Effect of ornidazole on fertility of male rats: inhibition of a glycolysis-related motility pattern and zona binding required for fertilization *in vitro*

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The effects of the male antifertility agent ornidazole on glycolysis as a prerequisite for fertilization were investigated in rats. Antifertility doses of ornidazole inhibited glycolysis within mature spermatozoa as determined from the lack of glucose utilization, reduced acidosis under anaerobic conditions and reduced glycolytic enzyme activity. As a consequence, cauda epididymidal spermatozoa from ornidazole-fed rats were unable to fertilize rat oocytes *in vitro*, with or without cumulus cells, which was not due to transfer of an inhibitor in epididymal fluid with the spermatozoa. Under IVF conditions, binding to the zona pellucida was reduced in spermatozoa from ornidazole-fed males and the spermatozoa did not undergo a change in swimming pattern, which was observed in controls. The block to fertilization could be explained by the disruption of glycolysis-dependent events, since reduced binding to the zona pellucida and a lack of kinematic changes were demonstrated by control spermatozoa in glucose-free media in the presence of respiratory substrates. The importance of glycolysis for binding to, and penetration of, the zona pellucida, and hyperactivation in rats is discussed in relation to the glycolytic production of ATP in the principal piece in which local deprivation of energy may explain the reduced force of spermatozoa from ornidazole-fed males.

Introduction

Ornidazole exerts a rapid and reversible antifertility effect in male rats (McClain and Downing, 1988a,b; Oberländer *et al.*, 1994; Cooper *et al.*, 1997). In dogs, humans and rats, one of the metabolites of ornidazole is the C3-chloro side-chain of the nitroimidazole ring (Schwartz *et al.*, 1979; Jones and Cooper, 1996) which may produce 3-chlorolactaldehyde and α -chlorohydrin, known inhibitors of the glycolytic enzymes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and triosephosphate isomerase (TPI) in spermatozoa (Jones *et al.*, 1981; Jones and Stevenson, 1983; Stevenson and Jones, 1985). In accordance with this view, Oberländer *et al.* (1996) reported a 32% inhibition of GAPDH and a 52% inhibition of TPI activities in spermatozoa when male rats were administered 400 mg ornidazole per kg per day for 10 days. Therefore, the infertility action of ornidazole appears to be a result of its effect on the ability of spermatozoa ultimately to obtain ATP by the glycolytic pathway. A reduction in kinematic parameters in spermatozoa from ornidazole-treated rats could only be observed when spermatozoa were released into medium containing glucose as the only

substrate (Yeung *et al.*, 1995; Cooper *et al.*, 1997), but not when pyruvate and lactate were also added (Yeung *et al.*, 1995), indicating that ornidazole affects the utilization of glucose.

As pyruvate and lactate are present in uterine and oviductal secretions (Leese, 1988), which may maintain sperm motility and ATP concentrations (Ford and Harrison, 1987) despite glycolytic inhibition, the infertility of ornidazole-fed males is unlikely to be a consequence of reduced sperm motility. This view is supported by the observation that a similar number of spermatozoa reach the oviductal ampulla of females mated with ornidazole- or vehicle-treated males (Yeung *et al.*, 1995). Nevertheless, as no fertilization occurred under these conditions, ornidazole must have brought about a fertilization block in the ampulla, other than that due to reduced progressive motility. Therefore, ornidazole and α -chlorohydrin could affect other sperm functions by reducing glycolysis.

The present study was conducted (i) to demonstrate the effect of the incomplete inhibition of GAPDH and TPI after ornidazole treatment in rats (Oberländer *et al.*, 1996) on glycolytic turnover in spermatozoa and (ii) to investigate the consequences of a glycolytic block on the fertilization of rat gametes *in vitro*.

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Materials and Methods

Chemicals, animals and treatments

All reagents were from Sigma (Deisenhofen) unless otherwise stated. Male (350–450 g) and female (180–220 g) Sprague–Dawley rats from Charles River Wiga GmbH (Sulzfeld) were housed under 12 h light:12 h dark (lights on 07:00 h) at 22°C and 50% relative humidity and had access to standard chow (Altrumin GmbH, Lage) and water *ad libitum*. Male rats were fed 400 mg ornidazole kg⁻¹ once a day by oral gavage between 08:00 h and 10:00 h for 14 days and controls were fed vehicle alone (0.2% (w/v) carboxymethylcellulose in water plus two drops Tween 20 per 100 ml) during the same time period.

