Energy metabolism and intracellular pH in boar spermatozoa

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The effect of energy metabolism on intracellular pH was studied in boar spermatozoa using nuclear magnetic resonance (NMR) spectroscopy and confocal microscopy with the pH-sensitive dye seminaphthorhodafluor (SNARF-1). Freshly ejaculated spermatozoa had a high adenylate energy charge (AEC = 0.8), which decreased to 0.6 under aerobic conditions and to 0.2 under anaerobic conditions. Correspondingly, no ATP resonances but high AMP resonance were visible in ³¹P-NMR-spectra of the spermatozoa. When an artificial oxygen buffer (Fluosol) and a purpose-built air supply system were used during ³¹P-NMR data acquisition, ATP resonances reappeared whereas the AMP resonance disappeared. Boar spermatozoa kept under aerobic conditions have intracellular compartments that differ markedly in pH, as demonstrated by both

³¹P-NMR spectroscopy and confocal microscopy. Using confocal microscopy, the midpiece of the flagellum in which all mitochondria are located was identified as an acidic compartment (pH_{i-mp} 6.7). The intracellular pH of both the head (pH_{i-h}) and the long principal piece of the flagellum (pH_{i-pp}) were 7.2 and, thus, only slightly below the extracellular pH (pH_e 7.3). Storage of spermatozoa in a glucose-free medium at 15°C when they are immotile slowly shifted the pH_{i-mp} from 6.7 to 6.9 within 20 h, whereas pH_{i-h} and pH_{i-pp} remained unchanged (pH 7.1–7.2). When glucose was present in the medium, all visible compartments of the spermatozoa as well as the medium were acidified to pH 6.2 within 20 h. Under these conditions a resonance at 4.8 ppm appeared representing glycerol 3-phosphate.

Introduction

Mammalian spermatozoa are highly specialized motile cells. They are elongated with a sequential arrangement of the different intracellular compartments. The driving force is generated by the long flagellum which consists of two parts: the relatively short midpiece containing all the mitochondria and the long principal piece that is free of mitochondria. Thus, oxidative phosphorylation is restricted to the midpiece. However, a central enzyme of glycolysis, a sperm-specific form of glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), is tightly bound to the fibrous sheath, a dense structure of the principal piece (Westhoff and Kamp, 1997; Bunch *et al.*, 1998), indicating that glycolytic ATP production occurs predominantly in the principal piece.

Sugars, such as glucose and fructose, lactate and fatty acids can fuel motility in boar spermatozoa, although under natural conditions fructose, glucose and lactate are the main substrates (Mann and Lutwak-Mann, 1981; Jones, 1997; Jones and Milmlow, 1997).

Unlike spermatozoa from many other vertebrates and invertebrates (Robitaille *et al.*, 1987; Kamp *et al.*, 1996), boar spermatozoa do not contain phosphorylcreatine and creatine kinase or any other phosphagens. However, it has been suggested that a phosphagen is necessary for the transport of energy-rich phosphate from the mitochondria in the midpiece to the distal dynein-ATPases (for review, see Kaldis *et al.*, 1997). The absence of a phosphagen indicates that the glycolytic ATP production in the principal piece is crucial for boar spermatozoa to function properly. The present study aims to compare mitochondrial and glycolytic ATP production in motile and immotile boar spermatozoa.

Boar spermatozoa are apt for this purpose because they have a peculiarity that facilitates studying the energy requirement for sperm motility: their motility can be reversibly switched off by cooling to 15–18°C. This treatment renders it possible to compare energy metabolism in motile and immotile spermatozoa. By convention, storage of semen at 15–18°C for at least 2 days is used in artificial insemination techniques (Weitze and Petzholdt, 1992). However, metabolic acidosis may prevent prolonged storage if no buffer is present. The

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present study examined pH changes in boar spermatozoa during rest (at 15°C) and motility (at 35°C) using ^{31}P -nuclear magnetic resonance (NMR) spectroscopy and fluorescence microscopy.

Changes in intracellular pH (pH_i) have already been reported in ram and boar spermatozoa by Gatti *et al.* (1993) who used the pH-dependent distribution of radioactive methylamine. These authors demonstrated that pH_i can be adjusted by the extracellular pH (pH_e). This finding could be part of the important mechanism by which sperm motility is triggered during ejaculation, when the slightly acidic epididymal fluid (Mann, 1964) is mixed with secretions from the accessory glands to result in a seminal fluid of pH 7.3 (Mann und Lutwak-Mann, 1981). The aim of the present study was to analyse the effect of energy metabolism on pH_i in fresh and stored boar spermatozoa.

