increase (%)b 30 min | EC50 (μM)a 120 min | Maximal increase (%)b 120 min |
|-----------------------|-------------------|-------------------------------|--------------------|-------------------------------|
| Motility              | 37 ± 3            | 9 ± 2                         | ND                 | 0                             |
| Progressive motility  | 18 ± 5            | 21 ± 1                        | 20 ± 6             | 15 ± 3                        |
| VCL                   | 43 ± 14           | 36 ± 7                        | 32 ± 5             | 26 ± 4                        |
| ALH                   | 64 ± 13           | 19 ± 3                        | 47 ± 4             | 13 ± 2                        |
| Hyperactive motility  | 58 ± 8            | 130 ± 26                      | 41 ± 1             | 66 ± 22                       |
| ATP                   | 34 ± 15           | 56 ± 14                       | 20 ± 10            | 27 ± 3                        |

Numbers are given as mean values ± SEM of four separate experiments. ND, not detectable.

aEC50 values (μM).
bMaximal increase (%) of sperm motility and ATP production by a titration of pyruvate after 30 and 120 min incubation.

Hyperactivation was defined according to criteria given by Burkman (1991). Addition of pyruvate enhanced all categories of sperm motility measured in a dose-dependent manner after 30 min (Fig. 1A–D). Maximal increase (%) and EC50 values (μM) induced by added pyruvate were calculated for motility, progressive motility, VCL, ALH and hyperactive motility and presented in Table I. Our findings...
demonstrate that exogenous pyruvate is a potent activator of sperm motility and hyperactivation.

**Exogenous pyruvate increases intracellular ATP- and tyrosine phosphorylation levels in human spermatozoa**

We next speculated that the observed increase in sperm motility and hyperactivation by exogenous pyruvate was correlated to endogenous ATP levels. A luminescence-based assay was used to measure endogenous ATP. Spermatozoa were incubated under capacitating conditions in the presence of increasing concentrations of pyruvate and 5 mM glucose. A dose-dependent rise in ATP concentrations by pyruvate was observed both after 30 and 120 min (Fig. 2A and B), with an average maximal increase of 56 ± 14 and 27 ± 3%, respectively (Table I). Successful capacitation was confirmed by immunoblotting using pY antibodies. Total tyrosine phosphorylation increased markedly under capacitating conditions (Fig. 2C, lanes 2 and 4) in comparison with spermatozoa incubated under non-capacitating conditions (lane 1). The presence of 5 mM pyruvate markedly increased tyrosine phosphorylation levels after both 30 and 120 min incubations (Fig. 2C, lanes 3 and 5). These results demonstrate that pyruvate-stimulated motility and hyperactivation correlate with increased tyrosine phosphorylation and endogenous ATP levels.

**A combination of pyruvate and glucose maintains ATP production in human spermatozoa blocked for mitochondrial respiration**

We next questioned whether the observed pyruvate-induced increase in ATP content was dependent on glycolysis alone or if oxidative phosphorylation was required. Human spermatozoa were treated with increasing concentrations of NaCN, an inhibitor of cytochrome c.
oxidase and the electron transport chain (ETC) in mitochondria (Way, 1984), in conditions promoting capacitation for 120 min. In the absence of metabolic substrates, the endogenous ATP concentrations were reduced by NaCN in a dose-dependent manner with \( \approx 100\% \) reduction at 1 mM NaCN (Fig. 3A). The decreased ATP levels by NaCN could not be restored when 5 mM of glucose, lactate or pyruvate was added separately (Fig. 3B–D). However, a combination of glucose and pyruvate restored intracellular ATP levels to a similar level to that seen in non-inhibited control cells (Fig. 3F). A combination of glucose and lactate could not reestablish the ATP levels under the same conditions (Fig. 3E). The experiment was repeated with two other inhibitors of mitochondrial respiration, rotenone and antimycin A, with similar results (Supplementary data, Fig. S1). These findings indicate that exogenous pyruvate enhances ATP production independently of mitochondrial respiration and that the mechanism is dependent on glucose.

