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Influence of oral administration of ornidazole on capacitation and the activity of some glycolytic enzymes of rat spermatozoa

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The chlortetracycline fluorescence assay was used to study the status of capacitation and the extent of induced acrosome reactions in cauda epididymidal spermatozoa from fertile and infertile rats fed, respectively, with vehicle or ornidazole (400 mg kg^{-1} day^{-1}) for 10 days. Uniform bright fluorescence over the whole head was classified as the uncapacitated pattern, whereas a postacrosomal dark band, and a uniformly weaker fluorescence over the acrosome, reflected patterns intermediate between the uncapacitated and acrosome-reacted states. Acrosome-reacted spermatozoa displayed a dark head but always retained fluorescence at their tip. There was no difference between experimental and control groups of rats with regard to the development of the chlortetracycline fluorescence patterns during incubation. Under basal incubation conditions, the acrosome reaction was slightly delayed in spermatozoa from ornidazole-treated animals. In contrast, more spermatozoa were acrosome reacted in this group after incubation for 5 h when the concentration of BSA was increased from 4 to 20 mg ml^{-1}. The Ca^{2+}-ionophore A23187 induced a similar stimulation of capacitation and acrosome reactions in spermatozoa from control and ornidazole-fed animals, but in the latter group A23187 caused strong immobilization of spermatozoa. In the capacitation medium containing 5 mmol lactate l^{-1} and 5 mmol glucose l^{-1}, the straight-line velocity of spermatozoa from ornidazole-treated rats was reduced by 50% compared with controls, irrespective of the concentration of BSA. Two glycolytic enzymes, triose phosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase, displayed reduced activity (48% and 68% of controls, respectively) in cauda epididymidal spermatozoa from ornidazole-fed rats, whereas the activities of hexokinase and lactate dehydrogenase remained unchanged. This finding suggests that the fertility-compromising action of ornidazole is due to a disturbed glycolytic pathway.

Introduction

The nitroimidazole derivative ornidazole (α-(chloromethyl)-2-methyl-5-nitroimidazole-1-ethanol) is used as a chemotherapeutic agent for clinical purposes against anaerobic bacteria and flagellates. It induces infertility in male rats within 3 weeks at a dosage of 400 mg kg^{-1} day^{-1} (McClain and Downing, 1988a, b), but, in contrast to other compounds of similar structure, it does not affect spermatogenesis or induce testicular atrophy at doses causing infertility. Detailed investigation, with a more stringent mating schedule, resulted in complete infertility after 6–10 days of treatment (Oberländer *et al.*, 1994; Yeung *et al.*, 1995). This rapid onset of infertility implies an action directed upon the epididymis rather than on the testis, most probably directly on the spermatozoa. Indeed, the progressive velocity (VSL) and the percentage of motile epididymal spermatozoa from the cauda region were found to be reduced in infertile male rats (McClain and Downing, 1988b). When the motility of such spermatozoa from different epididymal regions was analysed by computer-assisted sperm analysis (CASA) some velocity parameters were reduced, but not the percentage of motile cells, and impairment occurred only in spermatozoa from the distal regions of the organ (Oberländer *et al.*, 1994).

Since the amount of the major epididymal secretory products remain unchanged in ornidazole-treated animals, the compound is thought to act directly on the spermatozoa, rather than via manipulation of epididymal function (Oberländer *et al.*, 1994). Recovery of spermatozoa from the female genital tract 9 h after copulation revealed reduced numbers and lower velocities in the oviductal isthmus when ornidazole was administered to male rats for 10 days before mating (Yeung *et al.*, 1995). However, the number of spermatozoa arriving in the ampulla was the same as that after mating in control animals, although the spermatozoa from treated animals failed to penetrate the cumulus and none fertilized oocytes. Cauda epididymidal spermatozoa from the treated males also showed a reduced ability to penetrate viscous methylcellulose solutions *in vitro*, used as a surrogate for the cumulus, and the velocity of spermatozoa from males treated for 10 days was reduced when

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the incubation medium contained only glucose as exogenous substrate (Yeung *et al.*, 1995). The reduced ability of the spermatozoa to use glucose as substrate could be explained by a reduced metabolism of glucose.

The development of hyperactivated motility which enables spermatozoa to penetrate the visco-elastic cumulus and zona pellucida (Suarez and Dai, 1992) is associated with the capacitation of spermatozoa in the female genital tract (Fraser and Ahuja, 1988; Shalgi and Phillips, 1988). As glucose is necessary for hyperactivation (Cooper, 1984), the acrosome reaction (Fraser and Herod, 1990), penetration and fertilization (Hoppe, 1976; Fraser and Quinn, 1981; Niwa and Iritani, 1978), impaired glucose metabolism could explain the inability of spermatozoa from ornidazole-fed rats to fertilize eggs *in vivo* and their weakness in penetrating viscous medium *in vitro*. The present study, therefore, examined the activity of some glycolytic enzymes of cauda epididymidal spermatozoa from ornidazole-treated rats and evaluated with the chlortetracycline (CTC) fluorescence assay the extent of capacitation and acrosome reaction under basal and stimulating conditions. This method was chosen because it allows monitoring of capacitation as well as the acrosome reaction.

Materials and Methods

Chemicals

All chemicals were from Sigma Chemie (Deisenhofen, Germany) unless otherwise stated.

Animals

Twenty, adult male rats (Sprague Dawley strain, 375–445 g) were obtained from Charles River Wiga GmbH (Sulzfeld). Animals were kept at about 22°C under a 12 h light:12 h dark cycle and were provided with water and rat standard diet (Altromin GmbH, Lage) *ad libitum*.

