BIOLOGY OF REPRODUCTION (2012) **87**(3):75, 1–15
Published online before print 25 July 2012.
DOI 10.1095/biolreprod.112.102673

Metabolic Substrates Exhibit Differential Effects on Functional Parameters of Mouse Sperm Capacitation[1]

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ABSTRACT

Although substantial evidence exists that sperm ATP production via glycolysis is required for mammalian sperm function and male fertility, conflicting reports involving multiple species have appeared regarding the ability of individual glycolytic or mitochondrial substrates to support the physiological changes that occur during capacitation. Several mouse models with defects in the signaling pathways required for capacitation exhibit reductions in sperm ATP levels, suggesting regulatory interactions between sperm metabolism and signal transduction cascades. To better understand these interactions, we conducted quantitative studies of mouse sperm throughout a 2-h in vitro capacitation period and compared the effects of single substrates assayed under identical conditions. Multiple glycolytic and nonglycolytic substrates maintained sperm ATP levels and comparable percentages of motility, but only glucose and mannose supported hyperactivation. These monosaccharides and fructose supported the full pattern of tyrosine phosphorylation, whereas nonglycolytic substrates supported at least partial tyrosine phosphorylation. Inhibition of glycolysis impaired motility in the presence of glucose, fructose, or pyruvate but not in the presence of hydroxybutyrate. Addition of an uncoupler of oxidative phosphorylation reduced motility with pyruvate or hydroxybutyrate as substrates but unexpectedly stimulated hyperactivation with fructose. Investigating differences between glucose and fructose in more detail, we demonstrated that hyperactivation results from the active metabolism of glucose. Differences between glucose and fructose appeared to be downstream of changes in intracellular pH, which rose to comparable levels during incubation with either substrate. Sperm redox pathways were differentially affected, with higher levels of associated metabolites and reactive oxygen species generated during incubations with fructose than during incubations with glucose.

glycolysis, metabolism, sperm capacitation, sperm hyperactivation, sperm motility and transport

[1]Supported by the Eunice Kennedy Shriver NICHD/NIH through cooperative agreement U54 HD35041 as part of the Specialized Cooperative Centers Program in Reproduction and Infertility Research.
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Received: 8 June 2012.
First decision: 28 June 2012.
Accepted: 18 July 2012.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

INTRODUCTION

During epididymal maturation, sperm acquire the ability to achieve progressive motility and bind the zona pellucida [1]. However, sperm are not considered to be functionally mature until they have resided for a time in the female reproductive tract or in defined media mimicking this environment. During this time, sperm undergo a set of biochemical changes rendering them competent for fertilization [2]. These events, collectively termed capacitation, include acquisition of the ability to undergo the acrosome reaction and achieve hyperactivated motility.

Both sperm metabolism and signal transduction cascades are essential for the physiological changes that occur during capacitation. Sperm motility and hyperactivation require substantial ATP, estimated to be as much as 70% of the ATP produced in these cells [3]. ATP is also required for maintenance of sperm intracellular ion concentrations, including Ca^{2+} and H^{+}, which serve as key signals during capacitation [4]. To achieve capacitation, sperm must initiate appropriate signaling pathways, including bicarbonate-dependent stimulation of ADCY10 (also known as soluble adenylyl cyclase), protein kinase A (PKA)-dependent tyrosine phosphorylation of specific proteins, and inhibition of specific protein phosphatases [5, 6]. Reductions in sperm ATP levels and motility have been reported for mice deficient in capacitation-associated signaling components, such as Catsper1, ADCYC10, and the Cα2 subunit of PKA [7–11]. These reductions point to a tightly coupled association between metabolism and capacitation-associated signaling, although direct links between these processes are not well understood.

Both mitochondrial respiration and glycolysis are active in mammalian sperm. Oxidative phosphorylation yields more ATP per molecule of glucose, but sperm from different species show variable dependence on mitochondrial metabolism [12–15]. Despite these species differences, mammalian sperm typically exhibit a high rate of glycolysis that is correlated with motility [16, 17]. Gene targeting studies provide compelling evidence that glycolysis [18–20], rather than mitochondrial ATP production [21], is essential for maintaining sperm motility and male fertility in the mouse. Human sperm also appear to obtain a significant proportion of their ATP from glycolysis [14, 22]. Nevertheless, reports conflict regarding the maintenance of ATP levels and motility when mouse or human sperm are incubated in the absence of glycolysable substrates [14, 15, 22–28].

Sperm metabolism has several unique features. The glycolytic machinery is compartmentalized in the principal piece of the sperm flagellum [29, 30], whereas the mitochondria are localized in the midpiece. Multiple glycolytic enzymes in mammalian sperm are germ cell-specific isozymes, including glyceraldehyde phosphate dehydrogenase, spermatogenic (GAPDHS), phosphoglycerate kinase 2 (PGK2), and lactate dehydrogenase C (LDHC) [31–34]. Studies in the mouse have identified several additional components of metabolic pathways that are germline-specific isoforms, including hexokinase [35] and aldolase A [36] in the glycolytic pathway and cytochrome *c* and succinyl-coenzyme A transferase in mitochondria [37, 38]. In addition, some proteins typically confined to the mitochondria have been detected at locations outside the mitochondria in sperm. For example, the pyruvate dehydrogenase complex has been localized in the principal piece in hamster sperm, where it has been shown to undergo capacitation-associated tyrosine phosphorylation [39]. These alterations in metabolic enzymes suggest that the regulation of sperm metabolism may have distinctive features not found in somatic cells.

Previous studies of individual substrates used a variety of sperm isolation and incubation conditions and frequently did not investigate multiple parameters of sperm function that change during capacitation. To begin to understand interactions between sperm metabolism and signaling pathways, we conducted detailed analyses regarding the impact of glycolysable and nonglycolysable substrates on sperm functional parameters throughout a 2-h in vitro capacitation period. We monitored sperm energy production, capacitation-associated tyrosine phosphorylation, and total motility. To understand the changes in the types of motility, including hyperactivation, we conducted multiclass motility analysis using CASAnova, a tool developed to describe changes in sperm motility more precisely [40]. Because we observed differences in the ability of individual glycolysable or nonglycolysable substrates to support functional changes during capacitation, we compared ATP and motility parameters of sperm incubated in single substrates with either an inhibitor of glycolysis or an uncoupler of oxidative phosphorylation. We also explored differences in the effects of fructose and glucose in greater detail, determining complete metabolic profiles of sperm incubated in each substrate and monitoring their effects on intracellular pH (pH_i) and the production of reactive oxygen species (ROS).

MATERIALS AND METHODS

Reagents

Reagents for media preparations and sperm functional analyses were purchased from Sigma-Aldrich with the exception of sodium chloride, glucose, Tris, ethylenediaminetetra-acetic acid (EDTA), sodium dodecyl sulfate, glycerol, tris(2-carboxyethyl)phosphine (TCEP)-reducing agent, and Tween 20 (Thermo Fisher Scientific); sodium pyruvate (Invitrogen); sodium bicarbonate (EM Science); potassium chloride, magnesium sulfate heptrahydrate, and potassium phosphate (Mallinckrodt Chemical); and penicillin/streptomycin solution (Gemini Bioproducts).

Animals

Adult CD1 male mice were purchased from Charles River Laboratories and allowed to acclimatize before use. All procedures involving mice were approved in advance by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Sperm In Vitro Capacitation

All sperm incubations were performed in human tubal fluid (HTF) medium, which has been shown to support both mouse and human in vitro fertilization [41, 42]. HTF complete medium consists of 101.6 mM NaCl, 4.7 mM KCl, 0.37 mM KH_2PO_4, 0.2 mM $MgSO_4 \cdot 7H_2O$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 5 mg/ml of bovine serum albumin (BSA), 100 U of penicillin G, 0.1 mg of streptomycin, 2.78 mM glucose, 0.33 mM pyruvate, and 21.4 mM lactate, with pH 7.4. When bicarbonate was omitted, 21 mM Hepes was added to maintain the pH of the medium. For experiments without energy substrates, glucose, lactate, and pyruvate were omitted. HTF medium was prepared with individual substrates at appropriate concentrations to compare the ability of each substrate to support sperm capacitation. For all media, the osmolality was adjusted to approximately 315 mOsm/kg with 5 M NaCl using a Model 3300 micro-osmometer (Advanced Instruments).

Sperm were collected from the caudae epididymides of sexually mature (age, >8 wk) mice. Each cauda was carefully trimmed to remove adipose and other tissue, rinsed in PBS (140 mM NaCl, 3mM KCl, 4 mM $NaH_2PO_4 \cdot 7H_2O$, and 1.4 mM KH_2PO_4; pH 7.4), and placed in 1 ml of HTF medium lacking bicarbonate and energy substrates. Four to six cuts were made in each cauda using iris scissors, and sperm were released into the medium by incubation for 10 min at 37°C under 5% CO_2 and air. The tissue was removed, and the suspension was mixed gently by swirling. A 10-µl aliquot was reserved for calculating sperm concentration for ATP and Western blot analysis.

Control samples were diluted at least 1:10 in HTF complete medium and incubated for 2 h at 37°C under 5% CO_2 and air, conditions that support sperm capacitation. Other samples were diluted similarly in HTF medium containing 25 mM bicarbonate and individual test substrates. Multiple substrates were tested on sperm from each mouse. At appropriate time points, aliquots of sperm suspensions were taken for analyses of sperm motility, ATP levels, and tyrosine phosphorylation.

Sperm Viability

Sperm viability was measured after incubating for 2 h in HTF medium with appropriate substrates. Aliquots were taken, and propidium iodide (Invitrogen) was added to a final concentration of 12 µM [43]. Viability was determined by counting at least 100 cells for each condition and determining the proportion of membrane-intact sperm that exclude this DNA intercalating agent.