Collection of spermatozoa for estimation of glycolytic activity

After feeding rats ornidazole or vehicle for 14 days, the males were killed by cervical dislocation. Spermatozoa were obtained by retrograde flushing over 15 min through a PVC cannula (i.d. 0.5 mm, o.d. 0.8 mm; Critchley Electrical Products Pty Ltd, Silverwater, NSW) tied into the vas deferens and collected into a positive displacement pipette from a cut end of the tubule in the mid-cauda epididymidis. Flushing medium was warmed (37°C) medium G (Cooper *et al.*, 1997) for enzyme measurements or nuclear magnetic resonance (NMR) medium (90 mmol NaCl l⁻¹, 4.78 mmol KCl l⁻¹, 1.71 mmol CaCl₂ l⁻¹, 1.19 mmol MgSO₄ l⁻¹, 27.07 mmol NaHCO₃ l⁻¹, 10 mmol Hepes l⁻¹, 21.58 mmol sodium lactate l⁻¹, 0.5 mmol sodium pyruvate l⁻¹, 4 mg BSA ml⁻¹, 5 mmol phenylphosphonate l⁻¹; osmolality 305 mmol kg⁻¹, pH 7.2). After release, sperm concentration was estimated by nephelometry at 405 nm (Spectromax 250, Molecular Devices, Sunnyvale, CA) against a standard curve constructed by volumetric determination in a Neubauer improved chamber.

Measurement of sperm enzyme activities

The activities of two glycolytic enzymes (GAPDH: EC 1.2.1.12 and TPI: EC 5.3.1.1) known to be inhibited by α -chlorohydrin within intact spermatozoa were measured. The activity of hexokinase (EC 2.7.1.1) was also quantified to determine whether there was a non-specific detrimental effect on sperm enzymes. Enzyme activities were measured in sperm sonicates (KLN Ultraschallgenerator, Heppenheim (5 mm probe tip) and Vibracell™, Sonics and Materials Inc, Danbury (3 mm probe tip); 25 W, 20 kHz, 3 $\times$ 7 s, held on ice between) and assayed by photometry modified from Bergmeyer (1974) (GAPDH, hexokinase) and Ford *et al.* (1981) (TPI). For measurement of hyaluronidase (EC 3.2.1.35), the released sperm suspensions in medium G (500 μ l) were centrifuged for 7 min at 800 g through 5% (w/v) Ficoll in medium G. The washed sperm pellet was resuspended in 1 ml medium G and frozen for 3 h at -20°C. After thawing, the cells were diluted and the concentration was estimated as described above. Enzyme activity was measured by the method of Dorfman (1955) in which the

β -N-acetylhexosamine-[1 $\rightarrow$ 4] and β -glucuronic acid [1 $\rightarrow$ 3] glycosidic bonds are hydrolysed by the enzyme to be assayed, which reduces the extent of complexing of hyaluronic acid to albumin which is measured by turbidimetry at 600 nm. All enzyme assays were modified to be measured in 96-well plates.

NMR spectroscopy for estimation of glycolytic turnover

Glycolysis in spermatozoa under anaerobic conditions leads to an acidosis of spermatozoa, which results in an acidification of the exogenous medium (Hochachka and Mommsen, 1983; Smith *et al.*, 1985; Büsselmann, 1998). ³¹P-NMR spectroscopy was conducted using phenylphosphonate as a reference substance for pH changes in the medium, to estimate the glycolytic activity of spermatozoa. ¹³C-NMR spectroscopy was conducted to visualize the glucose consumption and lactate production of spermatozoa.

A Bruker AM 360 spectrometer (Karlsruhe) operating at 145.8 and 90.5 MHz for ³¹P and ¹³C, respectively, was used for the NMR experiments. Samples were measured in NMR tubes (10 mm o.d.) without spinning and without field or frequency lock. Spectra were recorded at 37°C. Spectral parameters for ¹³C-NMR and ³¹P-NMR were: impulse duration 13.6 and 12.7 μ s, relaxation delay 4 and 2 s, spectral width 22 and 7 kHz, 32 and 32 K data points, acquisition time 0.7 and 2.3 s, 250 and 500 transients and 10 and 10 Hz line broadening, respectively. ¹³C-NMR spectra were obtained with ¹H-CPD, ³¹P-NMR spectra without ¹H decoupling.