A combination of glucose and pyruvate generates ATP sufficient for sperm motility, hyperactivation and tyrosine phosphorylation in the presence of high doses of NaCN

Sperm motility, hyperactivation and tyrosine phosphorylation are energy-demanding processes. Hence, we wanted to elucidate whether the ATP regenerated by glucose and pyruvate in the presence of NaCN could support these processes. Purified human spermatozoa were incubated under capacitating conditions in the presence of high doses of NaCN (10 mM, giving maximal reduction of ATP in Fig. 3A–D) together with 5 mM glucose and 5 mM pyruvate alone or in combination. Spermatozoa treated with NaCN without any metabolic substrate showed a complete depletion of endogenous ATP (Fig. 4A). Incubation with either glucose or pyruvate could not restore ATP to control levels. In contrast, spermatozoa treated a combination of glucose and pyruvate reestablished their ATP levels in the presence of high doses of NaCN blocking mitochondrial respiration.

We further questioned whether these ATP levels were sufficient to support normal and hyperactive motility. Spermatozoa treated with 10 mM NaCN in the absence of metabolic substrates showed a decrease in all parameters of motility (Fig. 4B–E), with no progressive or hyperactive motility detected. The addition of either 5 mM glucose or 5 mM pyruvate to spermatozoa treated with NaCN did not recover the motility observed for the non-inhibited control cells (Fig. 4B–E). Importantly, these cells were not dead, as demonstrated by a reactivation experiment (Supplementary data, Fig. S3). NaCN-treated spermatozoa incubated in the presence of glucose and pyruvate in combination completely restored all categories of sperm movement measured and analyzed. Moreover, these spermatozoa were fully motile and performed the energetic swimming behavior of hyperactivation (Fig. 4E). (Videos and images with sperm tracks from CASA of human spermatozoa incubated under all the conditions presented in Fig. 4 are shown in the supplementary data, Fig. S2 and supplementary data, Video). The ability of glucose and pyruvate to stimulate tyrosine phosphorylation and thereby capacitation, in the presence of NaCN, was also investigated. With no mitochondrial inhibition, glucose and pyruvate supported tyrosine phosphorylation equally, whereas the addition of substrates in tandem led to improved phosphorylation levels (Fig. 4F). An inhibition of mitochondrial respiration by NaCN...
in the absence of metabolic substrates resulted in a lack of tyrosine phosphorylation. The presence of glucose or pyruvate alone could not restore the tyrosine phosphorylation levels. However, the addition of a combination of glucose and pyruvate resulted in tyrosine phosphorylation levels comparable to the non-inhibited control. Based on these results, we hypothesized the existence of an efficient mechanism of ATP generation in human spermatozoa, induced by glucose and pyruvate in concert, that fully supports motility and capacitation and that may well operate independently of oxidative phosphorylation.

Figure 4 A combination of glucose and pyruvate generates ATP concentrations sufficient to support motility, hyperactivation and tyrosine phosphorylation when mitochondrial respiration is blocked by NaCN. Purified human spermatozoa were incubated under capacitating conditions for 120 min in the absence (white bars) or presence (black bars) of 10 mM NaCN and 5 mM of the metabolic substrates glucose, pyruvate or a combination of glucose and pyruvate. (A) ATP concentrations measured by luminescence. (B–E) Sperm motility evaluated by CASA. (B) Motility (%), (C) progressive motility (%), (D) VCL (μm/s) and (E) hyperactive motility (%). Experiment A is presented as mean ± SEM from three separate experiments (n = 9). Experiment B–E are presented as mean ± SEM from five separate experiments (n = 10). (F) Total tyrosine phosphorylation levels detected by immunoblotting with pY antibodies, anti-α-tubulin antibodies were used as loading control. *P < 0.05, **P < 0.01 and ***P < 0.001, n.s., not significant.
Exogenous pyruvate increases the glycolytic flux in capacitated human spermatozoa