Materials and Methods

Sperm samples

Boar semen of proven fertility was collected manually using a dummy sow and provided by the Pig Breeders Association (GfS, Ascheberg). The gel fraction of the semen was removed by gauze filtration. Sperm concentration and motility were assessed by microscopic observation.

Chemicals and enzymes

Chemicals and enzymes were purchased from Roche (Mannheim), Merck (Darmstadt) and Sigma (München). Fluosol (Alpha Therapeutic, Thetford) was a gift of G. Klöck, Biozentrum Würzburg. Fluorescent probes were from Molecular Probes Inc. Europe (Leiden). Beltsville thawing solution (BTS, pH 7.3) comprised 205.0 mmol glucose l^{-1} , 20.6 mmol trisodium citrate l^{-1} , 15.0 mmol NaHCO_3 l^{-1} , 3.4 mmol EDTA l^{-1} , 10.0 mmol KCl l^{-1} , 0.6 g penicillin l^{-1} and 1.0 g streptomycin l^{-1} .

Measurement of oxygen consumption

Oxygen uptake by spermatozoa was measured at 37°C and at 15°C in a Yellow Springs oxygen monitor that had been calibrated with air-saturated BTS.

Number of spermatozoa

Semen samples were diluted with distilled water to immobilize the spermatozoa. The number of spermatozoa was determined in a Neubauer chamber.

Perchloric acid extraction

After incubation, semen was swiftly transferred into a mortar filled with liquid nitrogen and the frozen material was ground to a fine powder. Aliquots of the powder

were used for determining the number of sperm heads and the contents of some metabolites. In the latter case, the semen powder was weighed while still frozen, mixed with 3 mol cold perchloric acid l^{-1} and homogenized using an Ultra Turrax (Janke und Kunkel, Staufen). The homogenate was centrifuged (30 000 *g* at 4°C for 10 min) and the cold supernatant was neutralized with 3 mol KOH l^{-1} containing 0.4 mol KCl l^{-1} . KClO_4 was removed by centrifugation (10 000 *g* at 4°C for 10 min).

Analysis of adenine nucleotides

The contents of ATP, ADP and AMP in the neutralized perchloric acid extracts of boar spermatozoa were measured fluorometrically using a Jobin Yvon spectrofluorometer JY 3 D (150 Watt Xe-lamp) with a standard detector S5 (Hamamatsu photomultiplier R212 UH). Fluorescence of NADH and NADPH was detected at 457 nm after excitation at 340 nm by fluorometric enzymatic assays. The assays were run in 2 ml volume at 30°C.

The ATP assay (pH 8.1) comprised 30.0 mmol Tris-HCl l^{-1} , 1.0 mmol magnesium (acetate) $_2$ l^{-1} , 1.0 mmol dithiothreitol l^{-1} , 0.02–0.05 mmol NADP^+ l^{-1} , 0.1 mmol glucose l^{-1} and 0.03 U glucose-6-phosphate dehydrogenase ml^{-1} (EC 1.1.1.49). The reaction was started with 0.2 U hexokinase ml^{-1} (EC 2.7.1.1).

The combined assays of ADP and AMP (pH 7.0) comprised 30.0 mmol piperazine-*N,N'*-bis-2-ethanesulphonic acid (PIPES) l^{-1} , 75.0 mmol KCl l^{-1} , 5.0 mmol magnesium (acetate) $_2$ l^{-1} , 1.0 mmol dithiothreitol l^{-1} , 0.2–10.0 μmol $\text{NADH} + \text{H}^+$ l^{-1} , 10–20.0 μmol phosphoenolpyruvate l^{-1} and 1.0 U lactate dehydrogenase ml^{-1} (EC 1.1.1.27). For the determination of ADP, the reaction was started with 1 U pyruvate kinase ml^{-1} (EC 2.7.3.2). After complete transformation of ADP, 5.0 μmol ATP l^{-1} was added and AMP determination was started with 0.4 U adenylate kinase ml^{-1} (EC 2.7.4.3).

The adenylate energy charge (AEC) was calculated as $\text{AEC} = ([\text{ATP}] + 0.5 [\text{ADP}]) / ([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$ (Atkinson, 1968).