³¹P-NMR spectra were referenced to endogenous glycerophosphocholine (δ = 0.5), since glycerophosphocholine shows no variation in chemical shift at physiological pH (Büsselmann, 1998); the phenylphosphonate peak relative to that of glycerophosphocholine was localized at 12 p.p.m. A titration curve was constructed as a standard curve to derive the extracellular pH from the ³¹P-NMR shift. The ¹³C-NMR spectra were calibrated against external 3-trimethylsilyl[2,2,3,3-D]-1-propionate (TSP).

Spermatozoa from the distal cauda epididymidis of untreated males were treated without glucose (group a) or with [¹³C]glucose (5.56 mmol l⁻¹) (group b). Spermatozoa from ornidazole-treated males after the first recording (group c) were also provided with 5.56 mmol [¹³C]glucose l⁻¹. Spermatozoa were suspended in NMR medium without glucose. Approximately 150 $\times$ 10⁶ cells per 2 ml medium were placed in a 10 mm NMR tube at 37°C and an initial ³¹P-NMR measurement (t = 0 h) was made. After this reading, spermatozoa from groups b and c were given 100 μ l [¹³C]glucose and ¹³C-NMR spectra were recorded during 7.5 h. Sperm vitality was assessed for incubated spermatozoa using propidium iodide as vital dye.

Sperm vitality

After the incubations for NMR spectroscopy, 10 μ l sperm suspension was diluted in 400 μ l medium G (Oberländer *et al.*, 1994) containing 5 μ g propidium iodide ml⁻¹ and incubated at ambient temperature for 5 min in the dark. Approximately

10 000 sperm cells were analysed as dead (propidium iodide-positive) or viable in an EPICS XL flow cytometer (Coulter, Krefeld) by argon laser excitation at 488 nm.

Collection of spermatozoa for IVF

After 14 days of feeding, males were killed by CO₂ asphyxiation and spermatozoa were released by puncturing an excised cauda epididymidis into IVF medium (94.6 mmol NaCl l⁻¹, 4.78 mmol KCl l⁻¹, 1.71 mmol CaCl₂ l⁻¹, 1.19 mmol KH₂PO₄ l⁻¹, 1.19 mmol MgSO₄ l⁻¹, 25.07 mmol NaHCO₃ l⁻¹, 2 mg phenol red l⁻¹, 22.9 mmol sodium lactate l⁻¹, 0.5 mmol sodium pyruvate l⁻¹, 5.55 mmol glucose l⁻¹, 50 mg penicillin l⁻¹, 50 mg streptomycin l⁻¹, 4 g BSA l⁻¹). The tissue was removed after 2 min and after 15 min the sperm concentration was measured in triplicate as described above. The sperm suspension was diluted to 2.8 × 10⁶ ml⁻¹ and 800 µl was incubated under paraffin oil (BDH, Poole) before co-incubation with oocytes. The method of Toyoda and Chang (1974) was modified by using oocytes recovered from rats with natural oestrous cycles. Female rats were acclimatized for 1 week before the oestrous cycles were monitored by vaginal smears.

Collection of oocytes for IVF

IVF began between 03:00 h and 06:00 h because ovulation occurs about 03:00 h (Shalgi and Kraicer, 1978). Oestrous females were killed with CO₂ and oocytes were released within 30 min from the distal area of the oviductal ampulla by gentle tearing with fine needles. The oocytes with cumuli were transferred to 450 µl sterilized (0.22 µm filtered) IVF medium and incubated for 60 min before co-incubation with spermatozoa. Eggs were maintained at 37°C at all times. For fertilization of cumulus-free eggs, eggs were washed and transferred to a Petri dish containing 450 µl IVF medium and Type 1 S or Type IV S hyaluronidase (1.5 mg ml⁻¹, 480 U ml⁻¹) from bovine testis and gently dispersed to remove granulosa cells without causing mechanical damage to the eggs. After 20 min (at 37°C) the cells were dispersed and hyaluronidase was removed by washing four times for > 60 min in fresh IVF medium before transfer to the medium under oil for preincubation.