Treatment of male rats

Ornidazole (α-(chloromethyl)-2-methyl-5-nitroimidazole-1-ethanol) was given orally at a dosage of 400 mg kg^{-1} day^{-1} in a volume of 0.14 ml (100 g)$^{-1}$ body mass. Control animals received the same volume of vehicle, namely, 0.2% (w/v) carboxymethylcellulose in water (plus two drops Tween 20 per 100 ml). The duration of treatment was 10 days for the animals used for enzyme measurements and 11 days for those in the capacitation study.

Assessment of fertility

Male rats used for the measurement of enzyme activity had participated in another study in which their fertility was assessed after different mating schedules (Yeung *et al.*, 1995). From these experiments the most infertile ornidazole-treated males were chosen in the measurement of the activity of sperm enzymes and results were compared with the activities in spermatozoa from the control group. Before treatment, all males underwent mating tests with oviduct-ligated females to prove mating competence. One day before, and on scheduled days during treatment, males were mated with receptive intact females. Eleven to twelve days later the number of implantations and corpora lutea of the females were counted and fertility (%) was expressed as the number of implantations × 100/number of corpora lutea. For more details see Oberländer *et al.* (1994).

Medium for capacitation of rat spermatozoa (medium C)

The capacitation medium was modified from that of Toyoda and Chang (1974a). Since the physiological concentration of lactate in the ampulla of rabbits and mice lies between 3 and 5 mmol l^{-1} (Leese, 1988), this substrate was reduced to 5 mmol l^{-1}. As the capacitation time for rat spermatozoa can be shortened by a high K^+:Na^+ ratio (Toyoda and Chang, 1974b) and the K^+ concentration in the mouse ampulla shortly after mating is 25 mmol l^{-1} (Borland *et al.*, 1977), the final composition of the medium was 102 mmol NaCl l^{-1}, 25 mmol KCl l^{-1}, 1.7 mmol $CaCl_2$ l^{-1}, 2.4 mmol $MgSO_4$ l^{-1}, 1.2 mmol KH_2PO_4 l^{-1}, 25 mmol $NaHCO_3$ l^{-1}, 5 mmol D-glucose l^{-1}, 5 mmol sodium lactate l^{-1}, 0.25 mmol sodium pyruvate l^{-1} and 4 mg BSA ml^{-1} with an osmolality of about 300 mosmol kg^{-1}.

Preparation of spermatozoa

Experiments were performed with pairs of rats, and one control and one ornidazole-treated animal were processed at the same time. At the end of treatment, animals were killed by CO_2 asphyxiation. The epididymis of one side was placed in medium H (Yeung *et al.*, 1992) and the caudal region prepared free of fat and blood capillaries. A small part of the tubule in the mid-cauda was excised and placed on a plastic spatula. A drop of luminal content exuded from the cut tubule was transferred to 100 µl capacitation medium C (with 4 mg BSA ml^{-1}) or medium B (with 20 mg BSA ml^{-1}). After pre-incubation for 10 min at 37°C and 5% (v/v) CO_2, a 10 µl aliquot of sperm suspension was diluted 100 times and the sperm concentration was calculated from counts of spermatozoa within a defined area of a 40 µm deep slide chamber from a videomonitor. The concentration of spermatozoa was adjusted to 0.9–1.0 × 10^6 ml^{-1}. The timepoint of dilution was defined as the start of the capacitation incubation.

Incubation of spermatozoa and tests of different stimuli for capacitation

The incubation for spermatozoa in medium C lasted 5 h, the time required for the *in vitro* capacitation of rat spermatozoa (see Toyoda and Chang, 1974a, b). At different times (0, 2.5, 3.5 and 5 h) aliquots of spermatozoa were taken for the CTC assay and recording of motility. In preliminary studies, three conditions that promote capacitation and the acrosome reaction in untreated rat spermatozoa were assessed and the two conditions used in these experiments are described below.

(1) High BSA concentration (20 mg ml^{-1}): an aliquot of spermatozoa was released in medium B and incubated for 5 h. CTC-staining and a recording of motility were made at 0, 2.5 and 5 h.

(2) Ca^{2+}-ionophore A23187: after 3 h incubation, 2 µl of the stock ionophore solution (1.7 mmol l^{-1} in dimethyl sulfoxide (DMSO)) was added to 200 µl of sperm suspension so that the final concentration of ionophore was 17 µmol l^{-1} and DMSO 1%, v/v. After incubation for 30 min, spermatozoa were examined by CTC staining and motility was recorded.

Chlortetracycline (CTC) assay for capacitation and acrosome reaction

The staining procedure was a modification of that of Fraser and Herod (1990). Chlortetracycline was prepared freshly at a concentration of 1.5 mmol l^{-1} in 20 mmol Tris buffer l^{-1} containing 130 mmol NaCl l^{-1} and 5 mmol L-cysteine l^{-1}. The pH was adjusted to 7.8 and the solution was shielded from light at room temperature. A 50 µl aliquot of the sperm suspension was mixed with an equal volume of CTC solution in an Eppendorf tube. After 30 s, 8 µl 12.5% (w/v) paraformaldehyde in 0.5 mol Tris buffer l^{-1} (pH 7.4) was added. After mixing by gentle vortexing, 50 µl 0.22 mol 1,4-diazabicyclo[2.2.2]octane l^{-1} in glycerol was introduced and mixed well with the sperm suspension to retard fading of fluorescence. The samples were stored at 4°C for up to 2 days until they were examined. Fluorescence patterns of spermatozoa were analysed on a Photomikroskope III (Carl Zeiss, Oberkochem) with a × 10 ocular and × 63 (oil) objective and epifluorescence. The UV light passed through a band-pass filter of 390–440 nm with a reflector of 460 nm and a long-pass filter of 470 nm. Immediately before examination, the spermatozoa were centrifuged for 3 min at 3300 ***g***. Five microlitres of the concentrated sperm suspension was placed on a slide and covered with a 22 mm × 22 mm coverslip. In each sample, a total of 100 cells was assessed. The intra-assay coefficient of variation in each assay was calculated by counting 100 cells in duplicate from one sample. Values were 5.5% for assessment of uncapacitated pattern, 8.9% for the intermediate stage and 22.4% for acrosome reacted spermatozoa (calculated from arc-sine transformed percentages).