^{31}P -nuclear magnetic resonance spectroscopy

The ^{31}P -NMR spectra were collected with a Bruker AM 360 spectrometer operating at 145.8 MHz. Samples were measured in 10 mm sample tubes. NMR spectroscopy of live spermatozoa was performed without spinning and without field frequency lock. The spectra were obtained with 50 pulses, 7 kHz spectral width and an acquisition time of 2.3 s. No proton decoupling was applied and processing was carried out with 10 Hz line broadening. The chemical shift of the glycerophosphorylcholine signal (GPC) which is independent of pH was used as an internal reference and set to ppm, which is consistent with external calibration with 85% (v/v)

H₃PO₄ (Robitaille *et al.*, 1991). This internal standard was preferred to an external reference because salt in the solvents can shift values up to 1 ppm (Robitaille *et al.*, 1991).

pH determination from ³¹P-NMR spectra

As the chemical shift of P_i is affected by the ionic composition of the medium (Robitaille *et al.*, 1991), P_i was titrated in a sperm extract (Fig. 1). Spermatozoa from fresh semen were collected by centrifugation (500 *g* at 15°C for 25 min) and washed twice in 160 mmol NaCl l⁻¹. After centrifugation (3000 *g* at 4°C for 10 min), sedimented spermatozoa (5 g wet weight) were resuspended in 15 ml of cold distilled water and homogenized with an Ultra-Turrax (2 × 20 s) and subsequently sonicated with a sonifier (2 × 5 s). Cell fragments were removed by centrifugation (30 000 *g* at 4°C for 30 min) and the supernatant was lyophilized. Assuming an intracellular water content of 50% wet mass, the lyophilisate of 1 g sperm wet mass was dissolved in 0.5 ml distilled water. For assessing the effect of dilution on the chemical shift, the lyophilisate of 1 g sperm wet mass was diluted in 1.25 ml water. Phenylphosphonate (PPA, 2 mmol l⁻¹) was used as an extracellular pH indicator (Juretschke and Kamp, 1990). pH values were adjusted in these sperm extracts by adding small volumes of 1 mol HCl l⁻¹ or 1 mol NaOH l⁻¹, and measured with a microelectrode (Ingold). The chemical shift values of P_i and PPA at the different pH values were subsequently determined by ³¹P-NMR spectroscopy. The titration curves were fitted by the Levenberg–Marquardt algorithm to Henderson–Hasselbalch equations.

Oxygen provision during NMR spectroscopy

For adequate NMR spectra a high density of cells in the sample tube is required. This was achieved by centrifugation of boar semen at 500 *g* at 15°C for 20–30 min. The supernatant was discarded and the soft sperm sediment mixed with Ringer solution (1:1 v/v, see below). Owing to sperm respiration, the medium will readily become hypoxic without an oxygen supply. In the experiments of the present study, aerobic conditions during NMR measurements were achieved by using the oxygen buffer Fluosol and an automatic air supply system: a final concentration of 20% (v/v) Fluosol was added to the incubation medium while pH was kept at 7.4 and osmolarity at 320–360 mOsmol l⁻¹. Oxygen-saturated Fluosol maintained aerobic metabolism at 15°C for about 10 min. For extended NMR spectroscopy, air was pumped at regular intervals directly into the sample through a small silicon tube, but was switched off during data acquisition to achieve sufficient field homogeneity. A cycle of 2 min of air pumping followed by a 30 s pause and 5 min of data acquisition proved sufficient to maintain aerobic conditions for several hours.

Fig. 1. Effect of pH on chemical shift values of inorganic phosphate (P_i) and phenylphosphonate (PPA) in ³¹P-nuclear magnetic resonance (NMR) spectra. The calibrations of the chemical shifts of P_i and PPA were performed in boar sperm extract and in Beltsville thawing solution (BTS) buffered with piperazine-*N,N'*-bis-2-ethanesulphonic acid (PIPES) (5 mmol l⁻¹), respectively. The Henderson–Hasselbalch equations were calculated iteratively using the Levenberg–Marquardt algorithm. The pK_a for PPA (▼) in BTS was 7.05 and the limiting chemical shifts at high and low pH were 13.85 and 11.87, respectively. The pK_a for P_i (●) in the extract of spermatozoa was 6.59 and the limiting chemical shifts were 0.66 and 3.16, respectively. If sperm extract was diluted 2.5-fold (○) the chemical shift versus pH curve of P_i shifted to higher pH values.