In vitro fertilization

Diluted sperm suspension (150 µl) was transferred to the 450 µl IVF dishes containing 5–10 oocytes, giving 700 000 spermatozoa ml⁻¹. Gametes were co-incubated for 36 h when two-cell stage embryos were anticipated (Toyoda and Chang, 1974). At this time, all oocytes and zygotes were transferred to washing medium to remove excess spermatozoa, and the number of two-cell embryos was counted.

Testing epididymal fluid for effects on IVF

The contents of the distal cauda epididymidis were flushed into 2 ml IVF medium and the sperm concentration

was assessed. Sperm suspension (1 ml) was centrifuged through 5 ml of 5% (w/v) Ficoll in IVF medium at 700 g for 7 min at 4°C to remove most of the spermatozoa without damage. Completely sperm-free fluid was obtained by further centrifugation at 10 000 g for 7 min at 4°C. Approximately 8 µl of this supernatant (equivalent to the amount of epididymal fluid containing the same number of spermatozoa used in each IVF insemination) was transferred to the co-culture of eggs and spermatozoa from control animals.

Rat sperm–zona binding

Spermatozoa from vehicle- and ornidazole-fed rats, collected at 02:00 h and prepared as described for IVF, were incubated in IVF medium (with or without glucose) at 0.7 × 10⁶ ml⁻¹ for 5.5 h at 37°C in 5% CO₂. Sperm suspensions were transferred to 400 µl IVF medium containing a maximum of 12 cumulus-free eggs and incubated for 45 min. Oocytes were collected at 05:30 h from two female rats and the granulosa cells were removed with 0.375 mg hyaluronidase ml⁻¹ (280–560 U ml⁻¹) and washed three times before co-incubation with spermatozoa as described above. Eggs were transferred to 100 µl PBS containing 0.25% (v/v) glutaraldehyde and gently shaken in PBS to remove loosely bound spermatozoa, before the number of eggs to which spermatozoa were bound, and the number of spermatozoa bound to the zona pellucida, were recorded. Because primary binding of rat spermatozoa to the zona pellucida is weak, it is possible that spermatozoa were dislodged during this procedure. Therefore, the assay may monitor spermatozoa that are already penetrating the zona pellucida.

Motility of spermatozoa

As fertilization *in vitro* occurs between 4 and 6 h after insemination in rats (Toyoda and Chang, 1974), sperm motility was examined at 2, 4, 6 and 8 h after release from the epididymis and incubation in 40 ml of IVF medium without covering with oil. Computer-aided sperm analysis (CASA) was performed as described for rats by Yeung *et al.* (1992) but at 37°C and in 80 µm chambers (provided by Hamilton-Thorne) with some modifications for monitoring possible hyperactivated motility (HTM-IVOS version 10.8, 50 Hz, frames analysed ≥ 100, minimum contrast 10 pixels, minimal size 10 pixels, magnification 0.63, points per track 40–100). A green interference filter (Olympus, IF550) was used in addition to pseudo-dark-field optics provided by an Olympus BH-2 microscope with a ×4 objective and ×20 phase ring.

The following parameters were measured: curvilinear velocity (VCL, time-average velocity of a sperm head along its actual curvilinear trajectory); average path velocity (VAP, time-average velocity of a sperm head along its spatial average trajectory); straight line velocity (VSL, time-average velocity of a sperm head along the straight line between its first and last detected positions); amplitude of head displacement (ALH, magnitude of lateral displacement of a

sperm head about its spatial trajectory); beat cross frequency (BCF, time-average rate at which the VCL trajectory crosses the VAP trajectory); linearity (linearity of the curvilinear trajectory = $VSL/VCL \times 100$); wobble (a measure of the oscillation of the actual trajectory about its spatial average path = $VAP/VCL \times 100$); and straightness (linearity of the spatial average path = $VSL/VAP \times 100$).