Our initial observations demonstrated that pyruvate stimulates ATP production in human spermatozoa even in the presence of inhibitors of mitochondrial respiration. Glucose metabolism has been shown to be essential for sperm capacitation and we speculated that pyruvate indirectly affected glycolytic ATP production. The total production of $^{13}$C$_3$ pyruvate and $^{13}$C$_3$ lactate derived from $^{13}$C$_6$ labeled glucose was used as a measure of glycolytic flux. This was based on the assumption that glycolysis in spermatozoa is highly effective, creating negligible levels of glycolytic intermediates. The metabolism of substrates was traced by a combination of chromatography, separating lactate and pyruvate in time (Supplementary data, Fig. S4), and MS, separating the different isotopes of the metabolites by mass. We found that when capacitated human spermatozoa were treated with 1 mM $^{13}$C$_1$ pyruvate in addition to 1 mM $^{13}$C$_6$ glucose for 120 min, the glycolytic flux increased by more than 50% (Fig. 5A). Based on these findings and the fact that every pyruvate or lactate produced from glucose results in the synthesis of one ATP molecule, we speculated that the observed increase in ATP levels by exogenous pyruvate in our initial experiments was derived from glycolysis and not from oxidative phosphorylation. This hypothesis was supported by a second experiment where mitochondrial respiration was blocked by the treatment with 10 mM NaCN. Here, spermatozoa continued to produce glycolytic ATP in the presence of exogenous pyruvate (Fig. 5B). Interestingly, spermatozoa incubated with glucose as only metabolic substrate could not maintain glycolysis in the presence of NaCN. This finding indicates that mitochondria and oxidative phosphorylation have an important function in sperm energy production other than synthesis of ATP. Moreover, it seems that the mitochondrial function can be replaced by exogenous pyruvate. Spermatozoa are thus unique compared with healthy somatic cells in that they produce ATP mainly by glycolysis even in the presence of oxygen.

Human spermatozoa metabolize exogenous pyruvate into lactate

The LC-MS experiments demonstrated that the observed increase in sperm ATP concentrations, motility, hyperactivation and tyrosine phosphorylation levels by exogenous pyruvate may have resulted from an accelerated glycolytic flux. To investigate the molecular mechanism behind this observation, we repeated the experiment and traced the metabolism of $^{13}$C$_1$ labeled pyruvate. LDH is known to produce lactate from pyruvate under anaerobic conditions, but we hypothesized that this may be true for spermatozoa also under sufficient levels of oxygen. The conversion of exogenous $^{13}$C$_1$ pyruvate to $^{13}$C$_1$ lactate in capacitated spermatozoa after 120 min incubation was measured by LC-MS and is presented in Table II. This experiment showed that the added $^{13}$C$_1$ pyruvate was rapidly consumed by the sperm cells (≈0.39 mM = 1.95 nmol/10$^6$ spermatozoa after 120 min). Strikingly, all $^{13}$C$_1$ pyruvate consumed were converted to $^{13}$C$_1$ lactate. This shows that pyruvate does not enter the tricarboxylic acid cycle even at excess concentrations and under normal oxygen.

### Table II

| Measured metabolite | Start concentration (mM, t = 0) | End concentration (mM, t = 120 min) | Consumption (mM) | Production (mM) |
|---------------------|--------------------------------|------------------------------------|-----------------|-----------------|
| $^{13}$C$_1$ pyruvate | 0.91 ± 0.05                  | 0.52 ± 0.01                        | 0.39 ± 0.05     | 0.37 ± 0.04     |
| $^{13}$C$_1$ lactate | 0                              | 0.37 ± 0.04                        |                 |                 |

Concentration values (mM) represent total amount of metabolite in the sample (extra- and intracellular) and are presented as mean ± SEM from five different experiments ($n = 10$).
conditions. Although the conversion of pyruvate to lactate by LDH does not generate ATP, the reaction depends on NADH as a cofactor that is concomitantly oxidized to NAD$^+$ and necessary for a continued glycolysis. The concentration of NAD$^+$ is rate-limiting in glycolysis and an accessible pool derived from exogenous pyruvate may allow for an accelerated glycolytic flux. The established method using labeled metabolites demonstrates that exogenous pyruvate enhances glycolytic ATP production and makes spermatozoa independent of mitochondria.