Sperm Motility

The initial sperm suspension collected from the cauda epididymis sperm was diluted 1:20 to 1:60 with HTF medium containing appropriate substrates to a concentration (typically 2–4 $\times$ 10^5 sperm/ml) that yields approximately 50 sperm/microscope field for motility assessment by computer-assisted sperm analysis (CASA). Motility was assessed at 30-min intervals throughout the 2-h in vitro capacitation period. Initial time points for each condition were completed within 2 min of dilution. Quantitative parameters of sperm motility were recorded by CASA using the CEROS sperm analysis system (software version 12.3; Hamilton Thorne Biosciences) configured as described previously [40]. The default Mouse 2 analysis settings provided by Hamilton Thorne Biosciences were used, except that 90 frames were recorded and slow cells were counted as motile. Motile sperm tracks were also required to have a minimum of 45 points. Sperm were loaded into Leja slides (depth, 100 µm), and CASA motility parameters were recorded for least 10 fields/sample, with tracks and kinetic parameters saved for individual sperm in each field [40]. Individual database text (DBT) files were generated from the CASA images and used to generate motility profiles using CASAnova software (http://www.csbio.unc.edu/CASAnova/) [40].

Sperm ATP Levels

Sperm were diluted at least 1:10 from epididymal suspensions and incubated for 2 h in HTF medium with appropriate substrates. At relevant time points, sperm samples were swirled gently, and triplicate 50-µl aliquots were removed and diluted into individual tubes containing 450 µl of hot Tris-EDTA buffer (0.1M Tris-HCl and 4 mM EDTA; pH 7.75). The resulting suspensions were boiled for 5 min and then frozen in liquid nitrogen. Upon thawing, each sample was centrifuged at 15 000 $\times$ *g* for 5 min at room temperature. A 20-µl aliquot of the supernatant was mixed with 80 µl of water, and then 50 µl were utilized for quantifying ATP. The ATP content was determined using the ATP Bioluminescence Assay Kit CLS II (Roche Applied Science) according to the manufacturer's protocol.

Capacitation-Associated Tyrosine Phosphorylation

To assess the status of sperm tyrosine phosphorylation during capacitation, sperm were diluted at least 1:10 from epididymal suspensions into HTF medium containing various substrates. One-milliliter aliquots were removed

from each test medium at 0 and 2 h and centrifuged at 1000 × *g* for 3 min. The supernatant was removed, and the sperm pellet was resuspended in 1 ml of PBS, then centrifuged briefly at 10 000 × *g*. After removing the supernatant, the pellet was resuspended in 2× sample buffer (4.6% SDS, 125 mM Tris [pH 6.8], 18% glycerol, 1% bromophenol blue, and 50 mM TCEP) and boiled for 5 min. Equivalents of 10^6 sperm/sample were resolved by SDS-PAGE on 10% polyacrylamide gels. Proteins were then transferred to Immobilon-P membranes (Millipore), and tyrosine phosphorylation was detected using the antiphosphotyrosine 4G10 monoclonal antibody (Millipore) as described previously [44] with minor modifications. Briefly, blots were blocked overnight at 4°C in PBS plus 0.1% Tween 20 (PBST) and 5% fish gelatin and then incubated for 1 h at room temperature with 4G10 diluted 1:5000 in PBST with 5% fish gelatin. Blots were washed three times with PBST for 5 min each time and then incubated for 30 min at room temperature with horseradish peroxidase (HRP)-conjugated goat-anti-mouse secondary antibody (KPL) diluted 1:30 000 in PBST with 5% fish gelatin. Blots were washed in two changes of PBST for 1 h each time before detection of chemiluminescence using Supersignal West Pico (Pierce).

Analysis of Sperm Function in the Presence of Metabolic Inhibitors

To determine the effect of inhibition of metabolic pathways on sperm function, epididymal sperm were isolated as described above and incubated with HTF medium containing single substrates supplemented with either 10 mM α-chlorohydrin (ACH; supplied as 3-chloro-1,2-propanediol; Sigma-Aldrich) [25], 10 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Sigma-Aldrich) [15, 45], or dimethyl sulfoxide (DMSO) as a vehicle control. Aliquots were taken at 0 and 90 min for analysis of ATP levels, determination of percentage motility by CASA, and analysis of motility profiles generated with CASAnova.

Assessment of Sperm pH_i

To assess pH_i, sperm were collected as described above in HTF medium (1 ml/cauda) lacking bicarbonate, BSA, and substrates and supplemented with 3 μM 2′,7′-bis-2-carboxyethyl-5-6-carboxyfluorescein acetoxymethyl ester (BCECF-AM) [46]. The resulting suspensions were diluted to 10 ml with the same medium without BCECF-AM and centrifuged at 500 × *g*. The supernatant was removed, and the pellet was resuspended to determine sperm concentration.

Sperm were added to HTF medium containing either 2.78 mM glucose or 5 mM fructose at a final concentration of 1×10^6 sperm/ml and analyzed using an Aminco Bowman spectrofluorometer (Thermo Scientific). Emission ratios were calculated from recorded fluorescence at 535 nm following sequential excitation at 440 and 490 nm. Signals were averaged over 8 sec. Calibration of pH_i was performed after lysis of sperm with 0.1% Triton and addition of defined volumes of HCl [46].

Substrate Preincubation Experiments

Sperm from epididymal suspensions were diluted 1:10 into 6 ml of HTF medium containing either 2.78 mM glucose or 5 mM fructose and incubated for 30 min at 37°C under 5% CO_2 in air. Each suspension was then diluted to 30 ml with substrate-free HTF medium, divided in half, and centrifuged at 500 × *g* for 10 min at room temperature. Each sperm pellet was washed again with 5 ml of substrate-free HTF medium and centrifuged at 1000 × *g* for 5 min. The supernatant was removed, and sperm were gently resuspended in the remaining liquid (~150 μl). The sperm suspension was then evenly divided into 1 ml of HTF medium containing 2.78 mM glucose, 5 mM fructose, or no substrates. Sperm were incubated at 37°C under 5% CO_2 in air for 30 min before taking aliquots for CASA analysis. Additional time points were taken at 60 and 90 min after resuspension with substrates. The percentage of sperm undergoing hyperactivation was determined from motility profiles generated by CASAnova.

Preparation of Sperm for Metabolomic Analyses after Incubation with Glucose or Fructose

Sperm samples for metabolomic analyses were pooled from six CD1 mice after isolation in bicarbonate-free, substrate-free HTF medium (1 ml/cauda). The sperm suspension was mixed by gentle swirling, then diluted to 100 ml with PBS and centrifuged at 1000 × *g* for 10 min. The pellets were resuspended in residual PBS, divided equally into 25 ml of HTF medium with 2.78 mM glucose or 25 ml of HTF medium with 5 mM fructose and then incubated for 90 min at 37°C under 5% CO_2 in air. At the end of the incubation period, each sperm suspension was diluted to 50 ml with ice-cold PBS to quench metabolic reactions and then centrifuged at 1000 × *g* for 10 min at 4°C. After removing the supernatant, the pellet was transferred to a 1.5-ml microfuge tube and diluted to 1 ml with ice-cold PBS. The pellets were resuspended by gentle mixing, and 10 μl were reserved for sperm counting. Suspensions were spun briefly at 14 000 × *g* to pellet, the supernatants removed, and the pellets snap-frozen in liquid nitrogen and stored at −80°C until processing.

Sperm pellets from different experiments were combined to yield a minimum of 45×10^6 sperm/sample. A total of eight pools for each substrate were analyzed, comparing sperm from the same mice incubated with either glucose or fructose. Sperm metabolites were extracted from each pellet using 500 μl of ice-cold methanol-chloroform (ratio = 2:1; Fisher Scientific). The mixture was added to sperm pellets on ice, and pellets were resuspended and briefly sonicated. Samples were incubated for 30 min on ice and briefly sonicated again before the addition of 500 μl of chloroform-water (ratio = 1:1). After mixing by vortexing, samples were centrifuged at 15 000 × *g* for 20 min. Aqueous and organic phases were separated into glass crimp-top vials (Agilent) and stored at −80°C. Samples were shipped on dry ice to the North Carolina Research Campus in Kannapolis for analysis.

Metabolomic Analysis by High-Performance Liquid Chromatography Time-of-Flight Mass Spectrometry

Aqueous and organic layers from sperm samples were vacuum-dried in the same vial at room temperature, then reconstituted in 150 μl of methanol-water (ratio = 80:20, v/v; Optima LC/MS; Fisher Scientific). Samples were then filtered through 0.20-μm membrane (Millipore) for analysis by high-performance liquid chromatography (HPLC) time-of-flight (TOF) mass spectrometry (MS).

A 10-μl aliquot sample was injected onto an Agilent HPLC 1200 system equipped with a binary solvent delivery manager and a sample manager (Agilent Corporation), with chromatographic separations performed on an Agilent ZORBAX Eclipse XDB-C18 chromatography column (length, 150 mm; inner diameter, 4.6 mm; pore size, 5 μm). The elution conditions were optimized as described previously [47]. MS was performed using an Agilent model 6220 MSD TOF mass spectrometer equipped with a dual-sprayer electrospray ionization source (Agilent). The system was tuned for optimum sensitivity and resolution as described previously [47]. During metabolite profiling experiments, both plot and centroid data were acquired for each sample from 50–1000 Da over a 25-min analysis.

The resulting data files were centroided, deisotoped, and converted to mzData xml files using the MassHunter Qualitative Analysis Program (vB.03.01; Agilent). Following conversion, xml files were analyzed using the XCMS package (v1.24.1; http://metlin.scripps.edu), which runs in the statistical package R (v.2.12.1; http://www.r-project.org), to pick, align, and quantify chromatographic events corresponding to specific *m/z* values and retention times. The software was used in the default setting as described elsewhere (http://metlin.scripps.edu) except for xset (bw = 5) and rector (plottype = ''m''; family = ''s''). The created tsv file was exported into Microsoft Excel, and the resulting data sheet was used for further analysis.