Statistical analysis

For each sample, kinematic parameters were expressed as the median of at least 100 spermatozoa per animal. The data in the text and graphs are the mean values of each treatment group. All percentages were transformed by *arc-sin* square-root conversion for statistical evaluation and the retransformed values were plotted. Differences in motility and kinematic parameters between treatments were calculated by one-way ANOVA followed by least squared differences or Student–Newman–Keuls methods for multiple comparisons.

Results

Effects of ornidazole on glycolysis of rat spermatozoa

Activity of three glycolytic enzymes of rat spermatozoa. Spermatozoa from rats fed ornidazole at 400 mg kg⁻¹ for 14 days showed a reduced activity (mU per 10⁶ spermatozoa) compared with controls (mean $\pm$ SEM) of GAPDH (1.0 $\pm$ 0.15 versus 7.6 $\pm$ 0.90) and TPI (8.03 $\pm$ 0.80 versus 26.00 $\pm$ 3.03), whereas hexokinase activity was unchanged (5.93 $\pm$ 0.10 versus 5.70 $\pm$ 0.19).

³¹P-NMR spectroscopy for the measurement of acidosis under anaerobic conditions. Spermatozoa from control rats under anaerobic conditions in medium containing glucose displayed acidosis, as determined by the shift in phenylphosphonate peak towards higher p.p.m. values, indicating a decline in pH (Fig. 1b, Table 1). However, spermatozoa from ornidazole-treated males incubated under the same conditions did not display acidosis as there was no shift in pH (Fig. 1c, Table 1), yielding results similar to those of control spermatozoa incubated in the absence of glucose (Fig. 1a). The absence of the pH shift by spermatozoa from the ornidazole-treated rats was not due to cell death, as the percentage of vital cells in medium containing glucose was unchanged, although a decline in vitality of spermatozoa incubated in glucose-free medium was observed after 8 h of incubation (Table 1).

¹³C-NMR measurement for glucose utilization and lactate production. In these experiments, spermatozoa were incubated with 5.56 mmol [¹³C]glucose l⁻¹ as substrate under anaerobic conditions for up to 7.5 h. Control spermatozoa liberated detectable amounts of lactate after 1 h, which increased by 7.5 h when all of the glucose had been utilized (Fig. 2a). In contrast, production of lactate by spermatozoa from ornidazole-fed rats was markedly reduced even after 7 h and most of the glucose remained unutilized (Fig. 2b).

Table 1. Acidosis^a and vitality^b of rat sperm cells during anaerobic incubation with and without glucose

Duration of incubation (h)	Controls		Ornidazole-treated
	Without glucose	With 5.56 mol glucose l ⁻¹	With 5.56 mol glucose l ⁻¹
0 ^c	7.69 $\pm$ 0.13	7.72 $\pm$ 0.03	7.66 $\pm$ 0.03
3 ^c	7.67 $\pm$ 0.17	7.35 $\pm$ 0.03	7.65 $\pm$ 0.04
6 ^c	7.67 $\pm$ 0.16	7.16 $\pm$ 0.04	7.68 $\pm$ 0.04
8 ^d	36.3 $\pm$ 2.70	50.4 $\pm$ 2.40	49.6 $\pm$ 3.40

Values are mean $\pm$ SEM, *n* = 3.

^aAs determined by the chemical shift of phenylphosphonate in ³¹P-NMR spectroscopy.

^bAs determined from the exclusion of propidium iodide by flow cytometry.

^cResults for 0, 3 and 6 h of incubation are pH values.

^dResults for 8 h of incubation are vitality (%).

Effects of glycolytic inhibition on the stages of IVF

Fertilization of rat eggs incubated in vitro. Spermatozoa from four control rats fertilized a mean of 65.4 $\pm$ 8.8% of 165 cumulus-intact eggs after 36 h co-incubation, whereas no eggs (0/131) were fertilized under the same conditions by spermatozoa from four ornidazole-fed rats. Similarly, although fewer cumulus-free eggs were fertilized by spermatozoa from four control males (24.2 $\pm$ 7.7% of 108 ova), no ova (0/109) were fertilized by spermatozoa from four ornidazole-fed rats.

Hyaluronidase activity of spermatozoa. Hyaluronidase activity of spermatozoa was unchanged in five males fed ornidazole (6.58 $\pm$ 0.72 U per 10⁶ spermatozoa) compared with five males fed vehicle (5.73 $\pm$ 0.75 U per 10⁶ spermatozoa).