**NaCN inactivates ETC-dependent oxygen consumption in human spermatozoa**

Pyruvate can be oxidized in the process of mitochondrial respiration generating ATP. Thus, we next investigated whether the increase in endogenous ATP induced by the combination of glucose and pyruvate originated from insufficient inhibition of respiration by NaCN. The mitochondrial activities in capacitated spermatozoa were first assessed by monitoring the basal respiration rates during glucose and glucose/pyruvate administration. Then, the mitochondrial uncoupler FCCP was added to obtain total respiration capacity. Average total respiration capacity in the presence of glucose and pyruvate was $2.0 \pm 0.3 \text{ pmol } O_2/10^6 \text{ cells}/\text{s}$ (mean $\pm$ SEM) and with glucose alone it was $1.7 \pm 0.2 \text{ pmol } O_2/10^6 \text{ cells}/\text{s}$. Finally, cyanide was titrated to unravel sensitivity to cyanide as well as to evaluate ETC-independent oxygen consumption. Under normal capacitation, spermatozoa resired at a rate that constituted less than 40% of total respiration capacity (Fig. 6A). As seen in Fig. 6B, 1 mM NaCN was sufficient to inactivate mitochondrial respiration in spermatozoa metabolizing glucose, while 3 mM NaCN was required to completely block respiration during combined glucose/pyruvate administration. In comparison, 1 mM NaCN was sufficient to block succinate-supported respiration in freeze-thawed mouse heart mitochondria (data not shown). ETC-independent oxygen consumption (respiration activity during 10 mM NaCN) yielded ~8% of respiration capacity in the spermatozoa.

**Methylene blue rescues ATP and motility levels in human spermatozoa blocked for mitochondrial respiration**

Methylene blue is an oxidizing agent that can oxidize NADH to NAD$^+$ (Sevcik and Dunford, 1991; Sevcik and Dunford, 1995). Hence, methylene blue should, in similar manner to pyruvate be able to rescue the ATP levels and motility observed for mitochondria-inhibited human spermatozoa. This hypothesis was investigated by the treatment of spermatozoa with 10 mM NaCN, 10 $\mu$M rotenone or 1 $\mu$M antimycin A under capacitating conditions (including 5 mM glucose) for 120 min. The presence of mitochondrial inhibitors reduced both ATP levels and all parameters of motility measured. Co-incubation with 50 $\mu$M methylene blue partly or totally regained the ATP (Fig. 7A) and motility levels (Fig. 7B–E) compared with the levels obtained with 5 mM pyruvate. These findings support the hypothesis that exogenous pyruvate promotes ATP and capacitation through an increase of intracellular NAD$^+$ levels.

**Oxamate inhibits motility, hyperactivation and ATP production in human spermatozoa**

The metabolic tracing experiments demonstrated that human spermatozoa convert exogenous pyruvate exclusively to lactate, a reaction that is catalyzed by LDH in cells. An inhibition of LDH should therefore result in reduced motility and ATP levels. To test this, we treated human spermatozoa under capacitating conditions with increasing concentrations of oxamate, a known competitive substrate inhibitor of LDH (Adams et al., 1973; Wong et al., 1997), for 120 min. This resulted in a dose-dependent reduction in normal- and progressive motility, VCL, hyperactivation and ATP levels (Fig. 8). Maximum inhibition (%) and EC$_{50}$ values ($\mu$M) of oxamate were calculated for motility and ATP concentrations and presented in Table III. Oxamate reduced progressive and hyperactive motility by 94 and 95%, respectively. Normal motility and ATP levels were on the other hand not completely depleted by oxamate, which may have resulted from a competition by glycolysis-derived pyruvate. Our findings strengthen...
the theory of LDH as an important enzyme responsible for the pyruvate-induced increase of sperm motility and capacitation.

An increased and sustained ATP production by a combination of glucose and pyruvate is unaffected by the presence of high concentrations of exogenous lactate

Spermatozoa are surrounded by high concentrations of lactate present in the female oviductal fluid (Dickens et al., 1995; Gardner et al., 1996; Tay et al., 1997). Since high lactate concentrations favor the reverse reaction of LDH and consequently an inhibition of lactic acid fermentation, we wanted to investigate whether an excess of lactate would block ATP production in our setup. We repeated the experiment where capacitated spermatozoa were treated with a titration of NaCN, this time in the presence of 5 mM glucose and 0.4 mM pyruvate. ATP production was sustained in the presence of up to 25 mM NaCN (Fig. 9). Addition of 5- and 50-fold molar excess of lactate compared to pyruvate did not affect ATP production induced by exogenous pyruvate and glucose in the presence of NaCN. These results suggest that sperm motility and hyperactivation stimulated solely by glycolytic ATP synthesis and lactic acid fermentation may operate in vivo.