The Ringer solution (pH 7.4) for aerobic incubations comprised 30 mmol Hepes l⁻¹, 20 mmol KCl l⁻¹, 5 mmol MgCl₂ l⁻¹, 125 mmol NaCl l⁻¹, 20% Fluosol and 10 µl Silicon Anti Foam (Sigma) per 100 ml. Fluosol is a mixture of 17.5 g perfluorodecaline, 7.5 g perfluorotri-*n*-propylamine, 3.4 g poloxamer, 1.0 g glycerol USP, 0.5 g egg yolk phospholipids and 0.04 g potassium oleate in 100 ml H₂O.

Storage of semen with media containing glucose or inositol

BTS (pH 7.3) was prepared with 205 mmol glucose l⁻¹ (glucose–BTS) or inositol (inositol–BTS). Both media contained 20.6 mmol trisodium citrate l⁻¹, 15.0 mmol NaHCO₃ l⁻¹, 3.4 mmol EDTA l⁻¹, 10.0 mmol KCl l⁻¹, 0.6 g penicillin l⁻¹ and 1.0 g streptomycin l⁻¹. Semen

(100 ml at 15°C) was diluted with 200 ml BTS–glucose or BTS–inositol (15°C) and stored in a 300 ml tube sealed with parafilm. After 48 h the sperm cells had sedimented and the supernatant was removed. Avoiding perturbation, the sperm sediment was transferred into an NMR tube.

For more efficient data acquisition, fresh boar semen was concentrated by centrifugation before NMR measurement (500 *g* at 15°C for 20–30 min); the seminal plasma was removed and the sediment mixed with BTS–glucose or BTS–inositol (1:2, w/v) containing PPA (20 mmol l⁻¹).

Confocal laser scanning microscopy

A confocal laser scanning microscope LSM410 invert (Carl Zeiss Jena GmbH) equipped with a Plan-Neofluar × 100/1.3 oil objective was used according to Wiesner *et al.* (1998). The pH sensitive probe seminaphthorhodafluor (SNARF) was used for detection of pH_i. Spermatozoa were loaded with the acetoxymethyl ester (SNARF-1-AM solubilized in dimethyl sulphoxide 0.1%) at a final concentration of 9 μmol SNARF-1-AM l⁻¹ in BTS. SNARF-1-AM enters the spermatozoa where it is hydrolysed to SNARF by intracellular esterases. Adhesion of the spermatozoa to the glass surface and a temperature of 15°C ensured that spermatozoa remained immobile.

SNARF-1 was excited to fluorescence by an Argon–Krypton laser (488 nm). The excitation wavelength was set by a dichroic mirror (FT510). The fluorescence was split with a dichroic mirror (DT580) into two channels, and the fluorescence intensities were measured with a bandpass (BP515-565, green) in channel 1 (ch 1) and a longpass (LP590, red) in channel 2 (ch 2). The data can be displayed as overlay images with colour from red (low intensity in ch 1, high intensity in ch 2) to green (high intensity in ch 1, low intensity in ch 2). At equal intensities of both channels the resulting colour would be yellow.

Results

Oxygen consumption and lactate production by boar semen

Boar semen diluted with one volume of BTS showed vigorously motile spermatozoa consuming oxygen at a rate of 14.4 ± 2.4 nmol O₂ min⁻¹ 10⁻⁸ spermatozoa at 35°C (means ± SEM, *n*=4). This respiratory rate was maintained as long as the oxygen pressure was > 1 kPa. Cooling semen to 15°C rendered spermatozoa immotile and reduced the oxygen consumption by 90% to $1.4 \pm 0.2\%$. Under these conditions, lactate accumulated in the extracellular fluid at rates of 4.4 ± 0.8 and 0.5 ± 0.1 nmol min⁻¹ 10⁻⁸ spermatozoa (*n*=3) at 35°C and 15°C, respectively. During anoxia, lactate production was increased to 6.9 ± 1.7 at 35°C and to 0.8 ± 0.4 nmol min⁻¹ 10⁻⁸ spermatozoa at 15°C

(*n*=3), but the rates decreased with time owing to the accumulation of protons (see below). At between 5 and 10 h of anoxia at 35°C and 15°C, lactate accumulation decreased to 3.6 ± 1.0 and 0.5 ± 0.4 nmol lactate min⁻¹ 10⁻⁸ spermatozoa, respectively.