Effect of contaminating epididymal fluid on fertilization in vitro. There was no inhibitory effect of additional cauda epididymal fluid from the ornidazole-fed males on the fertility of spermatozoa taken from control males. Spermatozoa from control rats fertilized 34.8 $\pm$ 7.3% of 80 eggs (*n* = 4) when incubated in IVF medium and 43.1 $\pm$ 12.8% of 76 eggs (*n* = 4) when incubated in the presence of epididymal fluid from an ornidazole-treated male rat.

Number of spermatozoa associated with eggs. When spermatozoa from control males (*n* = 6) were used, 26.5% of 147 eggs contained spermatozoa within the perivitelline space or vitellus, whereas no sperm tails were observed within the zona pellucida of 76 eggs examined in four experiments using spermatozoa from ornidazole-fed males.

Sperm binding to the zona pellucida. Sperm released from vehicle-fed rats preincubated (capacitated) for 5.5 h in complete IVF medium bound 45 min later at their acrosomal region to the zona pellucida (Fig. 3, inset). Fifty-four of the 80 oocytes had more than four spermatozoa attached with a maximum of 69 spermatozoa bound to the zona. Most of the

Fig. 1. ^{31}P -NMR spectroscopy of rat spermatozoa during anaerobic incubation ($t = 0, 3$ and 6 h) under three conditions: (a) spermatozoa from control rats in NMR medium lacking glucose; (b) spermatozoa from control rats in NMR medium containing $5.56 \text{ mmol glucose l}^{-1}$; and (c) spermatozoa from rats fed $400 \text{ mg ornidazole per kg per day}$ for 14 days and incubated in NMR medium containing $5.56 \text{ mmol glucose l}^{-1}$. The extracellular pH calculated from the chemical shift of phenylphosphonate (PPA) is given at each time point for each condition.

Fig. 2. ^{13}C -NMR spectroscopy of spermatozoa from (a) control and (b) ornidazole-fed rats incubated for up to 7.5 h. The position of signals in the spectrum originating from glucose (α and β C1 carbons) and the C3 carbon of lactate are shown.

oocytes had 20–40 spermatozoa attached. In contrast, significantly fewer spermatozoa released from ornidazole-treated rats and incubated in glucose, or spermatozoa released from control rats and incubated in medium lacking glucose, were attached to the zona pellucida (Fig. 3).

Change of kinematic parameters of rat spermatozoa. During the zona pellucida binding assay, a different pattern of motility was observed in spermatozoa from the ornidazole-fed rats compared with the vehicle-treated rats at the time point of adding the oocytes to the sperm suspension (5.5 h after

Fig. 3. Number of spermatozoa from the distal cauda epididymidis bound to the zona pellucida of rat eggs collected 45 min after insemination. Spermatozoa from four vehicle- (control) and four ornidazole-fed males (400 mg per kg per day for 14 days) were pre-incubated for 5.5 h in normal IVF medium (with glucose and lactate) and spermatozoa from four control males were incubated in glucose-free medium. Box represents 25th and 75th percentiles, whiskers 10th and 90th, and the line the median. Outliers are indicated by circles. Plots bearing different letters are significantly different ($P < 0.05$). Inset shows a photomicrograph of a rat egg with several adherent control spermatozoa. Scale bar represents 50 μ m.

releasing the spermatozoa from the epididymis). Samples from both groups were highly motile, but spermatozoa from the vehicle-treated rats appeared more active, moved jerkily and tended to swim in circular paths, whereas in spermatozoa from ornidazole-treated males the motion was smooth and progression was greater. Some spermatozoa in the control group displayed a highly curved flagellar bending, which, as a subjective observation, could not be quantified.