Discussion

In this report, we demonstrate that sperm ATP levels, motility, hyperactivation and tyrosine phosphorylation increase in the presence of exogenous pyruvate in combination with glucose. Hence, pyruvate may promote male fertility. In the light of our findings, we suggest

Figure 7 The reduced levels of ATP and motility in human spermatozoa treated with mitochondrial inhibitors can be rescued by methylene blue. Spermatozoa were incubated under capacitating conditions supplemented with 5 mM glucose and 10 mM NaCN, 10 μM rotenone or 1 μM antimycin A for 120 min in the absence (black bars) and presence of 5 mM pyruvate (grey bars) or 50 μM methylene blue (white bars). (A) ATP concentrations measured by luminescence. (B–E) Sperm motility evaluated by CASA. The data are presented as mean ± SEM from three separate experiments (A; n = 9, B–E; n = 6).
new criteria for sperm capacitation. Pyruvate, together with already known capacitating agents such as glucose, HSA and HCO$_3^-$ should be included in standard IVF and laboratory protocols for capacitation of human spermatozoa. Furthermore, the effect of exogenous pyruvate could be developed into a clinical test for evaluation of glycolytic flux rates in human sperm samples of men having their fertility scrutinized.

ATP concentration and all parameters of motility measured increased at comparable EC50 values ($\sim 0.04$ mM) by the addition of pyruvate. These results suggest that endogenous ATP levels, motility and hyperactivation are coupled processes. After only 30 min incubation, we observed increased tyrosine phosphorylation and hyperactivation together with a peak in ATP levels in the presence of exogenous pyruvate. This indicates that the pyruvate-accelerated capacitation process is associated with an acute elevation in endogenous ATP levels. The hypothesis is in agreement with experiments performed by Ho et al. (2002) who concluded that the vigorous movement of hyperactivation requires increased ATP levels. It is possible that stimulation of spermatozoa with capacitating agents such as pyruvate and HCO$_3^-$ leads to an increase in endogenous ATP concentrations that further initiate hyperactivation and tyrosine phosphorylation. A connection between ATP content and capacitation is further supported by the observation that mouse spermatozoa lacking functional protein kinase A (PKA) showed reduced levels of tyrosine phosphorylation and endogenous ATP (Nolan et al., 2004). PKA is a serine/threonine kinase suggested to be a master regulator of sperm capacitation and tyrosine phosphorylation through Src (Baker et al., 2006).

Spermatozoa incubated with glucose, pyruvate or lactate showed depleted ATP levels in the presence of mitochondrial inhibitors. However, when treated with a combination of glucose and pyruvate, ATP levels were restored to non-inhibited control levels. With this combination, even spermatozoa treated with high doses (10 mM) of NaCN produced sufficient ATP to support normal levels of progressive motility, hyperactivation and tyrosine phosphorylation. Our oxygen

Table III  Effects of oxamate on motility and endogenous ATP levels of capacitated human spermatozoa.

| Sperm parameter | EC50 (mM)$^a$ | Maximal inhibition (%)$^b$ |
|-----------------|--------------|---------------------------|
| Motility        | 2.9 ± 0.7    | 31 ± 1                    |
| Progressive motility | 1.8 ± 0.4    | 94 ± 1                    |
| VCL             | 1.0 ± 0.3    | 42 ± 3                    |
| Hyperactive motility | 1.0 ± 0.3    | 95 ± 2                    |
| ATP             | 0.5 ± 0.1    | 82 ± 3                    |

Numbers are given as mean values ± SEM of three separate experiments.

$^a$EC50 values (mM).

$^b$Maximum inhibition (%) of sperm motility and ATP production by a titration of oxamate.
consumption experiments demonstrated a complete blockage of oxidative phosphorylation already at 3 mM NaCN, excluding any ATP contribution from mitochondria at 10 mM NaCN. Reports from the literature have been somewhat inconsistent regarding inhibitors of mitochondrial respiration and their effect on sperm motility (Ford and Harrison, 1981; Fraser and Quinn, 1981; Travis et al., 2001; Mukai and Okuno, 2004; Hung et al., 2008). To our knowledge, research on motility and capacitation of human spermatozoa has been performed in a variety of cell media, occasionally containing pyruvate (e.g. Biggers, Whitten and Whittingham and Ham’s F10) (Rogers and Perreault, 1990; Williams and Ford, 2001; Nascimento et al., 2008; Barbonetti et al., 2010). This may have masked the important role of pyruvate in promoting sperm capacitation and reduced the potency of mitochondrial inhibitors on sperm motility.