Assessment of sperm motility

A 21.8 µl aliquot of diluted sperm suspension was placed on a siliconized slide and a 21 mm × 26 mm coverslip was used to achieve a chamber depth of 40 µm. Recording of motility and analysis of motion parameters were performed as described by Yeung *et al.* (1992), except that the new CASA system from Hamilton Thorne (HTM Master C, version 10.4Q, Beverly, MA) that allows analysis of 30 frames at 13 frames s^{-1} was used.

Preparation of spermatozoa for enzyme assay

The animals were anaesthetized with urethane (1.1 g kg^{-1} body mass) and the epididymides were removed. The cauda region and the ductus deferens were cleaned of fat and blood vessels. The cannula (0.5 mm i.d., 0.8 mm o.d. PVC tubing, Dural Plastics and Engineering, Dural, NSW) with an inserted shaft of a 27 gauge needle was filled with PBS and inserted into the lumen of the ductus deferens and secured with a 5/0 suture thread. In the mid-cauda region, the capsule was opened and a small loop of the tubule exposed and cut. A 1 ml syringe displaced PBS buffer through the cannula and flushed out the luminal contents of the distal cauda region. Luminal contents were sucked from the cut end of the tubule into a 100 µl glass capillary tube and dispersed in 0.5–1 ml PBS in the Eppendorf tube. The spermatozoa were separated from epididymal fluid by centrifugation through 5 ml Ficoll (M_r 400 000, 5% (w/v) in PBS) for 5 min at 800 ***g***. The sperm pellet was resuspended in 0.4 ml PBS buffer and frozen at − 80°C until enzyme activities were determined.

Measurement of sperm concentration

The concentration of spermatozoa was determined by nephelometry. Aliquots of spermatozoa were diluted into the linear part of the standard curve range (up to 8×10^6 spermatozoa ml^{-1}) and the absorbance of a 100 µl aliquot in a 96-well plate at 405 nm was noted. The appropriate dilution for each assay was made with ice-cold 10 mmol K^+-phosphate buffer l^{-1} (pH 7.4) to lyse the plasma membrane (Ford *et al.*, 1981).

Measurement of enzyme activity

Measurements were performed with a Pye Unicam PU 8610 UV/VIS Kinetics Spectrophotometer (Unicam Analytische Systeme GmbH, Kassel) at 25°C in a total volume of 2 ml (excluding sample volume). The rate of reaction was determined by monitoring the NAD(P)H concentrations at 340 nm (Shonk and Boxer, 1964; Ford *et al.*, 1981). The possible decrease of enzyme activity between thawing of samples and the end of assay was normalized by parallel determination of control- and ornidazole-derived samples. The sperm concentrations used were chosen after preliminary experiments to optimize the sensitivity as well as linearity with time for each assay.

Hexokinase (EC 2.7.1.1). Reaction mixture contained 50 mmol triethanolamine l^{-1}, pH 7.4; 10 mmol $MgCl_2$ l^{-1}; 5 mmol EDTA l^{-1}; 7.5 mmol KCl l^{-1}; 10 mmol ATP l^{-1}; 0.59 mmol $NADP^+$ l^{-1}; 0.74 U glucose 6-phosphate dehydrogenase; 1.5×10^6 spermatozoa (0.1 ml). The reaction was started with 5 mmol glucose l^{-1}.

Triose phosphate isomerase (EC 5.3.1.1). The reaction solution contained 50 mmol Tris l^{-1}, pH 8.2; 5 mmol $MgCl_2$ l^{-1}; 0.2 mol KCl l^{-1}; 0.14 mmol NADH l^{-1}; 5 U glycerol 3-phosphate dehydrogenase; 0.9×10^6 spermatozoa (0.06 ml). The reaction was started by the addition of 1 mmol glyceraldehyde 3-phosphate l^{-1}.

Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12). The reaction mixture contained 50 mmol triethanolamine l^{-1}, pH 7.4; 10 mmol $MgCl_2$ l^{-1}; 75 mmol KCl l^{-1}; 4 mmol