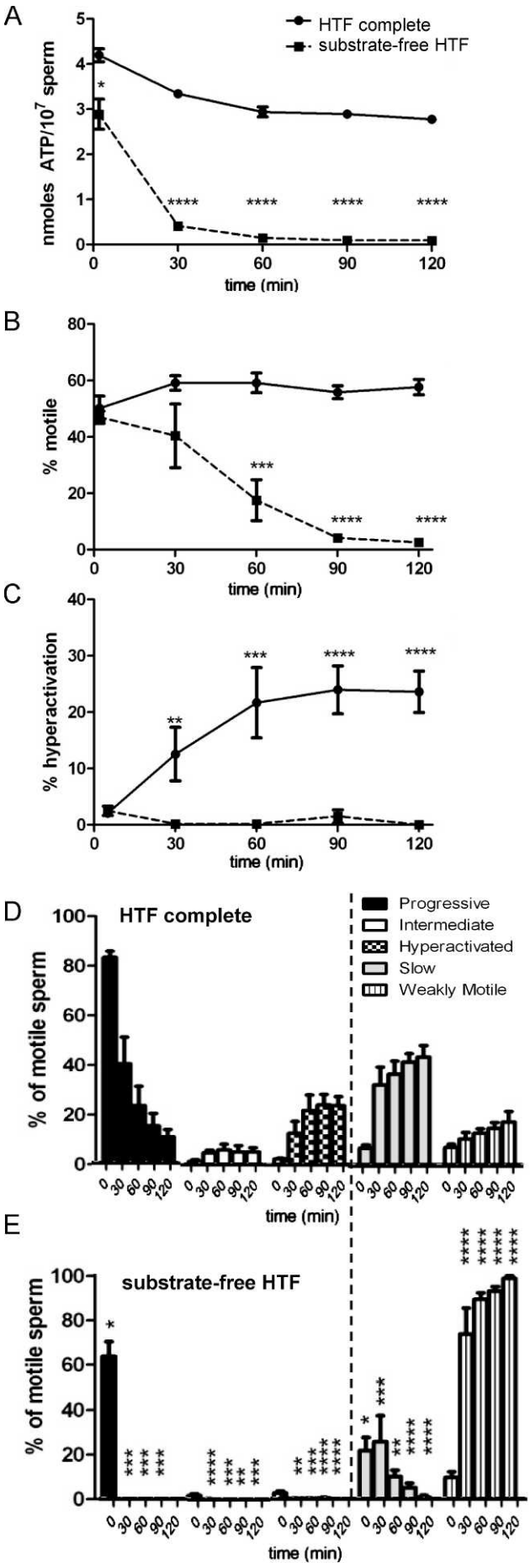
Metabolomic Analysis by Gas Chromatography TOFMS

After liquid chromatography (LC)-MS analysis, liquid from each sample was transferred into a glass vial and vacuum-dried at room temperature. The residue was derivatized using the following two-step procedure. First, 80 μl of methoxyamine HCl (15 mg/ml in pyridine; Sigma-Aldrich) were added to the vial and incubated at 30°C for 90 min. After the addition of 10 μl of retention index compounds (the mixture of C10-C40 alkanes, 50 μg/ml [Sigma-Aldrich]) and 80 μl of bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (Sigma-Aldrich) to the reaction vials, the samples were subjected to a 70°C derivatization reaction for 120 min.

Each 1-μl aliquot of derivatized solution was injected in splitless mode into an Agilent 7890N gas chromatograph coupled with a Pegasus HT TOF mass spectrometer (Leco Corporation), and sample analysis was performed as described previously [48]. The acquired data were analyzed using ChromaTOF software (v4.22; Leco Co.). Internal standards and any known artificial peaks were removed from the data set. Compound identification was performed by comparing the mass fragments with National Institute of Standards and Technology (NIST) 05 Standard mass spectral databases in NIST MS search 2.0 (NIST) software with a similarity of more than 70%. Identified compounds were verified by available reference compounds [48].

Detection of Substrate-Dependent ROS Production

To determine the impact of substrate utilization on ROS production, we measured sperm ROS levels using a luminol-HRP assay [45, 49–52]. Sperm



were isolated from the combined caudae epididymides of two CD1 mice in HTF medium (1 ml/cauda) lacking bicarbonate and substrates. The resulting epididymal sperm suspension was diluted 1:5 in the same medium and centrifuged at 500 × *g* for 10 min at room temperature. The pellet was resuspended in residual liquid, and sperm concentration was determined. Aliquots of the suspension were diluted to approximately 5×10^6 sperm/ml in HTF medium containing either 2.78 mM glucose or 5 mM fructose and divided into three 600-µl aliquots. Each aliquot also contained 50 µM luminol (Sigma) and 8.8 U of HRP (Type VI; Sigma). Sperm were incubated with luminol and HRP for 30 min at 37°C under 5% CO_2 and air before the initial measurement. ROS levels were measured using a TD 20/20 luminometer (Promega) with a 20-sec integration time. Medium lacking sperm was measured and used to determine background chemiluminescence. Sperm suspensions incubated with 100 µM *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP; Sigma) were also measured to confirm a lack of contamination by leukocytes. Sperm were maintained at 37°C under 5% CO_2 and air between measurements.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). All data are shown as the mean ± SEM. Statistical significance was determined using either two-tailed unpaired *t*-tests or one-way ANOVA after arcsine transformation of percentages. Differences were considered to be significant if $P < 0.05$.

RESULTS

Effects of Eliminating Endogenous Substrates on Sperm ATP Levels and Motility

Several studies have shown that HTF complete medium (containing 2.78 mM glucose, 21.4 mM lactate, and 0.33 mM pyruvate as energy substrates) supports tyrosine phosphorylation, hyperactivation, and other changes required for successful in vitro fertilization in multiple species [41, 42]. In the present metabolic studies, sperm from individual mice were divided into multiple aliquots so that functional parameters in each test medium were compared directly with those in HTF complete medium. Sperm were assessed every 30 min over a 2-h time course to determine steady-state ATP levels, percentage motility, and distribution of multiple patterns of motility.

Initial experiments determined conditions that reduce endogenous substrates to levels that do not sustain sperm ATP. When sperm were collected in substrate-free HTF medium (1 ml/cauda) and then further diluted at least 1:10 in the same medium, the mean ATP content fell to 12% of the initial level (2.89 ± 0.33 nmol per 10^7 sperm) within 30 min (Fig. 1A). ATP declined further throughout the incubation period, reaching mean values less than 5% of initial levels by 60 min. In contrast, mean ATP content was 4.20 ± 0.14 nmol per 10^7 sperm immediately after dilution into HTF complete medium and was maintained at greater than 65% of this level throughout the 2-h incubation period. Subsequent experiments evaluating single substrates used this same dilution strategy to minimize the contribution of epididymal energy substrates.

FIG. 1. ATP and motility parameters of sperm incubated in the presence or absence of energy substrates. Sperm were incubated in HTF complete medium (**A**–**D**) or in substrate-free HTF medium (**A**–**C** and **E**) and analyzed at 30-min time points over a 2-h in vitro capacitation period. **A**) Sperm ATP levels in the presence and absence of substrates. **B**) Total sperm motility assessed by CASA. **C**) The percentage of motile sperm displaying hyperactivated motility over the time course. **D**) Motility profiles of sperm incubated in HTF complete media. **E**) Motility profiles of sperm incubated without energy substrates. Data are represented as the mean ± SEM of sperm from seven mice. Differences between conditions at corresponding time points were analyzed using two-tailed, unpaired *t*-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

In parallel with ATP measurements, CASA was used to determine the effects of omitting substrates on sperm motility. In HTF complete medium, 50%–60% of sperm remained motile throughout the 2-h capacitation interval (Fig. 1B). Initial percentages of motile sperm in the absence of substrates were comparable to those in HTF complete medium. However, the mean percentage of motile sperm in substrate-free HTF medium declined significantly after 60 min and reached levels of less than 5% by 90 min. In the absence of added substrates, sperm ATP levels fell more rapidly than percentage motility, suggesting possible reductions in vigorous motility under these conditions.

We used CASAnova, a recently developed method for quantitative analyses of mouse sperm motility [40], to assess this possibility (Fig. 1, C–E). CASA tracks and DBT files for sperm analyzed in Figure 1B were used to compare motility profiles in substrate-free HTF medium with those observed in HTF complete medium. CASAnova assesses parameters of individual sperm tracks and assigns each sperm to vigorous (progressive, intermediate, or hyperactivated) or nonvigorous (slow or weakly motile) categories of motility. Analysis of percentage hyperactivation during incubation in HTF complete medium showed a steady increase in hyperactivated sperm, reaching mean values of 24.0% ± 4.3% after 90 min (Fig. 1C). No appreciable hyperactivation was observed at any time point when sperm were incubated in substrate-free HTF medium. Furthermore, comparison of all CASAnova categories reveals the rapid loss of all vigorous motility when substrates were omitted. As in previous studies [40], sperm in HTF complete medium exhibited a transition from predominantly progressive at time zero to other vigorous (intermediate or hyperactivated) and nonvigorous (slow or weakly motile) patterns of motility during capacitation (Fig. 1D). Sperm incubated in substrate-free HTF medium had a reduced percentage of progressive sperm and more slow sperm at the initial time point (Fig. 1E). Moreover, greater than 99% of the motile population in substrate-free HTF medium was classified as nonvigorous after 30 min and as weakly motile by the end of the 2-h time course. These results confirm the importance of examining changes in the types of sperm motility, rather than the overall percentage of motile sperm, when assessing the efficacy of metabolic substrates in supporting functional changes required for fertilization.

Effects of Single Substrates on Sperm Motility Parameters

Four substrates metabolized by glycolysis (glucose, mannose, fructose, and sorbitol) and four substrates metabolized by mitochondrial respiration (lactate, pyruvate, β-hydroxybutyrate [hydroxybutyrate], and citrate) were tested to determine their effects on motility transitions during capacitation. Sperm from at least four mice were tested with each substrate, with direct comparisons of each medium to HTF complete medium. Glucose (2.78 mM), lactate (21.4 mM), and pyruvate (0.33 mM) were initially tested at the concentrations found in HTF complete medium [42]. Other substrates were tested at concentrations shown previously to support sperm motility and other aspects of capacitation, including 5 mM mannose, 5 mM fructose, 5 mM sorbitol, 5 mM hydroxybutyrate, and 10 mM citrate [14, 25–27, 53–55]. We also tested glucose, lactate, pyruvate, and citrate at 5 mM but found that changing the concentration did not have significant effects on the sperm motility parameters described below (data not shown).

Motility profiles were first compared for the four substrates metabolized through the glycolytic pathway (Fig. 2). When sperm were incubated in HTF medium with glucose, mannose, fructose, or sorbitol for 2 h, no statistically significant differences were found in percentage motility assessed by CASA compared to HTF complete medium (Fig. 2A). However, motility decreased approximately 30% by 2 h in 5 of 11 samples incubated in HTF medium with fructose. CASAnova was used to classify the motility profiles of sperm analyzed in Figure 2A. HTF medium with 2.78 mM glucose or 5 mM mannose supported hyperactivation at mean levels that were comparable to or higher than those supported by HTF complete medium (Fig. 2B). Sperm incubated with 5 mM fructose as the sole energy substrate displayed significantly lower levels of hyperactivation throughout the incubation. Although 5 mM sorbitol supported a short burst of hyperactivation 30 min after substrate addition, the mean percentage of hyperactivated motility decreased steadily thereafter, generating a time course of hyperactivation markedly different from that of the HTF complete medium control.