The regeneration of ATP by exogenous pyruvate in spermatozoa blocked for mitochondrial respiration could be due to several mechanisms. Pyruvate might function as an antidote for the mitochondrial blockers (Way, 1984), an antioxidant against reactive oxygen species (ROS) (Andrae et al., 1985; de Lamirande and Gagnon, 1992; Brand and Hermfisse, 1997) or be utilized in alternative metabolic pathways. However, tracing of metabolites by LC-MS showed that spermatozoa treated with NaCN reestablished ATP levels via an enhanced glycolytic flux in the presence of pyruvate. In fact, all extracellular pyruvate consumed by spermatozoa was converted to lactate, even under aerobic conditions. This reaction is catalyzed by LDH in the final step of lactic acid fermentation with a concomitant production of NAD\(^+\) from NADH, which is necessary for a rapid glycolysis. This indicates that human spermatozoa display a slow glycolysis in the absence of exogenous pyruvate, possibly because of the low respiration rate observed in human spermatozoa (Storey, 1978) and thus a deficiency in the transfer of NAD\(^+\) to the cytosol.

Other authors have suggested that oxidative phosphorylation is an important source of ATP in spermatozoa based on experiments demonstrating that sperm motility and ATP levels are reduced when treated with mitochondrial blockers (and glucose is the sole metabolic substrate), (Ford and Harrison, 1981; Halangk et al., 1985; Halangk and Bohnensack, 1986; de Lamirande and Gagnon, 1992; Ruiz-Pesini et al., 2000). However, our findings show that blocking of mitochondrial respiration only inhibits glycolytic ATP production in the absence of pyruvate. Hence, human spermatozoa may operate independently of mitochondria in the presence of extracellular pyruvate. Since ATP levels, motility and hyperactivation are inhibited by NaCN in the absence of pyruvate, a possible role for mitochondria may be to deliver NAD\(^+\) to glycolysis under basal conditions in the seminal plasma where the demand for ATP is lower than under capacitation. Such a mechanism would also contribute to the prevention of premature capacitation. In addition, sperm mitochondria may also serve other functions such as supplying ATP to the spermatozoon head (Carey et al., 1981).

Spermatozoa from boar (Calvin and Tubbs, 1978) and mouse (Carey et al., 1981) utilize the glycerol-3-phosphate shuttle where cytoplasmic glycerol-3-phosphate dehydrogenase (G3PDH) reduces dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P) by oxidizing NADH to NAD\(^+\). The reaction is driven by a mitochondrial membrane bound glycerol-3-phosphate dehydrogenase (mGAP) which catalyses the reverse reaction (G3P to DHAP) by reducing one molecule of mGAP-bound FAD to FADH\(_2\). FADH\(_2\) then reduces co-enzyme Q and the electrons finally enter oxidative phosphorylation. The G3P-shuttle both regenerates NAD\(^+\) in the cytoplasm that can fuel glycolysis and produces ATP in the mitochondria through the donated electrons. In addition, intramitochondrial LDH-C has been identified in spermatozoa from several mammalian species like rabbit (Storey and Kayne, 1977) and bull (Van et al., 1977). Located inside the mitochondria, LDH-C is able to directly oxidize lactate by a concomitant reduction of NAD\(^+\) to NADH and donating two electrons to the ETC. The pronounced effect on ATP levels and capacitation observed by exogenous pyruvate in this study indicates that human spermatozoa are undersupplied in both these pathways.

This hypothesis is further strengthened by the observation that the addition of methylene blue practically restored the same swimming pattern and ATP levels as exogenous pyruvate in the presence of three different mitochondrial inhibitors (Fig. 7). This is in contrast to a recent paper by Odet and co-workers where the addition of 5 \(\mu\)M methylene blue did not have any effect on sperm ATP levels or motility in wild type or LDH-C KO-mice (Odet et al., 2011). We chose to perform the experiment with 50 \(\mu\)M methylene blue based on dose–response curves (data not shown). In our experiments, 5 \(\mu\)M methylene blue was not sufficient to recover ATP levels. The discrepancy might also be due to species differences between human and mouse spermatozoa.