Spermatozoa from vehicle- and ornidazole-fed rats were incubated in IVF medium with and without glucose and the kinematic parameters were analysed by CASA over 8 h to quantify motility. During incubation, spermatozoa released from control animals in IVF-medium containing lactate, pyruvate and glucose showed a time-dependent decrease in VSL, linearity and straightness, and an increase in BCF, which was not observed when lactate and pyruvate only were present (Fig. 4). No difference in VCL was observed in the presence or absence of glucose. In contrast, spermatozoa from the ornidazole-fed rats displayed no differences in kinematic parameters depending on energy substrates and the values

obtained were similar to those of control spermatozoa in the absence of glucose (Fig. 4). After 8 h, there was a significant decrease in VCL in the presence of glucose, but no change in the other parameters (ALH, wobble and VAP).

Discussion

The results of this study demonstrate a marked decrease in glycolytic turnover of spermatozoa in rats treated with antifertility doses of ornidazole and the consequences of such a block in glycolysis for the functions of spermatozoa that are required for fertilization. The results confirmed the inhibition of the sperm enzymes GAPDH and TPI by feeding ornidazole for 10 days as described by Oberländer *et al.* (1996) and showed an even greater inhibition of GAPDH after feeding ornidazole for 14 days. Further evidence for glycolytic inhibition was proved by NMR spectroscopy: the lack of a pH-shift under anaerobic conditions of incubation, indicating a markedly reduced glycolytic turnover, and the severely limited conversion of glucose to lactate.

Fig. 4. Kinematic parameters of cauda epididymal spermatozoa of vehicle-control and ornidazole-treated (ornidazole) rats during 8 h incubation in IVF medium with (●) or without glucose (○). Values are means $\pm$ SEM ($n = 3$). Asterisks indicate significant differences between treatments at the same time point ($P < 0.05$). ALH: lateral head displacement; BCF: beat cross frequency; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight line velocity.

Such glycolytic inhibition may explain the reduced sperm motility observed *in vitro* in the absence of lactate and pyruvate (Yeung *et al.*, 1995; Cooper *et al.*, 1997). However, the similar number of spermatozoa in the oviductal ampulla of females mated with ornidazole- and vehicle-treated rats (Yeung *et al.*, 1995) indicates that the transport of spermatozoa to the fertilization site is not hindered, implying that the effect of ornidazole is on the fertilization process occurring in the ampulla. In the present study, IVF was used to demonstrate inhibition of fertilization by spermatozoa taken from ornidazole-treated rats, although the IVF medium contained the respiratory substrates lactate and pyruvate. These results are in agreement with the other studies (Tsunoda and Chang, 1975; Niwa and Iritani, 1978) in which IVF in rats failed in medium lacking glucose but containing lactate and pyruvate, and demonstrate the almost complete abolition of sperm glycolysis after feeding with ornidazole. It is possible that the spermatozoa were unable to overcome the mechanical resistance of the cumulus: Yeung *et al.* (1995) demonstrated a reduced number of spermatozoa within the cumulus oophorus of females mated to ornidazole-fed rats compared with controls, and Oberländer *et al.* (1994) highlighted the

difficulty of sperm penetration in viscous media. However, this could not be proved *in vitro*, due to the removal of nearly all of the granulosa cells by the large number of spermatozoa used in the IVF procedure.

Theoretically, inhibition of hyaluronidase, a sperm enzyme involved in penetrating the cumulus *in vivo* (Lin *et al.*, 1994; Myles and Primakoff, 1997) and binding to the zona pellucida (Overstreet *et al.*, 1995; Hunnicutt *et al.*, 1996), could mediate a block to fertilization, but this enzyme was not inhibited in the infertile ornidazole-fed rats. Removal of cumulus and granulosa cells before co-incubation of spermatozoa and oocytes failed to enable fertilization by spermatozoa from ornidazole-fed rats, revealing that neither the granulosa cells nor their secretions mediate the ornidazole-induced infertility. Since ornidazole inhibits the motility of spermatozoa *in vitro* (Bone *et al.*, 1997), it is possible that ornidazole itself, or a metabolite, present in the epididymal fluid of the treated males, and transferred with the spermatozoa to the eggs, could exert direct effects on sperm-egg interaction *in vitro*. This was examined by adding epididymal fluid from the treated males to the IVF co-culture. There was no evidence that the fluid was inhibitory,

confirming that the infertility of ornidazole-treated rats is due to changes in the spermatozoa.