Adenylate energy charge of boar semen

Boar semen was directly ejaculated into liquid nitrogen to freeze fresh spermatozoa rapidly. The adenine nucleotides were measured in perchloric acid extracts of frozen semen by enzymatic assays (Table 1). The rapid fixation resulted in comparatively high ATP and correspondingly low ADP and AMP contents yielding an AEC value of 0.82. Storage of untreated semen at 35°C decreased the AEC to values as low as 0.24 within 1 h. The AEC was only slightly higher (AEC=0.33) when semen had been diluted with two volumes of the commercial extender BTS. At 15°C, spermatozoa were immotile and the AEC was maintained at about 0.6 for at least 1 h in untreated semen and for 48 h if two volumes of BTS were added.

Effect of oxygen supply on ³¹P-NMR spectra of boar semen

Spermatozoa in boar semen were concentrated by centrifugation at 500 *g* at 20°C for 20 min to about 10⁹ cells per ml. The rather soft sediment was then diluted with air-saturated oxygen buffer Fluosol (1:1, v/v) and heated to 30°C, which is the temperature at which spermatozoa become motile. Without further air supply during spectroscopy at 30°C, signals of three phosphorous metabolites were obtained (Fig. 2a): glycerophosphorylcholine (GPC at 0.5 ppm), P_i (appearing as two resonances at about 2 ppm) and AMP (at 3.5 ppm). No resonances from ATP were detected. The pH_i was 6.8 ± 0.2 as estimated from the chemical shift value of the P_i signal and the calibration curve (Fig. 1). The spectrum of the same sample, but with air supply during data acquisition, was markedly different (Fig. 2b). Under these conditions, ATP resonances became visible, whereas the AMP signal disappeared, and the P_i signal indicated an increase in pH_i to 7.1 ± 0.2 (*n*=3) compared with the anaerobic sample.

Effect of glucose supply on ³¹P-NMR-spectra of boar semen

For artificial insemination, boar semen is routinely stored without loss of fertility for at least 2 days in a glucose medium (BTS) at 15°C (Busch *et al.*, 1991). As boar spermatozoa become reversibly immotile at 15°C, they accumulate on the bottom of the vessel. This process of sedimentation by centrifugation (at 500 *g* at 15°C for 20 min) was accelerated and the effect of glucose

Table 1. Contents of adenine nucleotides in freshly ejaculated boar spermatozoa and in spermatozoa that were incubated under various conditions for different periods

Conditions	ATP	ADP	AMP	SUM	AEC
	(nmol per 10 ⁸ spermatozoa)				
Fresh semen ^a	18.6 ± 3.8	6.8 ± 1.7	1.5 ± 0.2	26.9 ± 2.2	0.82 ± 0.02
Semen kept at 35°C for 15 min ^b	9.5 ± 2.3	8.1 ± 1.2	6.6 ± 1.1	24.1 ± 4.3	0.56 ± 0.04
Semen kept at 35°C for 60 min ^b	2.5 ± 1.6	4.0 ± 2.7	18.5 ± 2.8	24.9 ± 7.2	0.17 ± 0.05
Semen diluted with twofold volumes BTS and kept at 35°C for 60 min ^b	4.6 ± 1.6	7.3 ± 1.2	12.9 ± 1.7	24.8 ± 2.1	0.33 ± 0.05
Semen cooled and kept at 15°C for 60 min ^b	12.6 ± 1.4	9.5 ± 1.3	7.6 ± 0.5	29.7 ± 4.3	0.58 ± 0.02
Semen diluted with ninefold volumes BTS and kept at 15°C for 48 h ^b	11.5 ± 2.5	8.3 ± 1.2	6.6 ± 1.0	26.3 ± 5.1	0.59 ± 0.03

*Boar semen was ejaculated directly into liquid nitrogen and powdered without thawing for fluorimetric analyses of nucleotides and sperm counting.

†Aliquots of boar semen were incubated in plastic tubes as indicated before freezing in liquid nitrogen. Samples incubated at 15°C were cooled from body temperature to 15°C within 30 min and then stored for the time indicated. After spermatozoa were removed by centrifugation (5000 *g* for 10 min) semen fluid did not contain significant amounts of adenine nucleotides (< 5% of whole semen, control experiment not shown). The values given are means of three ejaculates ± SEMs.