Spermatozoa possess several features that may favor glycolytic ATP production. Firstly, spermatozoa are reported to express several hexose transporter isoforms (Burant et al., 1992; Haber et al., 1993; Angulo et al., 1998) with different kinetic characteristics that support an efficient uptake of glucose and fructose. The tight organization of glycolytic enzymes along the FS (Krisfalusi et al., 2006; Kim et al., 2007) may further facilitate an efficient glycolytic flux (Mukai et al., 2010).
Furthermore, spermatozoa are single cells that can easily access nutrients and excrete excess intracellular levels of lactate to the surroundings. In this way, a high rate of ATP production through glycolysis can be favored at a cost of a lower total yield. Another reason for cells to rely on aerobic glycolysis is that they avoid the production of ROS from oxidative phosphorylation. Hence, low mitochondrial activity might be of extra importance for spermatozoa whose main goal is to deliver intact DNA for fertilization. Finally, the biochemical properties separating LDH-C from the other LDH isoforms may contribute to the high glycolytic flux. LDH-C has a low \( K_{m} \) for pyruvate (\( \approx 0.030 \text{mM} \)) and a high \( K_{m} \) for lactate (\( \approx 2.0 \text{mM} \)) (Clausen and Ovlsen, 1965; Coronel et al., 1983; LeVan and Goldberg, 1991; Wong et al., 1997). This implies that LDH-C has an affinity for pyruvate that is >60-fold higher than for lactate, suggesting that pyruvate turnover to lactate may be high even at high concentrations of endogenous or extracellular lactate. This is supported by our experiments where addition of excess lactate (50-fold excess in relation to pyruvate) did not influence ATP production in capacitating spermatozoa. The relative composition of glucose, pyruvate and lactate in the female reproductive tract, although varying with the day of menstrual cycle and the exact place, are found to be in the range of 0.5–3.2 mM, 0.1–0.2 mM and 4.9–10.5 mM, respectively (Dickens et al., 1995; Gardner et al., 1996; Tay et al., 1997). Since the female reproductive tract contains glucose in the mM range and pyruvate at concentrations well above our calculated EC50 values (Table I), we argue that an increased glycolytic flux driven by glucose and pyruvate may operate in vivo.

We conclude that exogenous pyruvate and glucose together speed up the glycolytic machinery to produce sufficient ATP to support progressive motility and capacitation. This metabolic peculiarity may be vital for human fertilization. The fact that this process is independent of mitochondrial respiration indicates that human sperm cell motility and fertility are unaffected by low levels of oxygen as long as glucose and pyruvate are concurrently present at sufficient concentrations, as is the case in the female reproductive tract. Our findings emphasize how survival and function of spermatozoa require great flexibility as they are transcriptionally silent and exist in a constantly changing environment. Finally, the effect of exogenous pyruvate on human sperm ATP levels and capacitation is another example on how constituents of the female reproductive tract determine the metabolism of the conspecific spermatozoa.

**Supplementary data**

Supplementary data are available at [http://humrep.oxfordjournals.org/](http://humrep.oxfordjournals.org/).

**Authors’ roles**

T.H.H. designed the study, carried out and analyzed the experiments and wrote the manuscript. K.B.P.E. designed, performed and analyzed the LC-MS experiments. F.H.C. gave essential assistance in the LC-MS experiments and participated in critical discussions. L.E. performed the sperm respiration experiments. T.J. provided critical input on the interpretation of data and drafting of the manuscript. B.S.S. participated in critical discussions and drafting of the manuscript. K.R.R. gave essential assistance in the experiments, participated in the design of the study and critical discussions and wrote the manuscript. All authors approved the final manuscript.

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**Conflict of interest**

T.H.H. is an employee and holds stock options in Spermatech AS. K.R.R. is an employee and holds stock options in Spermatech AS. T.J. is a board member and holds stock options in Spermatech AS. B.S.S. is a shareholder in Spermatech AS.

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