Complete CASAnova motility profiles were compared at 90 min (Fig. 2C), the time point at which hyperactivated motility typically reaches maximum levels [40]. The mean percentages of vigorous (progressive, intermediate, or hyperactivated) and nonvigorous (slow or weakly motile) motility patterns in HTF medium with glucose or mannose were comparable to those seen in HTF complete medium. Sperm incubated in HTF medium with fructose displayed higher levels of progressive motility at 90 min, suggesting that sperm retain the progressive pattern of motility for longer periods when hyperactivation is inhibited. Unlike other glycolysable substrates tested, sorbitol did not sustain vigorous motility patterns throughout the incubation period. Greater than 93% of motile sperm were classified as slow or weakly motile after a 90-min incubation in HTF medium with sorbitol.

We also assessed the ability of nonglycolysable substrates (lactate, pyruvate, hydroxybutyrate, and citrate) to support sperm motility (Fig. 3). In HTF medium with pyruvate or lactate, approximately half the mice tested showed a 25% decrease in the percentage of motile sperm over time. However, we found no statistically significant reductions in percentage motility compared to HTF complete medium when sperm were incubated in HTF medium with 0.33 mM pyruvate, 21.4 mM lactate, or 5 mM hydroxybutyrate, except at the 60-min time point with pyruvate (Fig. 3A). In contrast, percentage motility declined significantly after incubation in HTF medium with 10 mM citrate for 60 min and reached mean levels of approximately 20% by the end of the incubation period. The viability of sperm in HTF medium with citrate at this time point was comparable to that in HTF complete medium (mean $\geq$ 54% in both media), indicating that the reduction in percentage motility did not result from a loss of cell viability.

None of the nonglycolysable substrates supported hyperactivation at the levels observed in HTF complete medium (Fig. 3B). Within 2 min of dilution into HTF medium containing citrate, a small but significant portion of sperm displayed hyperactivated motility (mean = 8.0% vs. 2.36% in HTF compete medium). However, hyperactivation was not observed at all subsequent time points in this medium. We also compared complete CASAnova motility profiles after incubation for 90 min with different nonglycolysable substrates (Fig. 3C). Although hyperactivated motility was significantly lower in HTF medium with pyruvate, lactate, or hydroxybutyrate, these substrates maintained progressive motility at comparable or higher mean levels than HTF complete medium. Of the low percentage of sperm (mean = 30.4%) that remained motile after a 90-min incubation in HTF medium with citrate, greater than

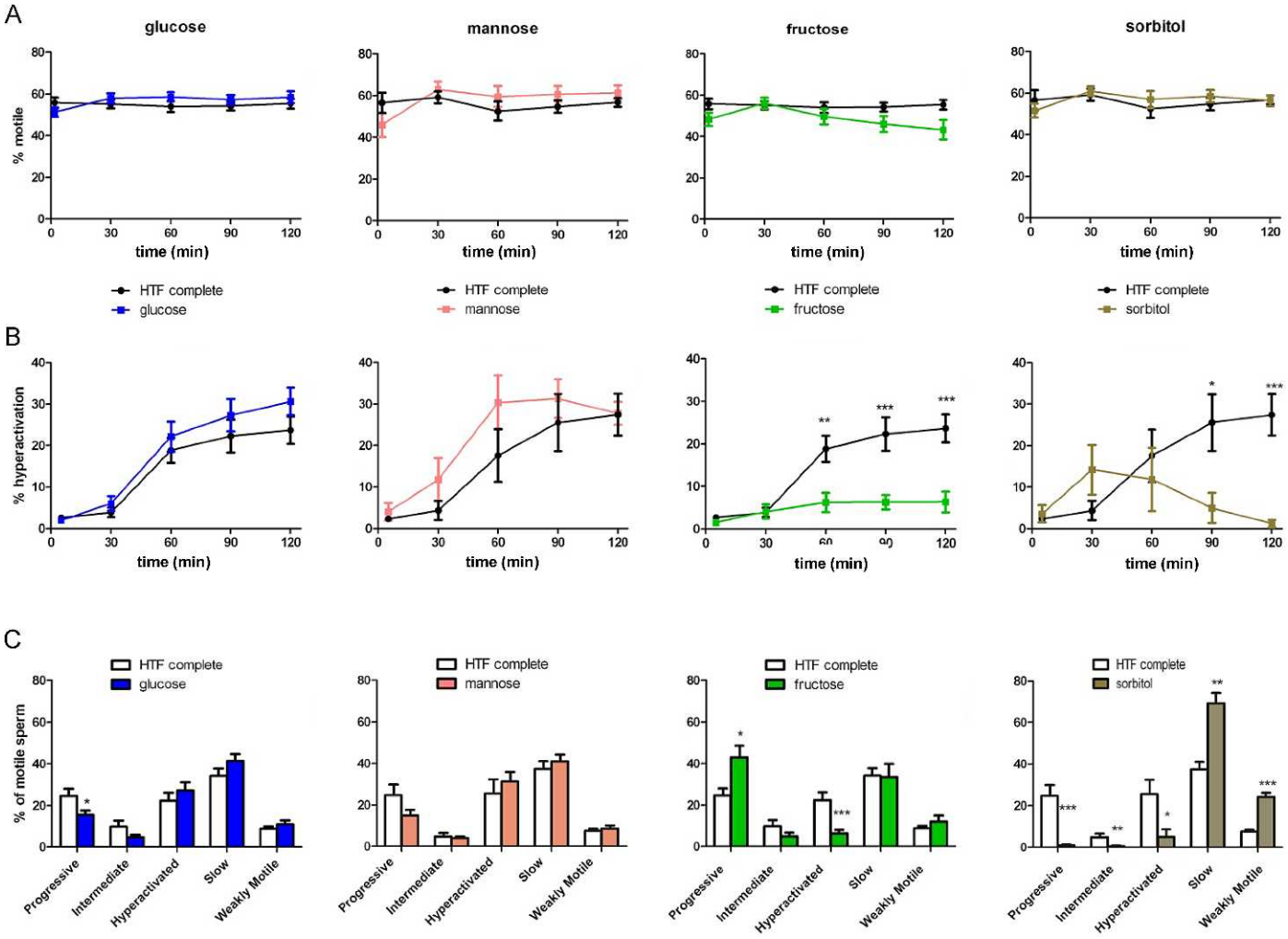


FIG. 2. Total motility, hyperactivation, and motility profiles of sperm incubated with glycolysable substrates. Sperm were incubated in HTF complete medium or HTF medium with 2.78 mM glucose (blue), 5 mM mannose (pink), 5 mM fructose (green), or 5 mM sorbitol (olive) as the sole energy substrate. **A** and **B**) At the indicated time points, sperm were analyzed for total motility (**A**) and percentage hyperactivation (**B**). **C**) Motility profiles of sperm incubated in HTF complete medium (open bars) or HTF medium with a glycolysable substrate (colored bars) for 90 min. Data are represented as the mean ± SEM of sperm from five or more mice. Differences between conditions at corresponding time points were analyzed using two-tailed, unpaired *t*-test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

99% were classified in the nonvigorous categories of slow or weakly motile.

Our basic CASA analyses demonstrated that all single substrates examined, except citrate, supported comparable percentages of sperm motility. CASAnova analysis of sperm motility profiles revealed additional differences in the ability of these substrates to support capacitation-associated changes in sperm motility. Vigorous motility was not supported by sorbitol or citrate, and hyperactivation was fully supported only by glucose and mannose.

Effects of Single Substrates on Capacitation-Associated Tyrosine Phosphorylation

Sperm capacitation is correlated with the tyrosine phosphorylation of a distinct subset of proteins [56], previously reported to occur at highest levels in the presence of glucose [27, 57]. We incubated sperm from individual mice or from pools of two to three mice (to simultaneously assess the effect of all single substrates on the sperm population) for 2 h in HTF medium with single substrates and examined tyrosine phosphorylation patterns by Western blot analysis using the phosphotyrosine-specific antibody 4G10. Three glycolysable substrates (glucose, mannose, and fructose) consistently supported the full, robust tyrosine phosphorylation pattern seen in HTF complete medium (Fig. 4A). However, sorbitol did not support capacitation-dependent tyrosine phosphorylation, even though sorbitol dehydrogenase present in mouse sperm flagella [27] should convert this monosaccharide to fructose.

We observed greater variability in the tyrosine phosphorylation patterns of sperm incubated in media containing nonglycolysable substrates (Fig. 4, B and C). Hydroxybutyrate supported the full pattern of tyrosine phosphorylation, comparable to HTF complete medium, in most experiments, but the signal intensity was reduced in others. Lactate and pyruvate supported partial tyrosine phosphorylation in some experiments, although the intensity of the signal was usually weaker than that for sperm incubated in HTF complete medium and the phosphorylated doublet with apparent molecular weights of 84 000 and 88 000 (arrows in Fig. 4, B and C) usually was not observed. Citrate did not support capacitation-dependent tyrosine phosphorylation.