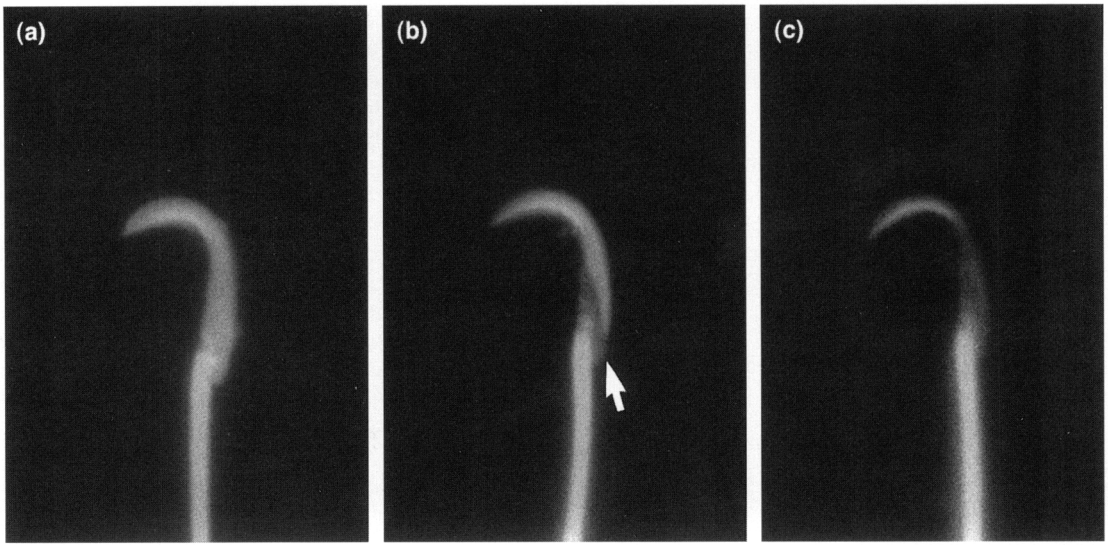

Fig. 1. Chlortetracycline fluorescence patterns of rat spermatozoa during capacitation. (a) Uncapacitated cell with uniform bright fluorescence over the head. (b) Intermediate pattern I (IM I) with a dark band (arrow) in the postacrosomal region of the sperm head. (c) Acrosome-reacted cell with dark head except for the tip, which retained some fluorescence.

ATP l^{-1}; 0.14 mmol NADH l^{-1}; 9 U phosphoglycerate kinase; 3×10^6 spermatozoa (0.1 ml). The reaction was started by the addition of 5 mmol D-glycerate-3-phosphate l^{-1}.

Lactate dehydrogenase (EC 1.1.1.27). The reaction mixture contained 50 mmol triethanolamine l^{-1}, pH 7.4; 10 mmol $MgCl_2$ l^{-1}; 75 mmol KCl l^{-1}; 0.14 mmol NADH l^{-1}; 0.5×10^6 spermatozoa (0.1 ml). The reaction was started by the addition of 0.5 mmol sodium pyruvate l^{-1}.

The net change of absorbance was determined by subtraction of the initial from the subsequent rate. The amount of substrate consumed was calculated from the equivalent amount of NAD(P)H oxidized or $NAD(P)^+$ reduced, using the extinction coefficient of NAD(P)H (6.22×10^6 cm^2 mol^{-1}) and results were expressed as nmol substrate converted by 10^8 spermatozoa min^{-1}. Each sample was measured in duplicate from which the intra-assay coefficients of variation were calculated; these were 2.8% for hexokinase, 7.6% for triose phosphate isomerase, 8.8% for glyceraldehyde 3-phosphate dehydrogenase and 1.6% for lactate dehydrogenase.

Statistical analyses

For CTC and motility data one-way analysis of variance was used by first comparing within the same incubation medium between different time points and between control and ornidazole-treated samples, and then comparing between different incubation media within each time point of incubation. Statistical significance was accepted if $P < 0.05$. Ratios were transformed by arc-sine square-root conversion. For expression of these data, the retransformed mean value is given and the SEM was calculated from the difference between the retransformed (mean + SEM) and the retransformed mean values. For each enzyme assay Student's t test was used to compare data of spermatozoa from control and ornidazole-treated males.

Results

Chlortetracycline (CTC) staining patterns in rat epididymal spermatozoa

The CTC staining of rat spermatozoa showed different patterns in the head region (Fig. 1). According to Saling and Storey (1979) and Ward and Storey (1984) bright fluorescence over the whole head is classified as the uncapacitated status and this classification was accepted in the present work (Fig. 1a). The acrosome reacted pattern was seen as darkness over the whole head, as in murine spermatozoa, except for the tip, which always retained some fluorescence (Fig. 1c). Patterns between these two extremes were classified as intermediate and two categories of these patterns (IM I and IM II) were distinguished. IM I (Fig. 1b) was characterized by an obvious dark band in the postacrosomal region and probably reflects the capacitated stage as found for murine spermatozoa (Saling and Storey, 1979; Ward and Storey, 1984). IM II showed weaker fluorescence over the whole head, but not as weak as in the acrosome reacted pattern, so that the postacrosomal dark band was just discernible. It is not certain whether IM II occurred before or after IM I. For presentation of data, IM I and II were grouped together and considered as the general intermediate stage between uncapacitated spermatozoa and cells displaying the acrosome-reacted status. The retention of CTC fluorescence at the tip of the sperm head most probably represents fluorescence emanating from the separate 'acrosomal sac' that is found on the ventral surface of the perforatorium in rats (Pikó, 1969).

Capacitation and acrosome reaction of spermatozoa

The development of capacitation and the acrosome reaction in control spermatozoa from untreated rats during incubation in medium C are shown (Fig. 2). The percentage of

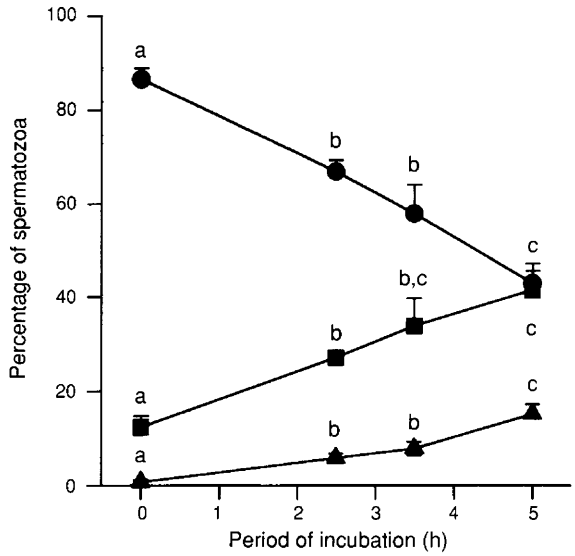

Fig. 2. The development of (●) the uncapacitated, (■) the intermediate and (▲) the acrosome-reacted pattern of chlortetracycline fluorescence over 5 h of incubation of cauda spermatozoa from untreated control rats. The medium contained 4 mg BSA ml^{-1} (medium C). Different superscripts denote significant differences between time points for each pattern. Values are means + SEM.

uncapacitated spermatozoa decreased significantly over incubation for 5 h, whereas the IM and acrosome reacted patterns showed significant increases. Under these basal conditions no difference was found between spermatozoa from the control and ornidazole group (Fig. 3a). The only exception was that, in spermatozoa from ornidazole-treated animals, the development of the acrosome–reacted pattern appeared to be delayed, with significantly fewer acrosome-reacted spermatozoa at 2.5 h, yet reaching the same percentage as in control spermatozoa after 5 h.