Examination of the inseminated eggs for evidence of fertilization revealed no spermatozoa from ornidazole-treated males beyond the zona pellucida. This is in agreement with the results of IVF in rats when glucose is absent from the medium (Tsunoda and Chang, 1975; Niwa and Iritani, 1978). Examination of the zonae pellucidae at 45 min after co-incubation with capacitated spermatozoa, when 80% of eggs are fertilized (Shalgi *et al.*, 1983, 1986), revealed that the ability of spermatozoa from ornidazole-fed rats to bind to the zona pellucida in the presence of glucose was greatly impaired compared with control spermatozoa. This impairment was similar to that of control spermatozoa in the absence of glucose, indicating a requirement for glucose in binding to the zona pellucida in rats. The binding of spermatozoa to the zona pellucida in the present study resembled that reported by Gaddum-Rosse *et al.* (1984). In contrast, Urner and Sakkas (1996) showed that glucose is not required for binding of murine spermatozoa to the zona pellucida, and this could reflect differences in incubation conditions or species differences.

Another capacitation-related event, hyperactivation, may be inhibited by ornidazole treatment and thereby prevent fertilization. In rats, this is characterized by increased bending of higher amplitude (with prominent R-bend formation) and less forward progression (Shalgi and Phillips, 1988). A higher amplitude of bending could be observed in only a few control spermatozoa in glucose-containing IVF medium, whereas the forward progression was decreased. Examination by CASA revealed that control spermatozoa had lower VSL, straightness and linearity, and higher BCF, than spermatozoa incubated in the absence of glucose; there were no changes in VCL, VAP or ALH. The changes in linearity, VSL and BCF are in agreement with the observations by Cancel and Perreault (1998) for hyperactivated rat spermatozoa. Although the large flagellar bends, which are characteristic of hyperactivated motility of spermatozoa in several mammalian species (Yanagimachi, 1994; Si, 1997), were only rarely observed in control spermatozoa incubated in glucose-containing medium, the kinematic parameters evaluated may reflect hyperactivation of rat spermatozoa, as a result of the following: (i) the onset of this pattern of motility occurred at time points at which rat spermatozoa are capacitated (Shalgi and Kraicer, 1978); (ii) the decreased VSL was due to a tendency of spermatozoa to swim in circles, which reflects asymmetric bending, which is also characteristic of hyperactivation (Yanagimachi, 1994; Si, 1997); (iii) the spermatozoa appeared to be more vigorous, agile and jerky, as reflected in the increased BCF, which Yanagimachi (1994) considered to be more important in hyperactivation than a particular pattern of tail movements *per se*; and (iv) the changes in linearity, straightness, BCF and VSL (but not VCL) are in agreement with kinematic values for hyperactivated rat spermatozoa determined by Cancel and Perreault (1998). As spermatozoa from ornidazole-treated rats did not show this type of hyperactivated motility, even in the presence of glucose, glycolytic turnover must be required for hyperactivation of rat spermatozoa.

The dependence of hyperactivation on a glycolytic source

of ATP, despite the inefficiency of this process compared with respiration (Hammerstedt and Lardy, 1983), may be a consequence of the peculiar compartmentation of energy metabolism within the flagellum. A sperm-specific isoenzyme of GAPDH (Welch *et al.*, 1992; Bunch *et al.*, 1998) is bound by covalent linkage to the fibrous sheath of the principal piece in a complex with TPI (Westhoff and Kamp, 1997; Bunch *et al.*, 1998), whereas respiration is localized in the mid-piece. Mammalian spermatozoa have limited phosphagen (Kamp *et al.*, 1996) that restricts their utilization of respiratory ATP. If the ATP formed in the principal piece is utilized in that location by dynein ATPases during hyperactivation, glycolysis may be crucial to recruitment of dynein-tubulin bridges in the axoneme which generates the high frequency, large bends that are characteristic of hyperactivation. Therefore, the failure of hyperactivation in spermatozoa from ornidazole-treated males may be explained by a local deprivation of ATP in the principal piece leading to low frequency, small bend, co-ordinated flagellation providing progressive motion rather than vigorous thrust. Thus, although inhibition of glycolysis would be unlikely to alter the total sperm ATP content (Ford and Harrison, 1987), it may be crucial to the failure of spermatozoa to develop sufficient force required for penetrating the zona pellucida.

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