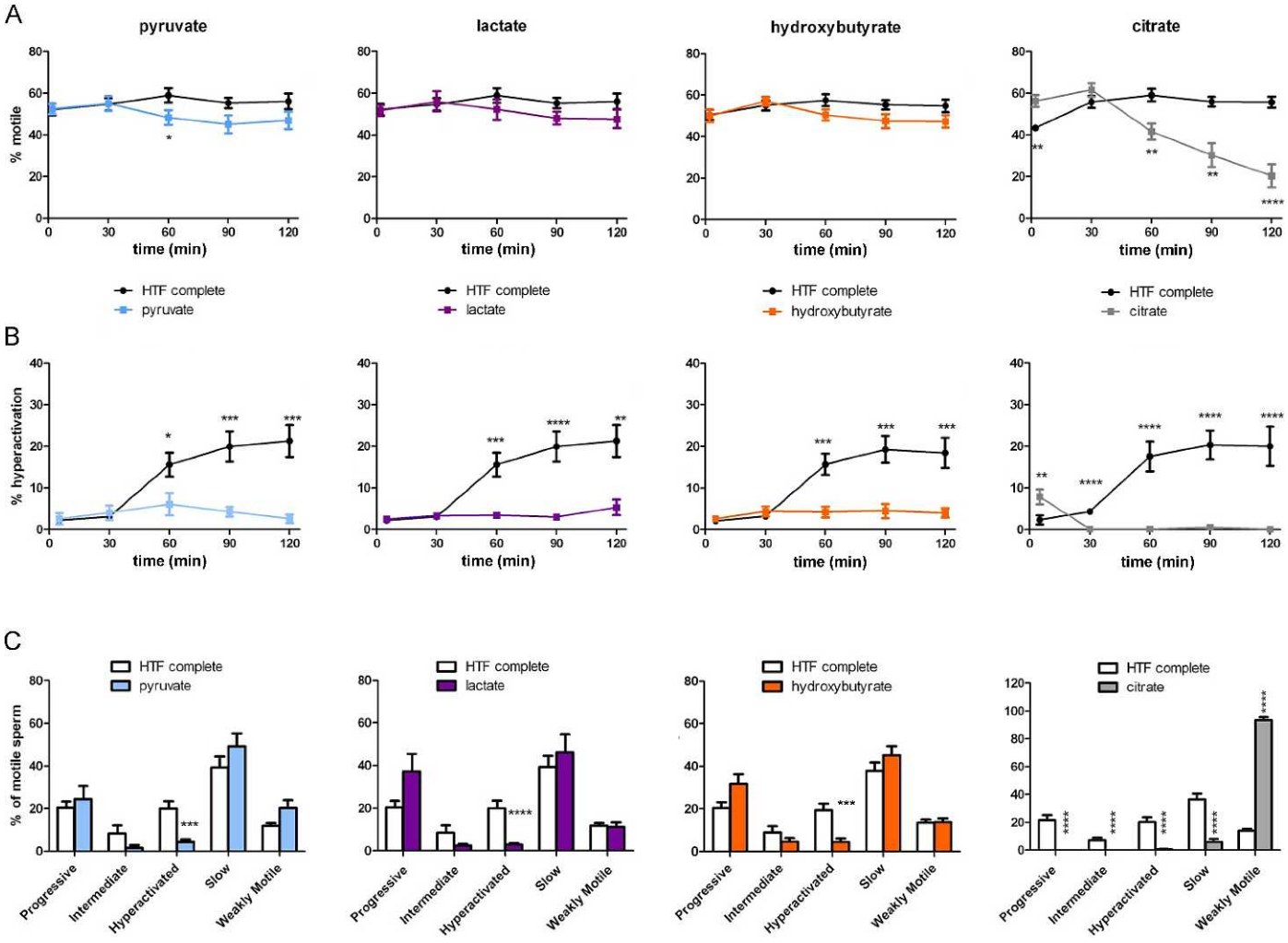


FIG. 3. Total motility, hyperactivation, and motility profiles of sperm incubated with nonglycolysable substrates. Sperm were incubated in HTF complete medium or HTF medium with 0.33 mM pyruvate (light blue), 21.4 mM lactate (purple), 5 mM hydroxybutyrate (orange), or 10 mM citrate (gray) as the sole energy substrate. **A** and **B**) At the indicated time points, sperm were analyzed for total motility (**A**) and percentage hyperactivation (**B**). **C**) Motility profiles of sperm incubated in HTF complete medium (open bars) or HTF medium with a glycolysable substrate (colored bars) for 90 min. Data are represented as the mean ± SEM of sperm from eight or more mice. Differences between conditions at corresponding time points were analyzed using two-tailed, unpaired *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Effects of Inhibitors of Glycolysis and Mitochondrial Function on Sperm ATP Levels and Motility

The present study indicates that both glycolysable and nonglycolysable substrates maintain sperm motility throughout 2-h in vitro incubation periods and that both types of substrates support at least partial capacitation-dependent tyrosine phosphorylation. However, individual substrates within these two categories have variable capacities to sustain motility or support the molecular and physiological changes associated with sperm capacitation. To assess these substrate differences in more detail, we assayed sperm ATP and motility parameters in the presence of the ACH (Fig. 5), a glycolytic inhibitor, or CCCP (Fig. 6), an uncoupler of oxidative phosphorylation. These experiments compared two glycolysable substrates (glucose and fructose) that varied in their ability to support hyperactivation and two nonglycolysable substrates (pyruvate and hydroxybutyrate) that varied in their ability to support capacitation-dependent tyrosine phosphorylation.

We first monitored ATP levels throughout 2-h in vitro capacitation intervals and found no statistically significant differences between sperm incubated in HTF medium with any single substrate and sperm incubated in HTF complete medium (data not shown). We then tested the ability of these substrates to support ATP and motility in the presence of 10 mM ACH or DMSO as a vehicle control. As anticipated, ATP levels of sperm incubated with glycolysable substrates (2.78 mM glucose or 5 mM fructose) were dramatically reduced after 90 min in the presence of ACH (Fig. 5A). The percentage of motile sperm also declined under these conditions, although the reduction was not statistically significant with fructose as the sole substrate (Fig. 5B). However, examination of CASAnova motility profiles demonstrates that greater than 90% of motile sperm incubated with glucose (Fig. 5C) or fructose (Fig. 5D) were classified as weakly motile when glycolysis was inhibited by the addition of ACH.

When sperm were incubated with nonglycolysable substrates (0.33 mM pyruvate or 5 mM hydroxybutyrate), the addition of ACH caused no significant changes in ATP levels (Fig. 5A) or percentage motility (Fig. 5B). Motility profiles of sperm incubated in hydroxybutyrate plus ACH were also unaltered compared to those for the vehicle control (Fig. 5F). However, a significant increase was found in the percentage of weakly motile sperm after incubation for 90 min in pyruvate

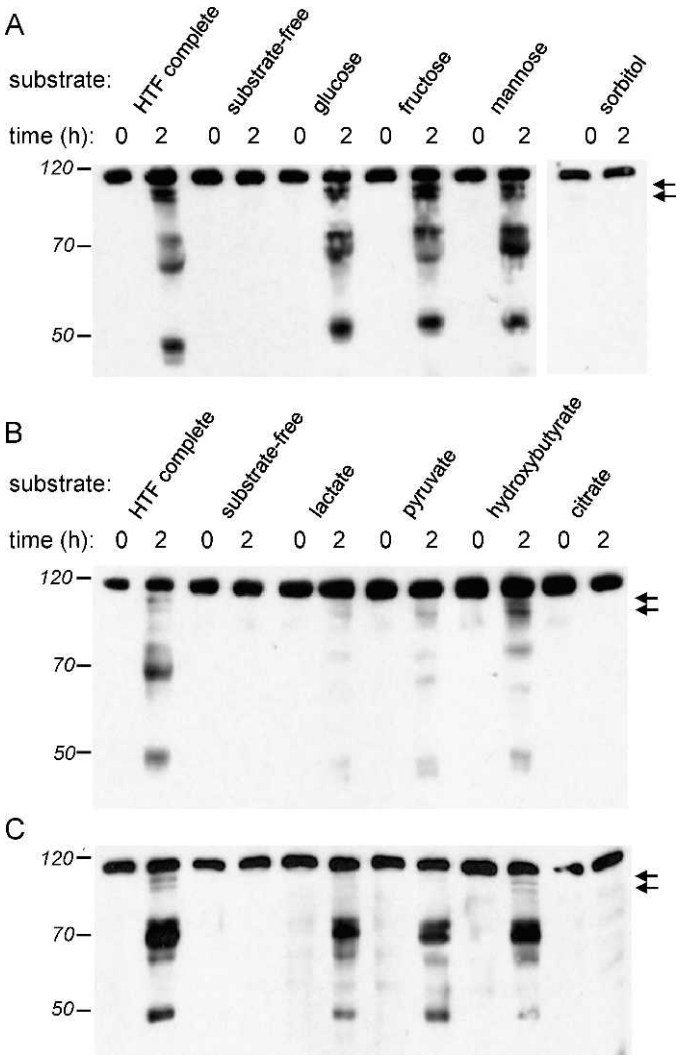


FIG. 4. Capacitation-associated tyrosine phosphorylation of sperm incubated in glycolysable or nonglycolysable substrates. Pooled sperm from two mice were divided and incubated for 0 or 2 h in HTF complete medium, substrate-free medium, or HTF medium with individual glycolysable substrates (**A**) or non-glycolysable substrates (**B** and **C**). Sperm were lysed and subjected to SDS-PAGE, followed by Western blot analysis with 4G10. Experiments were repeated a minimum of seven times for each substrate. Blots in **A** and **B** are from the same pool of sperm, except sorbitol. The blot in **C** is from a different pool of sperm to illustrate the observed variability of tyrosine phosphorylation in the presence of nonglycolysable substrates. Arrows denote a phosphorylated doublet with apparent molecular weights of 84 000 and 88 000.

plus ACH, suggesting that inhibition of sperm glycolysis has an impact on pyruvate metabolism.

Analysis of CASAnova motility profiles also revealed unexpected differences when sperm were incubated for 90 min with single substrates in the presence of CCCP. As expected, this uncoupling agent reduced both ATP levels (Fig. 6A) and percentage motility (Fig. 6B) to near zero when added to sperm incubated with pyruvate or hydroxybutyrate, substrates typically metabolized in the mitochondria. Sperm that retained motility under these conditions were classified as weakly motile (Fig. 6, E and F). ATP levels and percentage motility were unaffected when CCCP was added to sperm incubated with glycolysable substrates (glucose or fructose) (Fig. 6, A and B). When motility profiles were compared, sperm incubated in glucose plus CCCP displayed only small, but significant, reductions in the mean percentages of progressive and intermediate motility patterns (Fig. 6C). The addition of CCCP to sperm incubated with fructose caused more substantial shifts in motility profiles. The mean percentage of hyperactivated sperm increased to 30% in fructose plus CCCP (Fig. 6D), comparable to the levels achieved with glucose, mannose, or HTF complete medium. This change was accompanied by decreased progressive and increased nonvigorous motility profiles, similar to the motility transitions observed during capacitation in HTF compete medium (Fig. 1D).