Stimulation of capacitation and acrosome reaction

The high concentration of BSA (20 mg ml^{-1}) in medium B stimulated the acrosome reaction of spermatozoa from control and ornidazole-treated males (Fig. 3b). This effect was visible and significant for both groups only after 5 h of incubation. The stimulation was greater for spermatozoa from the ornidazole-fed males, so that significantly more (39%) spermatozoa of this group showed the acrosome reacted pattern compared with the control (27%). Concomitantly, the capacitation pattern decreased from $59.6 \pm 1.8\%$ of cells to $44.9 \pm 4.6\%$ ($P < 0.05$), whereas there was no change in the percentage of uncapacitated spermatozoa (12.6 ± 1.9 versus $14.5 \pm 4.5\%$). In contrast to the results obtained in control medium (4 mg BSA ml^{-1}), no delay in the acrosome reaction was observed in spermatozoa from the ornidazole-treated group with the increased BSA concentration.

Incubation for 30 min with 17 μmol Ca^{2+}-ionophore A23187 l^{-1} caused a significant decrease of the uncapacitated stage from 58% to 12% and 51% to 15% for spermatozoa from control and ornidazole-fed rats, respectively (Fig. 4). In both groups, there was a marked increase in the number of

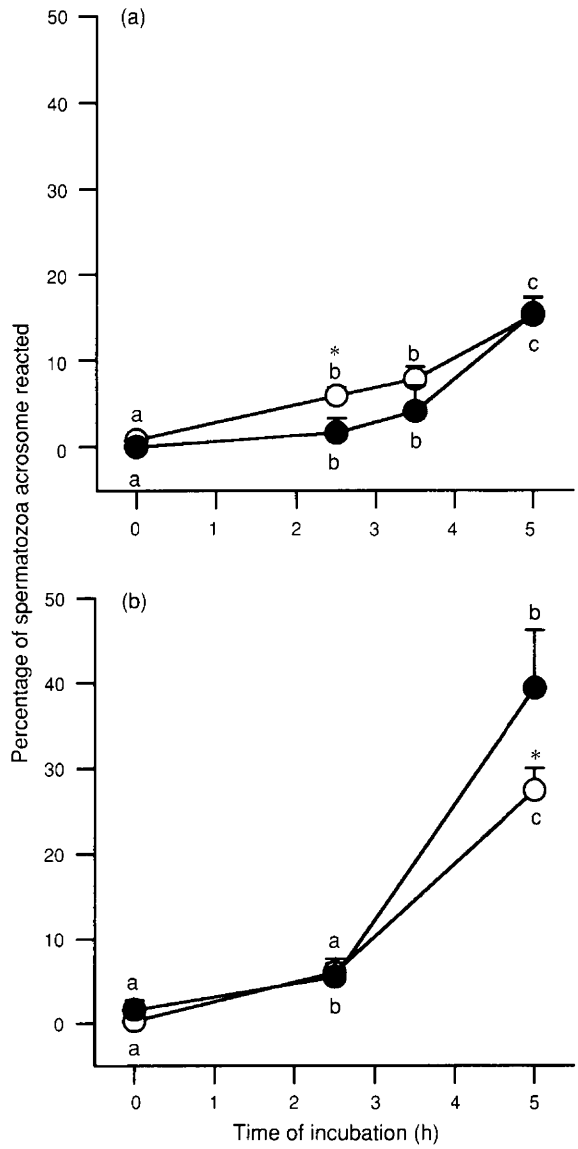

Fig. 3. Percentage of rat spermatozoa undergoing the acrosome reaction in the presence of (a) 4 mg ml^{-1} and (b) 20 mg ml^{-1} BSA. (○) Control and (●) ornidazole-treated male rats. Within treatment groups, different superscripts denote statistically significant differences between time-points; *denotes significant differences between control and ornidazole-treated animals at the same time point. Values are means + SEM.

spermatozoa showing the IM pattern (up to 70%) and the acrosome reaction was also significantly stimulated by the ionophore. No difference was observed between spermatozoa from ornidazole-fed or control animals with regard to the change in CTC pattern resulting from treatment with A23187 which depressed the percentage motility of spermatozoa from both control and ornidazole-treated rats, but significantly more in the latter group.

Motility of spermatozoa in low and high BSA

Motility and velocity parameters of spermatozoa were analysed after incubation in medium C (4 mg BSA ml^{-1}) and in