AEC: adenylate energy charge; BTS: Beltsville thawing solution; SUM: sum of ATP, ADP and AMP contents.

on metabolism of boar spermatozoa at the temperature of storage (15°C) studied. The sedimented spermatozoa were washed and suspended either in BTS medium containing 205 mmol glucose l⁻¹ or in BTS medium in which glucose was substituted by inositol. As a marker for extracellular pH (pH_e, Fig. 1), PPA was added to the medium. The experimental treatment and PPA did not affect sperm motility as tested after warming the sperm solution to 30°C. Boar spermatozoa suspended in glucose-BTS or in inositol-BTS were incubated in the magnet for 20 h at 15°C without air supply (Figs 3 and 4). As in all spectra ATP resonances were not visible, the negative chemical shift range is cut off in the figures. If glucose was supplied with the medium, AMP was detected together with another phosphomonoester that had previously been identified as glycerol 3-phosphate by Kalic (1997) (Fig. 3a). Incubation of spermatozoa in BTS containing 1-[¹³C]-glucose showed that glycerol 3-phosphate was derived from glucose (¹³C-NMR spectra not shown). Semen suspended in inositol-BTS showed no G3P resonance (Fig. 4).

The P_i-resonances obtained from semen were markedly different depending on whether semen was suspended in glucose-BTS or in inositol-BTS. In glucose-BTS, spermatozoa gave rise to two separate P_i-resonances (Fig. 4a). These resonances shifted downfield indicating intracellular acidosis and overlapped completely within 19 h of incubation (Fig. 3b,c). In contrast, spermatozoa suspended in inositol-BTS showed two P_i resonances that shifted upfield during incubation indicating intracellular alkalization (Fig. 4b,c). The extracellular fluid did

not contain inorganic phosphate and, hence, produced no P_i-resonance, as confirmed in a control ³¹P-NMR experiment after removing spermatozoa by centrifugation at 3000 *g* at 15°C for 10 min. Consequently the two P_i resonances must have derived from intracellular compartments of boar spermatozoa (Figs 3 and 4).

Both pH_i and pH_e decreased with time when spermatozoa were incubated in glucose-BTS at 15°C for 20 h (Fig. 5). The time course of acidosis was dependent on the concentration of spermatozoa and the buffer capacity of the extracellular fluid (data not shown). An intracellular acidosis (pH about 6.5) was not harmful because after being warmed to body temperature the stored cells were moving like fresh spermatozoa. The pH_i was always lower than the pH_e as would be expected when protons are produced by cell metabolism and released to the extracellular space.

Fluorescence microscopy and pH_i

Confocal microscopy and the pH indicator SNARF-1 were used to test whether the two P_i-resonances seen in NMR spectra of live spermatozoa (Figs 3 and 4) represent discrete intracellular compartments with different pH values (Fig. 6). This appears to be the case, as images of live spermatozoa show the presence of two compartments that differ in pH. The sperm head and the principal piece of the flagellum are red, which corresponds to a pH of 7.2, whereas the midpiece of the flagellum is green to yellow (corresponding to pH_{i-mp} = 6.7). Hence, the

Fig. 2. The effects of air supply on phosphorous metabolites in boar spermatozoa as shown by ³¹P-nuclear magnetic resonance (NMR) spectroscopy *in vivo*. Fresh boar semen was centrifuged (500 *g*, 20 min) and resuspended in a Hepes–Fluosol-ringer solution. Spectra are based on 1000 scans with a relaxation delay of 2 s at 30°C. The first measurement (a) was performed in a closed NMR tube with no air supply and subsequently air was bubbled through the sample for intervals of 2 min followed by data acquisition (b). Spectra of non-aerated samples lacked the signals from ATP. Oxygen supply caused ATP resonances to appear while the AMP resonance disappeared. The prominent signal of glycerophosphorylcholine (GPC) at 0.5 ppm was used as the internal standard. P_i: inorganic phosphate.

midpiece, where all the mitochondria are concentrated, must be the acidic compartment that was indicated by ³¹P-NMR spectroscopy of boar spermatozoa.

Fig. 3. ³¹P-nuclear magnetic resonance (NMR) spectra of boar spermatozoa in Beltsville thawing solution (BTS) as a function of the time of incubation at 15°C. Semen was concentrated by centrifugation at low speed (20 min, 500 *g*) and the fluffy sediment diluted with BTS (1:1, w:v) containing glucose (205 mmol l⁻¹). Spectra were collected after (a) 1 h, (b) 3 h and (c) 19 h of incubation. Two phosphomonoesters, AMP and glycerol-3-phosphate (G3P) could be identified. The chemical shifts of the phenylphosphonate (PPA) and inorganic phosphate (P_i) resonances indicate that both extracellular and intracellular acidification occurs if incubation is prolonged, as PPA shifted from 12.5 to 13.6 ppm, while P_i shifted from 2.5 to 1.2 ppm during 19 h. GPC: glycerophosphorylcholine.