Effect of Glucose and Fructose on Sperm pH_i

An increase in sperm pH_i occurs during capacitation and is required for hyperactivation and fertilization [46, 58, 59]. In a model of rat hepatocyte anoxia, fructose reduced pH_i compared to glucose [60]. We determined the pH_i of sperm loaded with BCECF-AM and incubated in HTF medium containing glucose or fructose compared to that under noncapacitating conditions in which bicarbonate was omitted from the medium (Fig. 7). Noncapacitating conditions maintained a constant pH_i over the time course, as expected [61]. Sperm incubated with glucose or fructose displayed an increase in pH_i of approximately 0.38 pH units, from approximately 6.5 to 6.9, and did not differ significantly at any time point. This calculation is based on a standard curve of BCECF fluorescent ratios at several calibrated pH values [46]. Because BCECF undergoes a spectral shift in sperm [61] and variable amounts of residual dye remain after washing, the absolute pH values should only be considered in reference to this particular set of experiments; it is more important to note the relative pH unit increase over time in comparison to other studies of pH_i [61–63]. These results suggest that the functional differences between glucose and fructose occur downstream of intracellular alkalinization in capacitation.

Substrate Preincubation Experiments

Previous studies reported that glucose is required for the initiation of sperm capacitation but may not be necessary for the completion of functional transitions leading to fertilization [64]. We incubated sperm sequentially in HTF medium with glucose or fructose to determine if glucose priming was sufficient to induce hyperactivation. Sperm were preincubated for 30 min in HTF medium containing either 2.78 mM glucose (Fig. 8A) or 5 mM fructose (Fig. 8B), followed by washing and resuspension in medium containing no substrates, glucose, or fructose. Hyperactivation levels were determined at 30, 60, and 90 min after resuspension in each medium. In the absence of energy substrates, sperm preincubated in glucose maintained some hyperactivation at all time points analyzed (Fig. 8A), whereas sperm preincubated in fructose did not (Fig. 8B). After preincubation in glucose, sperm resuspended in glucose achieved levels of hyperactivation comparable to those observed in HTF complete medium (Fig. 8A). Sperm resuspended in fructose after glucose preincubation did not exhibit increased hyperactivation above the levels observed following resuspension in substrate-free medium. Hyperactivation levels also remained low in sperm preincubated and resuspended in fructose (Fig. 8B). However, preincubation in fructose did not prevent sperm from achieving high levels of hyperactivation when resuspended in glucose (Fig. 8B). These results suggest that continuous glucose metabolism is required for stimulating hyperactivation to the levels typically achieved in HTF complete medium.

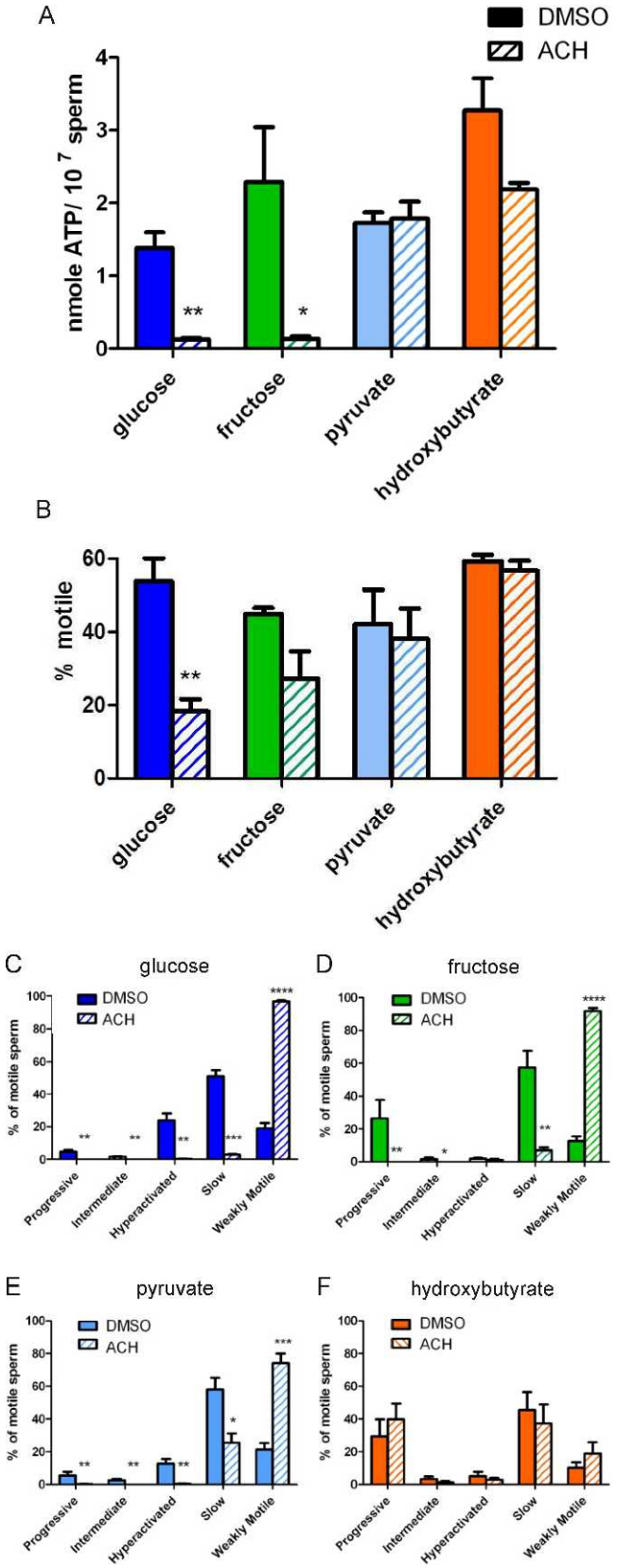


FIG. 5. ATP levels, percentage motility, and motility profiles incubated in the presence of an inhibitor of glycolysis. Sperm were incubated in HTF medium containing 2.78 mM glucose, 5 mM fructose, 0.33 mM pyruvate, or 5 mM hydroxybutyrate in the presence of either 10 mM ACH or the vehicle control (DMSO). After 90 min of incubation, sperm were analyzed for ATP content (**A**), percentage motility (**B**) and motility profiles (**C**, glucose; **D**, fructose; **E**, pyruvate; and **F**, hydroxybutyrate). Data are represented as the mean ± SEM of sperm from three or more mice. Differences between conditions at corresponding time points were analyzed using two-tailed, unpaired *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Analysis of Metabolic Differences Between Sperm Incubated in Glucose or Fructose

To further explore differences between the ability of fructose and glucose to support hyperactivation, we analyzed the metabolic profiles of sperm incubated in each of these substrates (Table 1). For these assays, eight sperm samples pooled from multiple mice were divided in half for parallel, 90-min incubations in HTF medium with either 2.78 mM glucose or 5 mM fructose. We analyzed both the aqueous and organic phases of extracts, and metabolites were detected using both gas chromatography-MS and LC-MS. Significantly altered peaks were identified ($P < 0.05$), and metabolites were confirmed using biochemical standards. Our analyses showed that flux through the glycolytic pathway was not significantly different between the two substrates, because lactate production was comparable (glucose:fructose ratio = 1.08, $P = 0.37$). Metabolic profiles also revealed an increase in the abundance of four confirmed metabolites in fructose-incubated samples: azelaic acid, mannose, taurine, and carnosine. The most striking difference between glucose- and fructose-metabolizing sperm involved azelaic acid, which was 40-fold higher when sperm were incubated in HTF medium with fructose. Azelaic acid is an end product of linoleic acid peroxidation and possesses a number of properties, including tyrosinase inhibition [65], inhibition of mitochondrial enzymes [66], ROS scavenging [67], inhibition of anaerobic glycolysis [68], and inhibition of thioredoxins in melanoma cells [69]. Whereas the antimitochondrial and antiglycolytic properties of azelaic acid are potential reasons for the difference between glucose and fructose effects on sperm, metabolic profiling did not detect significant differences in tricarboxylic acid (TCA) cycle intermediates.

Taurine was also more abundant in sperm incubated in medium containing fructose. Taurine is an end product of cysteine metabolism and is a key organic osmolyte [70]. Taurine is present at high levels in both human semen and sperm [71], and it has been shown to inhibit sodium-potassium ATPase activity in hamster sperm [72]. In addition, taurine protected rabbit sperm from lipid peroxidation and maintained sperm motility [73]. It is also possible that taurine is evidence of increased antioxidant activity in these sperm, because the antioxidant hypotaurine interacts with free radicals in a complex reaction that results in the production of taurine [71].

Carnosine, a natural dipeptide composed of β-alanine and histidine [74], was elevated in sperm incubated with fructose. It has been tested in Tris-based diluents for the storage of ram sperm [75], and it is a powerful antioxidant capable of inhibiting lipid peroxidation [76–78]. Carnosine was 70% more abundant in sperm metabolizing fructose, whereas its precursor, histidine, was elevated twofold in sperm metabolizing glucose, suggesting that there may be processes that induce alterations in redox processes in sperm metabolizing fructose.

In addition to histidine, only one other metabolite, dimethylsuccinate, was increased in sperm utilizing glucose. Whereas dimethylsuccinate has been used to supply an exogenous source of succinate, its endogenous function is unknown [79, 80].