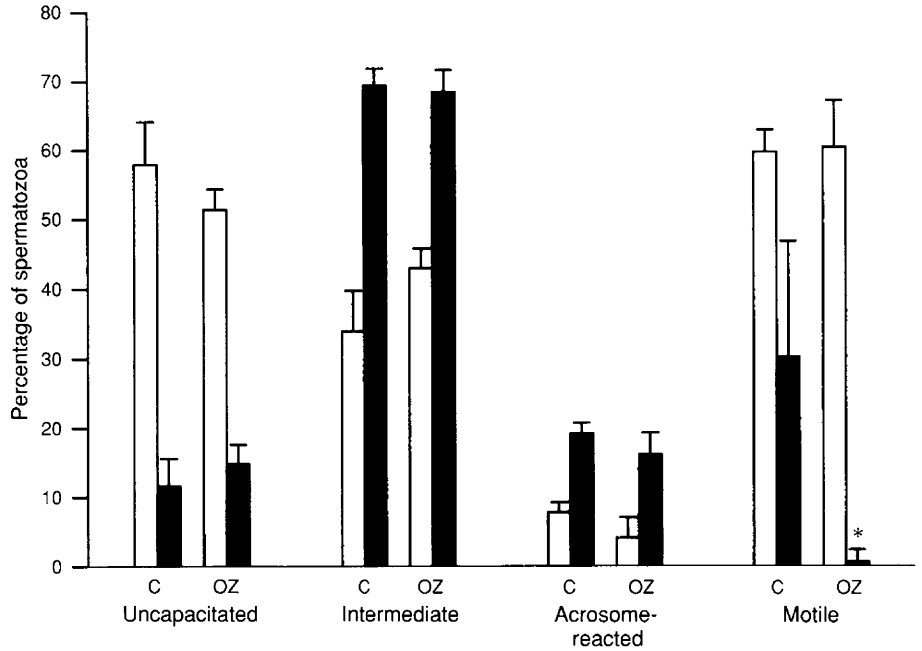

Fig. 4. Influence of incubation with the Ca^{2+}-ionophore A23187 (17 μmol l^{-1}) for 30 min on the capacitation status, intermediate forms, acrosome-reacted cells and motility of spermatozoa from (C) control and (OZ) ornidazole-treated rats in the (□) absence and (■) presence of A23187. *Denotes a significant difference between the control and ornidazole-treated animals. Values are means + SEM.

Table 1. Motility parameters of rat spermatozoa incubated in a capacitation medium with low or high BSA concentrations

BSA	Treatment	Incubation period (h)	Motility (%)	VSL (μm s^{-1})	VAP (μm s^{-1})	VCL (μm s^{-1})	STR (%)	ALH (μm)
4 mg ml^{-1}	Control	0.0	85.7 ± 3.0^{a}	55.5 ± 3.0	75.1 ± 2.4	105.5 ± 3.6^{a}	80.3 ± 2.6	25.2 ± 1.0^{a}
		2.5	72.6 ± 5.9^{a}	59.7 ± 4.4	72.2 ± 4.1	97.6 ± 3.1^{a,b}	88.3 ± 2.6	21.5 ± 1.5^{a,b}
		5.0	46.9 ± 6.5^{b}	50.8 ± 6.2	61.2 ± 5.1	86.6 ± 3.7^{b}	87.6 ± 3.7	18.8 ± 1.8^{b}
	Ornidazole-treated	0.0	87.4 ± 2.9^{a}	34.4 ± 9.2	51.0 ± 7.0^{t}	95.1 ± 2.9	67.8 ± 7.2	24.9 ± 1.9
		2.5	63.0 ± 7.5^{b}	29.5 ± 11.2^{t}	43.8 ± 11.7^{t}	89.4 ± 3.8	76.3 ± 6.8^{t}	20.2 ± 1.4
		5.0	46.0 ± 12.7^{b}	29.3 ± 12.3	43.0 ± 11.8	87.4 ± 6.8	72.1 ± 8.1	20.7 ± 1.4
20 mg ml^{-1}	Control	0.0	84.0 ± 2.4^{a}	56.8 ± 7.6	73.6 ± 5.7^{a}	102.8 ± 5.1^{a}	81.0 ± 5.0	24.4 ± 1.4^{a}
		2.5	64.6 ± 4.8^{b}	50.5 ± 5.0	59.3 ± 4.7^{a,b}	85.2 ± 3.0^{b}*	87.8 ± 2.4	20.0 ± 0.8^{b}
		5.0	25.2 ± 5.8^{c}	41.7 ± 5.5	52.7 ± 3.3^{b}	79.9 ± 1.9^{b}	85.5 ± 3.9	19.5 ± 1.3^{b}
	Ornidazole-treated	0.0	80.8 ± 4.0^{a}	29.4 ± 7.8^{t}	46.6 ± 6.1^{t}	91.1 ± 1.9^{t}	64.5 ± 7.0^{t}	25.4 ± 1.2
		2.5	52.4 ± 5.2^{b}	17.3 ± 5.0^{t}	34.7 ± 8.0^{t}	85.1 ± 2.7	63.6 ± 3.1^{t}	21.4 ± 1.8
		5.0	30.2 ± 9.6^{c}	20.0 ± 7.6^{t}	36.4 ± 9.6	83.8 ± 5.8	68.2 ± 9.1^{t}	23.6 ± 2.6

Velocity data are expressed as means ± SEM ($n = 5$); percentage motility and the ratio STR are expressed as mean ± SEM of retransformed data ($n = 5$).
VSL: straight-line velocity; VAP: average path velocity; VCL: curvilinear velocity; STR: straightness; ALH: amplitude of lateral displacement.
Significant differences ($P < 0.05$) over time within one group are indicated by different superscripts.
^{t}Indicates a significant difference ($P < 0.05$) between ornidazole-treated and relevant control group within the same BSA concentration.
*Indicates a significant difference ($P < 0.05$) in the same group between high and low BSA concentrations.

medium B (20 mg BSA ml^{-1}). At no time point with either concentration of BSA was there a difference in the percentage of motile spermatozoa between the ornidazole treated and control group (Table 1). The high BSA medium, although stimulating capacitation and the acrosome reaction, did not change any velocity parameter of the spermatozoa from control or ornidazole-treated rats (Table 1) with the exception that the curvilinear velocity (VCL) of control spermatozoa after 2.5 h in medium B was significantly lower than in the medium C. The results show that the percentage motility decreased significantly with time in control and ornidazole-treated samples with no difference between treatments. The VCL of spermatozoa from control and ornidazole-fed animals decreased over 5 h in both media, although significance was reached only in the control group owing to the higher initial values. Under each condition and treatment there was only a