Fig. 4. ^{31}P -nuclear magnetic resonance (NMR) spectra of boar spermatozoa kept in Beltsville thawing solution in which glucose was substituted by inositol. (For details of sperm incubation see Fig. 3). Spectrum after (a) 1 h, (b) 3 h and (c) 19 h of incubation. Phenylphosphonate (PPA) and inorganic phosphate (P_i) resonances did not indicate extracellular and intracellular acidification during incubation. The P_i -resonance was split into two signals which narrowed with incubation time. GPC: glycerophosphorylcholine.

Discussion

Ejaculated spermatozoa require permanent ATP production to maintain cell structures, intracellular ion com-

Fig. 5. Changes of intracellular (pH_i) and extracellular pH (pH_e) as measured by ^{31}P -nuclear magnetic resonance (NMR) spectroscopy of washed intact boar spermatozoa stored in Beltsville thawing solution at 15°C , which renders them immotile for 20 h. After incubation, spermatozoa regained their initial motility when warmed up to 35°C . The pH_e was continuously monitored from the chemical shift of phenylphosphonate (■) and occasionally measured with a pH electrode (□). From two P_i resonances (▲), two curved lines of pH_i were obtained which overlapped after 10 h of incubation.

Fig. 6. Confocal laser scanning micrograph of an intact boar spermatozoon at 15°C loaded with seminaphthorhodafluor 1 (SNARF-1). Green and yellow area indicates a more acidic intracellular pH than the red area. The head of the spermatozoon and the principal piece of its flagellum appear red; the flagellar midpiece, where all the mitochondria are concentrated, appears yellow and green.

position and, most of all, motility. Mature boar spermatozoa, like other mammalian spermatozoa, can oxidize various extracellular (Mann and Lutwak-Mann,

1981) and intracellular substrates (Jones and Bubb, 2000), but they contain neither phosphorylcreatine nor glycogen (Brooks, 1971; Kamp *et al.*, 1996). The main catabolic pathway in ejaculated boar spermatozoa appears to be fermentation of extracellular glucose or fructose to lactate and, if oxygen is available, the oxidation of lactate to CO₂ and H₂O (Jones, 1997). The latter pathway is initiated by a specific isoenzyme of lactate dehydrogenase that is localized in the mitochondria (Gallina *et al.*, 1994), thus cutting short the transport of hydrogen from the cytoplasm into the mitochondria (Milkowski and Lardy, 1977; Jones 1997).

³¹P-NMR spectra of boar semen at 35°C showed no ATP resonances, which is in line with studies by Robitaille *et al.* (1987) and Kalic *et al.* (1997). An oxygen supply system during NMR spectroscopy was used to demonstrate that ATP can be regenerated from AMP. Consequently, semen at 35°C requires an oxygen supply to maintain high AEC values in boar spermatozoa.

Glucose fermentation cannot compensate for the deficiency in aerobic ATP production. On the assumption that glucose was the only substrate, the aerobic ATP production from oxygen consumption and lactate production at 35°C was calculated to be about 95 nmol ATP min⁻¹ 10⁻⁸ spermatozoa (of which 5 nmol ATP min⁻¹ 10⁻⁸ spermatozoa was derived from glycolysis). Under anoxic conditions, when ATP can be generated only by fermentation to lactate, about 6.9 nmol ATP min⁻¹ 10⁻⁸ spermatozoa were produced. Hence, rather than increasing, catabolism of glucose decreases from 4.6 to 3.5 nmol min⁻¹ 10⁻⁸ when oxygen is lacking. Unlike many other cells, spermatozoa do not show the Pasteur effect (Schmidt and Kamp, 1996).

Owing to their high affinity for oxygen, respiration of boar spermatozoa is constant down to very low oxygen tensions (≥ 1 kPa), which ensures aerobic ATP production and consequently sperm motility at pO₂ concentrations regarded as severely hypoxic for most tissues. However, during anoxia, AEC values in boar spermatozoa decrease to 0.2 and may cause reversible loss of sperm motility.