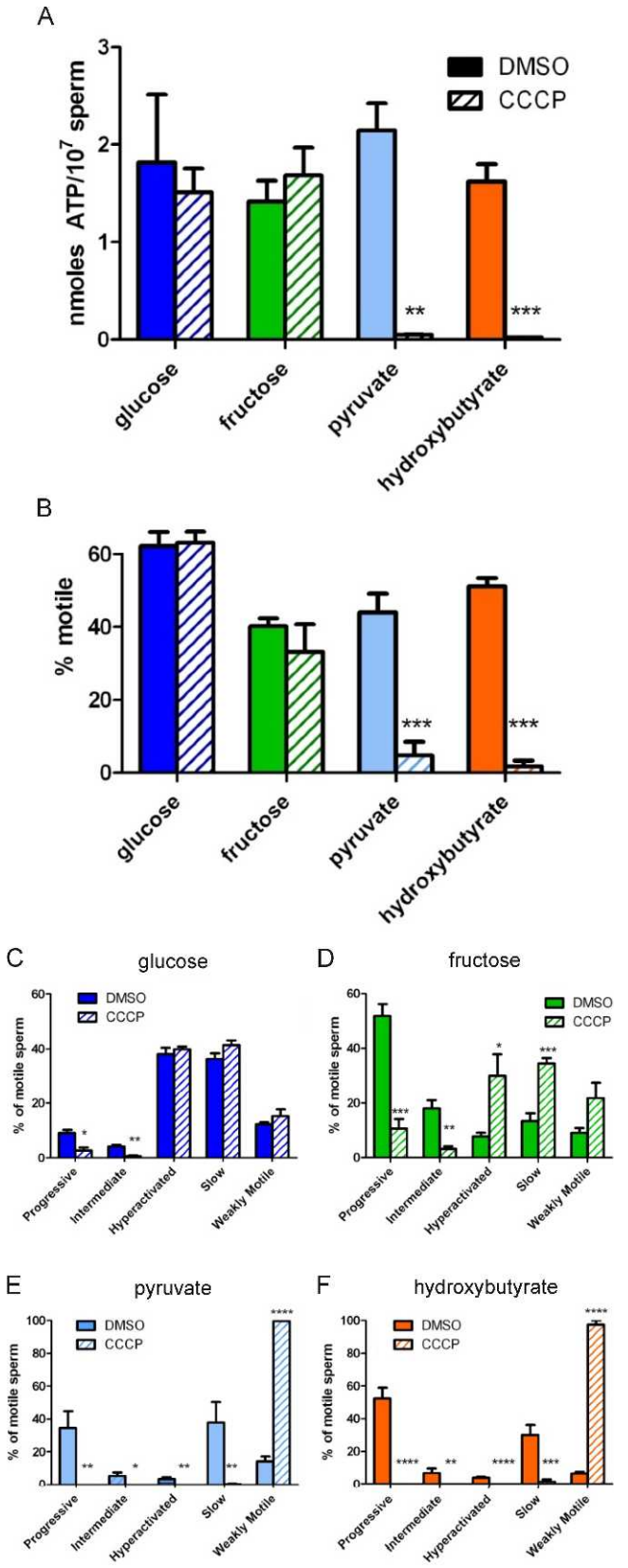


FIG. 6. ATP levels, percentage motility, and motility profiles incubated in the presence of an uncoupler of oxidative phosphorylation. Sperm were incubated in HTF medium containing 2.78 mM glucose, 5 mM fructose, 0.33 mM pyruvate, or 5 mM hydroxybutyrate in the presence of either 10 μM CCCP or the vehicle control (DMSO). After 90 min of incubation, sperm were analyzed for ATP content (**A**), percentage motility (**B**), and motility profiles (**C**, glucose; **D**, fructose; **E**, pyruvate; **F**, hydroxybutyrate). Data are represented as the mean ± SEM of sperm from three or more mice. Differences between conditions at corresponding time points were analyzed using two-tailed, unpaired *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Analysis of ROS Production in Sperm Metabolizing Glucose or Fructose

Endogenous ROS production influences sperm capacitation and hyperactivation in multiple species, including mouse [49, 50, 52, 81–83]. Because metabolites with redox properties were higher in the presence of fructose, we assessed endogenous ROS levels of sperm metabolizing glucose or fructose during in vitro capacitation. Whereas variations in the overall levels of ROS production were found among mice, presumably due to biological variation among individuals [50], ROS levels increased steadily over time in the presence of both substrates (Fig. 9). Sperm metabolizing fructose had endogenous ROS levels approximately twice those detected with glucose over the capacitation period in sperm from the six mice examined. This increased ROS production correlates with the increased presence of redox-associated metabolites when sperm metabolize fructose, suggesting that redox pathways are differentially affected by these glycolytic substrates.

DISCUSSION

To explore interactions between sperm metabolism and capacitation-associated signaling pathways, we investigated the impact of individual substrates on mouse sperm throughout an in vitro capacitation period using an experimental system that both avoids contamination by endogenous substrates and performs detailed analyses of sperm motility patterns. We examined individual sperm processes rather than the end point of fertilization, because the egg has its own metabolic requirements [84, 85]. Our results indicate that both glycolysable and nonglycolysable substrates can maintain steady-state ATP levels and percentage motility throughout a 2-h capacitation period. However, these substrates exhibit differential abilities to support hyperactivation and tyrosine phosphorylation. These results highlight the differential consequences between generating ATP in the midpiece mitochondria versus glycolysis in the principal piece of the sperm flagellum and reiterate the importance of compartmentalized ATP production in these highly polarized cells.

Sperm incubated with substrates metabolized within mitochondria were not capable of efficiently undergoing hyperactivated motility. These results were expected, because several reports have appeared that glycolysis is required for hyperactivation [14, 26, 86]. However, the impact of nonglycolysable substrates on tyrosine phosphorylation was much more variable. Hydroxybutyrate supported the full pattern of tyrosine phosphorylation more consistently than either lactate or pyruvate. Whereas glycolysis has been reported to be required for tyrosine phosphorylation [57, 87], the ability of hydroxybutyrate to support tyrosine phosphorylation indicates that there may be other modes of regulation linking metabolism to this step in capacitation. When the full pattern of tyrosine phosphorylation was not maintained, the most consistent result was loss of the doublet at molecular weights of approximately 84 000 and 88 000. The identity of these proteins is not known, although a number of proteins that undergo tyrosine phosphorylation have been identified in this range in sperm from multiple species [88–92].

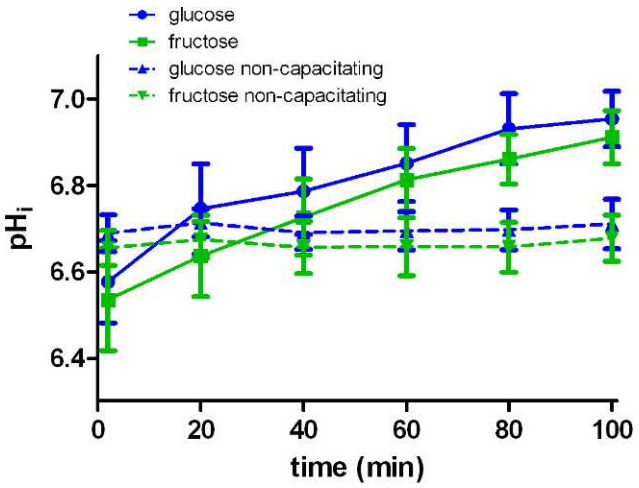


FIG. 7. Intracellular pH of sperm incubated in glucose or fructose-containing HTF medium. Sperm were loaded with 3 μM BCECF-AM and incubated in medium containing HTF with 2.78 mM glucose (blue, solid lines) or 5 mM fructose (green solid lines). Sperm were also incubated with these substrates in noncapacitating media (dashed lines). The pH_i was determined from BCECF fluorescence ratios at each time point and calculated against a standard curve of known pH values. Data are represented as the mean ± SEM of sperm from five mice.

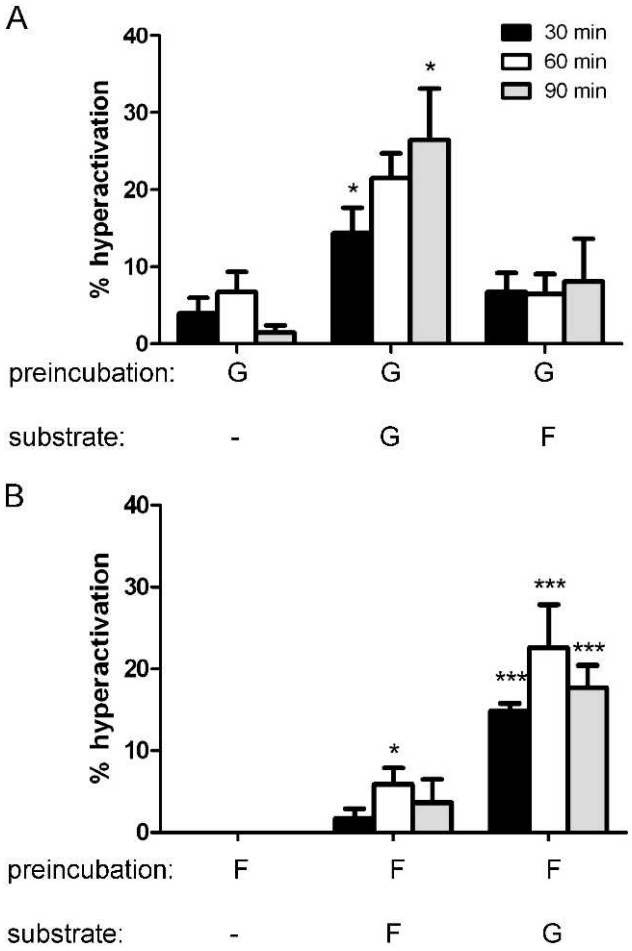


FIG. 8. Hyperactivation after preincubation in glucose or fructose-containing HTF medium. Sperm were preincubated in medium containing HTF with 2.78 mM glucose (**A**) or 5 mM fructose (**B**) for 30 min, washed, and then resuspended in either substrate-free HTF medium, HTF medium with 2.78 mM glucose, or HTF medium with 5 mM fructose. The percentage of the motile population displaying hyperactivated motility was determined 30 min (black bars), 60 min (open bars), or 90 min (gray bars) after resuspension. Data are represented as the mean ± SEM of sperm from four mice. Differences between conditions at corresponding time points were analyzed using one-way ANOVA. $^{*}P < 0.05$, $^{***}P < 0.001$.

Differential effects of individual substrates were also observed in the presence of metabolic inhibitors, further highlighting the complex integration of metabolism and signaling. As expected, ACH had no effect on hydroxybutyrate metabolism and did not change ATP levels or percentage motility of sperm incubated with pyruvate. However, a marked reduction in vigorous motility and increase in weakly motile sperm were found when ACH was added with pyruvate. This raises the possibility that inhibition of sperm glycolysis, perhaps by altering NAD^+:NADH ratios, may influence the metabolism of substrates typically utilized by the mitochondria. These results also suggest that multiple pathways regulate functional changes in sperm motility that occur during capacitation.