Table 2. Activity of some glycolytic enzymes in spermatozoa and the fertility of rats after treatment with ornidazole for 10 days

Enzyme	Control ($n = 5$)	Ornidazole (400 mg kg^{-1} day^{-1}) ($n = 5$)
Hexokinase	668 ± 24	726 ± 62
Triose phosphate isomerase	3436 ± 233	1634 ± 89*
Glyceraldehyde 3-phosphate dehydrogenase	316 ± 29	214 ± 16*
Lactate dehydrogenase	1616 ± 198	1540 ± 224
Fertility (%)	94.4 + 2.7	3.5 + 3.5*

Enzyme activity is expressed as nmol substrate converted 10^{-8} spermatozoa min^{-1} (mean ± SEM).
Fertility is expressed as mean (+ SEM) of retransformed data (number of embryos × 100/number of corpora lutea).
*Significantly different from control ($P < 0.05$).

small and insignificant decrease of straight line velocity (VSL) over time. However, spermatozoa from ornidazole-treated rats consistently moved more slowly than did the relevant controls, with a reduction of about 50%. The velocity along the average path (VAP) changed with time only in the control group with high BSA concentrations and was significantly lower for spermatozoa from ornidazole-treated males at the beginning and after 2.5 h of incubation in both BSA concentrations. The straightness (STR) (the ratio VSL:VAP expressed as %) remained stable over time under each condition, but in the medium with high BSA concentrations, it was always significantly lower for spermatozoa from the ornidazole-treated group than from the controls. A time-dependent decrease in the amplitude of lateral displacement (ALH) was found in control samples at both BSA concentrations but no difference was noticeable between the two treatments.

Activity of glycolytic enzymes

Two of the four measured enzymes showed significantly lower activities in spermatozoa from ornidazole-treated rats (Table 2). The activity of triose phosphate isomerase was reduced by 52%, whereas glyceraldehyde 3-phosphate dehydrogenase showed a less marked (32%), but still significant, inhibition. The activities of hexokinase and lactate dehydrogenase were not altered in spermatozoa from rats treated with ornidazole for 10 days. The fertility in this group was markedly reduced with complete inhibition of fertility occurring in two of five animals.

Discussion

The chlortetracycline assay for determining capacitation and acrosome reactions has been used for mice (Ward and Storey, 1984), men (Lee *et al.*, 1987), boars (Wang *et al.*, 1995) and bulls (Fraser *et al.*, 1995) but not for rat spermatozoa. The CTC patterns of rat spermatozoa undergoing capacitation were marked by the disappearance of fluorescence from the head and showed similarities to the patterns described in mice. Whereas in mice two uncapacitated patterns could be distinguished, this was not obvious in the rat spermatozoa, although two patterns featuring a dark band in the postacrosomal region (IM I and IM II) appeared with time and in parallel. However, it was often difficult to distinguish between these two patterns. One reason for this difficulty is the narrower hook shape of the rat sperm head which renders different patterns less discernible than they are in mice. In addition, the heads of rat spermatozoa that were considered to be acrosome reacted were dark except for a small part of the tip that always remained fluorescent. The anatomical structure in this ventral location in the rat sperm head is the 'acrosomal sac', which Pikó (1969) showed to be distinct from the main acrosomal structure on the dorsal surface of the head and from which fluorescence disappeared in what was considered to be the acrosome-reacted spermatozoon. The persistence of the fluorescence at this site suggests that this acrosomal sac does not fuse with the outer plasma membrane. Although present in the mouse sperm head (Pikó, 1969), it is not so extensive as in the rat and this may explain why retention of CTC fluorescence is not seen in mouse acrosome-reacted spermatozoa.

It has not been proved directly, with egg-binding studies, whether the IM stages reflect capacitation, as described by Saling and Storey (1979). Owing to its small size, the presence or absence of the acrosomal cap of rat spermatozoa cannot be determined by normal phase contrast microscopy (Miyake *et al.*, 1989), as it can be in mice (Fraser and Herod, 1990) and confirmation that an acrosome reaction had occurred was not possible because of the lack of available dyes for this species (Cross and Meizel, 1989).

There was no marked difference in the CTC patterns displayed by spermatozoa from control and ornidazole-treated rats during incubation under capacitation conditions. In the basal medium, the acrosome reactions of spermatozoa from ornidazole-treated rats were delayed briefly but reached control values after 5 h of incubation. Considering that normal rat spermatozoa reside for much longer than this in the female tract and after 8.5 h have fertilized only 60–80% of the eggs (Shalgi and Phillips, 1988; Yeung *et al.*, 1995), it is doubtful that this observed delay is of physiological relevance. The effect of BSA in stimulating capacitation and the acrosome reaction is generally thought to be due to cholesterol or lipid transfer between the spermatozoa and BSA (for review see Meizel, 1985). This stimulation was found to be higher after 5 h

incubation in spermatozoa from ornidazole-treated rats than in control rats. Although in this case degenerating spermatozoa, if any, were not eliminated from the analysis to exclude any false acrosome reactions, the percentages of such spermatozoa in both groups would be the same since the percentages of motile cells were the same in both groups. This higher stimulation in ornidazole-treated animals could indicate that these spermatozoa undergo the acrosome reaction in the female tract prematurely. This could explain their loss of fertilizing capacity *in vivo*, since, although the same numbers of spermatozoa enter the ampulla as in controls, they do not penetrate the cumulus (Yeung *et al.*, 1995). Treatment with the Ca^{2+}-ionophore was equally effective in stimulating the IM and acrosome-reacted patterns of spermatozoa for both groups of rats.