Spermatozoa may encounter low pO₂ *in vivo*, for example in the uterus, when the large volume of ejaculate (about 300–450 ml in boars) may hamper oxygen supply from female blood, but anoxia is probably a non-physiological situation. However, the prevention of ATP supply in boar spermatozoa due to anoxia could hamper storage of liquid semen as applied in artificial insemination techniques. The present study has shown that the AEC of boar spermatozoa is markedly reduced within 15 min from 0.82 to 0.56, and reaches a value of 0.2 after 1 h if semen is kept unstirred at 35°C in closed tubes. Calculation of oxygen consumption of the sperm-rich fraction of boar semen (5 × 10⁸ spermatozoa per ml), which is used for artificial insemination, indicates that oxygen is completely depleted at 35°C within 10 min. Cooling semen to about 15°C reduces oxygen

consumption by 98.6% and maintains the AEC at 0.6 for several hours. If semen is diluted 1:1 with BTS and cooled to 15°C, the AEC is maintained at 0.6, for at least 2 days and the motility can be regained by re-warming.

The present study supports the findings of Mann (1964) and Jones (1997) that spermatozoa produce lactate even under aerobic conditions. The reason for aerobic lactate production is probably the strict compartmentalization of respiratory ATP production in the midpiece of the sperm flagellum, the only site where mitochondria are located. It is hypothesized that glycolysis is active in the principal piece, the mitochondria-free part of the flagellum. This hypothesis is based on the discovery that the central glycolytic enzyme, GAPDH, is tightly bound in the principal piece (Westhoff and Kamp, 1997) and that boar spermatozoa lack a phosphorylcreatine–creatine kinase shuttle system to transport energy-rich phosphate from the mitochondria to the distal dynein ATPases (for review, see Kaldis *et al.*, 1997).

Anaerobic glucose metabolism also results in an accumulation of glycerol 3-phosphate in the spermatozoa which is prevented if glucose is substituted by inositol. This finding indicates the presence of glycerol 3-phosphate dehydrogenase in boar spermatozoa. Increasing concentrations of dihydroxyacetone phosphate and NADH + H⁺ during glycolysis will result in the accumulation of glycerol 3-phosphate, particularly under anaerobic conditions when the reoxidation of NADH + H⁺ in mitochondria is not possible. Accumulation of glycerol 3-phosphate is common in cells that rely on glycolytic ATP production, such as working vertebrate muscle (Krause and Wegener, 1996). In muscle, such an accumulation is regarded as an additional means for NAD⁺ regeneration, which is particularly important in the early phase of exercise when glycolysis is not yet fully activated. As anaerobic glucose metabolism is rather an artificial situation in spermatozoa, glycerol 3-phosphate production from glucose may not occur *in vivo*. Otherwise, glycerol 3-phosphate increases also under aerobic conditions if phospholipids are degraded in boar spermatozoa (Jones and Bubb, 2000).

Intracellular pH can be monitored by ³¹P-NMR spectroscopy because the chemical shift of the P_i resonance depends on pH. A good indicator of extracellular pH is PPA, which does not penetrate plasma membranes, is non-toxic and shows pH-dependent chemical shift values about +12 ppm that are well separated from signals produced by cellular phosphorous compounds. Catabolism of glucose produces protons, which are released into the extracellular space. Correspondingly, the proton gradient is formed from the intracellular to the extracellular space, and the intracellular and extracellular buffer capacity defines the extent of acidosis. This finding confirms previous results obtained with human and rat spermatozoa (Hamamah *et al.*, 1996; Bone *et al.*, 2000).

NMR spectroscopy and fluorescence microscopy have shown two compartments with different pH in boar spermatozoa of freshly collected semen. The midpiece is significantly more acidic than the principal piece and the head. The fact that the midpiece is densely filled with mitochondria, and the cytosolic fraction is minor, indicates that the low pH reflects mitochondrial activity. Protons are produced in the matrix of mitochondria by the citric acid cycle, releasing CO₂ and reducing equivalents. CO₂ diffuses along its concentration gradient into the extracellular space, whereas reduction equivalents are used for respiratory ATP synthesis. An accumulation occurs if the citric acid cycle is more active than CO₂ diffusion and if the respiration chain produces more protons into the intra-mitochondrial space as ATP is synthesized. Consequently a high reduction potential and an accumulation of CO₂ in the mitochondria could be responsible for the low pH observed in the midpiece.

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