Our analysis of glycolytic substrates in the present study further refines our understanding of the role of glycolysis in supporting events leading to fertilization. Both glucose and mannose supported hyperactivation and tyrosine phosphorylation at levels comparable to HTF complete medium. The effectiveness of 2.78 mM glucose is consistent with previous studies reporting capacitation-associated changes at micromolar concentrations of this monosaccharide [57, 93]. Fructose did not support hyperactivation, as in previous studies [26, 86], but did support the full pattern of tyrosine phosphorylation comparable to HTF complete medium. Because we only performed one-dimensional analysis of tyrosine phosphorylation, we cannot rule out the possibility of differential phosphorylation patterns with different substrates that could be visualized using two-dimensional analysis. In contrast to a previous study [27], sorbitol did not support tyrosine phosphorylation or maintain hyperactivation after 30 min, and the loss of hyperactivated motility was correlated with the loss of all patterns of vigorous motility. Differences between sperm isolation conditions, particularly related to reduction of endogenous epididymal substrates, may have contributed to these contrasting results, because the earlier study observed hyperactivation without any added substrates [27].

The differences we observed in sperm functional parameters suggest that capacitation-dependent signaling pathways are differentially stimulated during the transport or metabolism of individual substrates. To explore these differences in more detail, we focused subsequent experiments on comparisons between glucose and fructose. Like glucose, fructose can enter the top half of glycolysis via phosphorylation to fructose-6-phosphate. However, fructose can also be metabolized to fructose-1-phosphate via ketohexokinase and enter glycolysis at the GAPDHS step via the Hers pathway, bypassing the initial steps in glycolysis [94, 95]. Any signaling that arises from the initial portion of the pathway would be bypassed under these circumstances if the Hers pathway is active in sperm. Low levels of ATP and weak motility were observed when ACH was added in the presence of either glucose or fructose. Under these conditions, small amounts of ATP may be derived from the metabolism of endogenous phospholipids, resulting in the generation of mitochondrial substrates such as glycerol-3-phosphate [96].

We found that fructose supports hyperactivation when mitochondrial function is uncoupled by CCCP. This compound affects cells in multiple ways, including disruption of pH_i and alteration of ROS levels in a tissue and substrate-specific

TABLE 1. Confirmed metabolites differentially present in sperm incubated with glucose or fructose.

Metabolite	Abundance[a]	Average glucose signal[b]	Average fructose signal[b]	Glucose:fructose ratio	*P* value
Substrate-dependent					
Azelaic acid	Fructose	0.44 ± 0.05	20.44 ± 7.6	0.021	0.02
Mannose	Fructose	0.03 ± 0.005	0.1 ± 0.02	0.262	0.003
Taurine	Fructose	0.002 ± 0.001	0.004 ± 0.0005	0.576	0.024
Carnosine	Fructose	0.002 ± 0.0003	0.004 ± 0.0004	0.592	0.005
Dimethyl succinic acid	Glucose	0.0014 ± 0.0001	0.001 ± 0.00006	1.312	0.046
Histidine	Glucose	0.001 ± 0.0003	0.0073 ± 0.0001	2.007	0.02
Glycolysis					
Lactate	N/A	42.73 ± 2.73	39.3 ± 2.54	1.087	0.374
Pyruvate	N/A	0.18 ± 0.03	0.12 ± 0.013	1.502	0.088
TCA					
Citrate	N/A	0.14 ± 0.04	0.48 ± 0.31	0.303	0.300
Fumarate	N/A	0.28 ± 0.06	0.46 ± 0.07	0.595	0.070
Succinate	N/A	1.28 ± 0.22	0.98 ± 0.06	1.306	0.205
Malate	N/A	0.5 ± 0.1	0.32 ± 0.02	1.595	0.092

[a] N/A, not applicable.
[b] Signal values are shown as the average ± SEM of the area under the peak for each metabolite normalized to the total area under all peaks per sample.

manner [97–99]. Because elevation of pH_i is required for capacitation [61, 100], we hypothesized that CCCP induction of hyperactivation might be due to alterations in pH_i when sperm are incubated with fructose. However, we did not find any difference in the pH_i of sperm incubated with these substrates. It is important to note that the vast majority of the BCECF signal in sperm emanates from the head and not the flagellum [61, 101]; thus, compartmentalized changes in pH_i may not be apparent. For example, CatSper channel activity is predicted to be highest at pH 7.0–8.0, above the levels detected in our experiments [102]. The flagellar pH_i may indeed reach this level in hyperactivated sperm, but it cannot be detected using our approach of whole-cell averaging of pH_i in sperm populations. In addition, we cannot rule out the possibility that transient changes in pH_i could occur immediately upon the initiation of substrate metabolism and affect downstream signaling. However, in addition to decoupling mitochondria, CCCP will also alter pHi by allowing proton flux across the plasma membrane. Any CCCP effect caused by alterations in pHi would be predicted to be the same for both glucose and fructose. This suggests that the differences observed in the presence of CCCP are not via pH_i and its regulation of the CatSper channels.

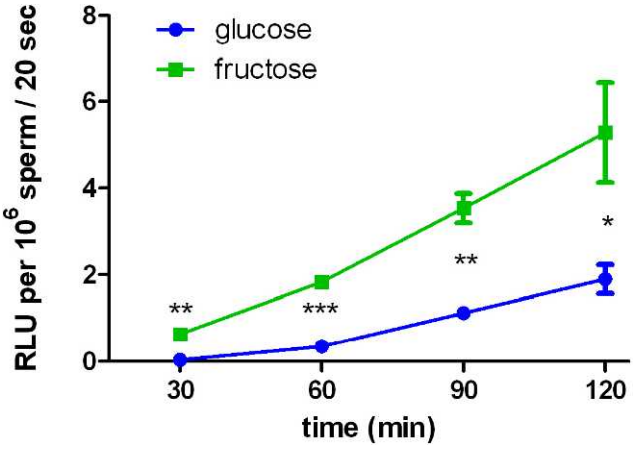


FIG. 9. Luminol-dependent ROS production in sperm incubated in the presence of glucose or fructose. Sperm from two mice were pooled and incubated in medium containing either 2.78 mM glucose (blue) or 5 mM fructose (green) along with 50 μM luminol and 8.8 U of HRP. ROS levels were determined by measurement of luminol signal over a 20-sec integration period. The graph is representative of six experiments. Data are represented as mean ± SEM of triplicate measurements. Differences between conditions at corresponding time points were analyzed using two-tailed unpaired *t*-test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Earlier studies found that sperm could fertilize oocytes in the absence of substrates if they were previously incubated in glucose, leading to the hypothesis that glucose ''priming'' was sufficient for sperm to become fertilization competent [64, 86]. In our experiments, sperm preincubated in glucose for 30 min did not exhibit increased hyperactivation when transferred to fructose-containing medium. Fraser and Quinn [86] preincubated sperm in glucose-containing medium for 2 h, a period that is typically sufficient for the development of maximal levels of hyperactivation, before mixing with oocytes. Okabe et al. [64] preincubated sperm in glucose medium for 40 min and observed zona penetration within 10 min of mixing with oocytes. However, these experiments did not quantitate the percentage of sperm undergoing hyperactivation. In control conditions where sperm were preincubated in HTF medium with glucose and then resuspended in substrate-free HTF medium, we observed that sperm were capable of maintaining some hyperactivated motility after being resuspended in media without substrates. This residual hyperactivity may be due to the remaining intracellular glucose or its metabolites still functioning in the sperm. Similar residual hyperactivation could contribute to the zona penetration observed by Okabe et al. We observed high levels of hyperactivation only when glucose was present in the medium, suggesting that active glucose metabolism is required for both the initiation and the maintenance of hyperactivated motility during in vitro capacitation.

Because our preincubation experiments suggested the signaling cascades that stimulate hyperactivation require the continuous metabolism of glucose, we examined the metabolic profiles of sperm utilizing glucose or fructose. Analysis of these profiles identified several significant differences. Because we used unlabeled sugars, we were unable to determine which metabolites were direct products of substrate utilization. However, this approach produces a universal profile of both primary and secondary metabolic pathways. The metabolites we identified have many activities. For example, the elevation of taurine in fructose-incubated sperm may indicate increased osmotic stress in these cells due to altered ion-channel function or could result from the conversion of hypotaurine to taurine in redox reactions. Overall, however, the results indicate that sperm incubated with fructose generated higher levels of metabolites associated with redox activities.

This increased abundance of redox metabolites correlates with increased levels of ROS in sperm utilizing fructose, in agreement with previous studies of rat sperm and other cell types [50, 103, 104]. Although high levels of ROS have been associated with infertility [105–108], low levels of ROS facilitate capacitation in both human and mouse sperm [49, 82, 83]. The levels of ROS detected in the presence of fructose may exceed the optimal levels for capacitation, accounting for the failure of these sperm to hyperactivate. In addition to serving as a substrate for glycolysis, glucose can be metabolized via the pentose phosphate pathway, thereby generating reducing equivalents in the form of NADPH [109, 110]. This cofactor promotes regeneration of reduced glutathione, which plays an important role in protecting mouse sperm from lipid peroxidation [111]. Metabolism of fructose via the Hers pathway would bypass the NADPH-regenerating phase of the pentose phosphate pathway, which is reported to be important in capacitation and fertility [109, 110]. Loss of NADPH-reducing equivalents in the presence of fructose would facilitate the elevation of ROS in sperm. This may partially explain the ability of CCCP to restore hyperactivation in sperm utilizing fructose, because ROS levels were reduced in sperm incubated with fructose and CCCP compared to fructose alone (data not shown). Further studies are needed to elucidate the mechanism by which CCCP induces hyperactivation in the presence of fructose, but CCCP has been shown to reduce ROS levels in a tissue- and substrate-dependent manner [99].

The present results suggest that glucose and fructose may exert their differential effects on capacitation through alterations in redox pathways. Studies in several mammalian species provide evidence that redox signaling plays an important role in regulating sperm function, including the changes that occur during capacitation [49, 51, 52, 112–114]. Further investigation of interactions between metabolism and redox pathways are likely to enhance our understanding of the molecular mechanisms that regulate capacitation and may uncover new targets for modulating sperm function.

ACKNOWLEDGMENT

The authors thank Drs. Harvey Florman, Fanny Odet, and Polina Danshina for their helpful discussions of the data; Dr. Ana Maria Salicioni for her assistance with technical issues; and Dr. Michael O'Rand for providing generous access to laboratory equipment.

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Summary: Both glycolytic and nonglycolytic substrates exhibit differential effects on sperm motility, hyperactivation, and tyrosine phosphorylation during capacitation, and differences between glucose and fructose are correlated with alteration of redox pathways.