Under the conditions used in this study, spermatozoa from control and ornidazole-fed animals showed little or no difference in their ability to undergo capacitation and the spontaneous acrosome reaction. However, the marker used, CTC, binds to divalent cations in a nonpolar environment (Saling and Storey, 1979) and may therefore address only the aspect of capacitation that is connected with changes in the membrane. Ornidazole treatment may impair intracellular processes that cannot be detected by CTC. The Ca^{2+}-ionophore A23187 caused almost complete immobilization of spermatozoa from ornidazole-treated animals in contrast to spermatozoa from the control group. This immobilization may indicate a disturbed response, caused by ornidazole, to increased intracellular calcium concentrations. Another possibility is that ornidazole alters membrane properties so that the incorporation of ionophore leads to cell damage.

In many species capacitated spermatozoa display hyperactivation, which is marked by high amplitude of the lateral head displacement with increased curvilinear velocity but decreased linearity. *In vivo*, this type of movement occurs in the oviduct and enhances the capacity of spermatozoa to penetrate viscoelastic media such as the cumulus complex surrounding the ovum (Suarez and Dai, 1992). Rat spermatozoa express this pattern in the oviductal ampulla, which is the site of fertilization (Shalgi and Phillips, 1988). This hyperactivation is not expressed by rat spermatozoa *in vitro* and therefore cannot be used as an indicator for capacitation. Yeung *et al.* (1995) found that significantly fewer spermatozoa from ornidazole-treated rats could penetrate the cumulus *in vivo* and that penetration of a viscous medium *in vitro* was impaired in this group. In the same study, measurement of kinematic parameters of spermatozoa from the oviductal isthmus showed decreased velocities (VSL, VAP, VCL) and amplitudes of the lateral displacement of spermatozoa from ornidazole-treated rats.

It has been shown that at the end of capacitation in mice (Hoppe, 1976; Fraser and Herod, 1990) and rats (Niwa and Iritani, 1978) glucose is required as an exogeneous substrate for fertilization. Spermatozoa from ornidazole-treated animals display a marked decline in velocity if lactate and pyruvate are omitted from the incubation medium and glucose is the only substrate (Yeung *et al.*, 1995), but when 12.5 mmol lactate l^{-1} is present, they exhibit the same velocity as do control spermatozoa over an incubation for 7 h. In the capacitation medium of the present study, the lactate concentration was reduced to 5 mmol l^{-1}, to mimic physiological concentrations. Under these conditions, spermatozoa from ornidazole-treated animals moved with lower straight line velocities than did control spermatozoa, especially in medium with the higher BSA concentration. Thus, their forward progression seemed to depend on the amount of lactate present, and glucose was unable to promote high velocity movement. The cells may thus have a defect in glycolysis rendering them dependent on oxidation of lactate for the generation of ATP. The activity of two of four glycolytic enzymes (triose phosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase) was lower in spermatozoa from ornidazole-treated rats than in the controls, whereas the activities of hexokinase and lactate dehydrogenase were unaffected.

It is possible that ornidazole itself does not cause the observed effects in spermatozoa, since it is extensively metabolized in rats and less than 4% of the drug is excreted unchanged (Schwartz *et al.*, 1979). These authors described the conversion of ornidazole into five different metabolites in which two had lost the chloropropanol side-chain, which could be liberated as an α-chlorohydrin-like molecule. A much higher dose of ornidazole is required for its antifertility action compared with the required dose of α-chlorohydrin; this is consistent with the metabolism of ornidazole to an active compound. α-Chlorohydrin, administered *in vivo*, inhibits glyceraldehyde 3-phosphate dehydrogenase activity in epididymal spermatozoa of several species (Ford and Harrison, 1983). Pig spermatozoa incubated with α-chlorohydrin and glucose suffer a rapid decrease in ATP concentration and an accumulation of glycolytic intermediates, an effect which can be prevented by the addition of lactate (Ford and Harrison, 1986).

The effects of ornidazole treatment on glycolytic enzymes of spermatozoa are similar to, but weaker than, the results obtained when male rats are fed with the antifertility agent 6-chloro-6-deoxyglucose. Spermatozoa from these infertile animals also cannot sustain motility in lactate-free medium (Ford and Waites, 1980). Treatment of rats with 6-chloro-6-deoxyglucose also leads to a strong inhibition of triose phosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase with less inhibition of hexokinase and lactate dehydrogenase (Ford and Waites, 1980; Ford *et al.*, 1981), whereas oxygen uptake is not affected (Ford *et al.*, 1981). It is known that 6-chloro-6-deoxyglucose and the *S*-enantiomer of α-chlorohydrin are converted into (*S*)-3-chlorolactaldehyde which impairs isomerization of trioses and the activity of glyceraldehyde 3-phosphate dehydrogenase (Jones and Ford, 1984). The rapid infertility induced by these compounds, the reduced sperm motility and the impairment of glycolytic enzymes in cauda spermatozoa are all characteristics consistent with the action of ornidazole revealed in the present study.

Further results from the study reported here indicate that when rats are treated with an antifertility dose of ornidazole there is no marked or persistent effect on capacitation or the acrosome reaction *in vitro* as determined with the CTC assay. An effect on capacitation not detected with the CTC staining but in terms of hyperactivation remains a possibility. The activities of two rate-limiting glycolytic sperm enzymes were impaired in infertile, ornidazole-fed rats; this is consistent with the decreased velocities of these spermatozoa *in vitro* and *in vivo*. Thus, infertility seems to occur because spermatozoa cannot generate enough energy, resulting in a decrease in motility in the oviduct and a concomitant failure to penetrate

the cumulus oophorus (Yeung *et al.*, 1995). Fertilization studies *in vitro* could be used to examine the fertilizing ability of spermatozoa from ornidazole-treated rats in more detail. Knowledge of the mechanism of action of ornidazole will advance the post-testicular approach to a fast-acting and reversible contraceptive.

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