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OUTSTANDING CONTRIBUTION

X-ray microscopy of human spermatozoa shows change of mitochondrial morphology after capacitation

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Using X-ray microscopy two morphologically distinct states were observed of the human spermatozoan mitochondria: (i) compact and tightly wrapped around the axoneme, and (ii) morphologically transformed, i.e. with circular areas of high X-ray transmission, either loosely wrapped around the axoneme or distended. The spermatozoa were examined at two stages of their post-ejaculation maturation process, i.e. as present in fresh ejaculated semen and after in-vitro capacitation. X-ray microscopy allowed sample preparation that was as simple as for conventional light microscopy whilst giving high resolution (30 nm) imaging of samples in liquid media compatible with the requirements of live biological specimens. The specimens were not fixed, stained or metal coated. These features make X-ray microscopy useful in the study of cells, particularly cells in suspension. The relative frequencies of the two morphological states of the mitochondria in seminal plasma and after in-vitro capacitation were compared. In seminal plasma, almost all spermatozoa had compact and tightly wrapped mitochondria. After harvesting by swim-up technique, an increase in the morphologically transformed state had occurred. However, the greatest increase in the morphologically transformed state occurred when the sample had been incubated under capacitating conditions. In this case almost all spermatozoa had morphologically transformed mitochondria.

Key words: capacitation/mitochondria/spermatozoa/X-ray microscopy

Introduction

The biological significance of the maturation of spermatozoa outside the male organs was revealed in studies by Austin (1951) and Chang (1951) who showed that the ability of spermatozoa to fertilize was acquired only after residing for a specific period of time in the female genital tract. The changes which the spermatozoon undergoes during this period have been designated 'capacitation' as a collective term for a complex series of events involving changes in motility, membrane properties and intracellular metal ion concentrations.

Hyperactivation, a vigorous but poorly progressive state of motility, is associated with capacitation. A formal characterization of this mode of sperm motility has been difficult and no consensus has so far been reached (for reviews see de Lamirande *et al*., 1997; Mortimer, 1997). However, advanced mathematical modelling of trajectories collected in computer-assisted sperm analysis (CASA) has improved classification of hyperactivation (Mortimer *et al*., 1996).

There is evidence of change in the membrane density of cholesterol during the progression of sperm maturation, and studies have linked a cholesterol efflux from the spermatozoon with capacitation (Ravnik *et al*., 1990). Also, it has been suggested that membrane bound proteins such as lectins appear on the sperm membrane after capacitation which thus may serve to expose putative zona pellucida binding proteins (Ahuja, 1985; Singer *et al*., 1985; Cross and Overstreet, 1987; Benoff *et al*., 1993).

The intracellular calcium concentration has been demonstrated to increase stepwise during sperm maturation (Baldi *et al*., 1991), and other divalent metal ions such as zinc may play a role in capacitation (Fraser, 1995). Studies point to Ca^{2+}-ATPase activity as an important regulator of the intracellular calcium concentration during capacitation (DasGupta *et al*., 1994) and this activity has been shown to be localized in head membranes (Adeoya-Osiguwa and Fraser, 1996). Voltage-dependent calcium channels appear to be involved in Ca^{2+} regulation in the terminal stage of the maturation process (Florman *et al*., 1992) which ultimately include the loss of the acrosome.

The large number of studies on the capacitation process, apart from highlighting the multifarious biochemistry involved, also emphasize the difficulty in delineating capacitation as a distinct stage on the path to maturation of the spermatozoon. Although staining techniques are available which can distinguish between non-capacitated, capacitated and acrosome-reacted spermatozoa (Lee *et al*., 1987), these are indirect means of evaluating the state of maturity when compared to the morphological change, the loss of the acrosomal cap, of acrosome-reacted spermatozoa.

Electron microscopy (EM) has revealed fine structural

details of the spermatozoon and has contributed much to our understanding of the processes transforming the immature spermatozoon into a potentially fertilizing gamete (Hafez, 1971). In the 1960s it was concluded from EM studies that capacitation did not include a morphological characteristic with a distinctness similar to the loss of the acrosome (Adams and Chang, 1962; Bedford, 1969). Subsequent reviews have thus stated that 'no major morphological changes are induced by capacitation' (Langlais and Roberts, 1985) or emphasized events at the molecular level as important (de Lamirande *et al*., 1997). Sample treatment required for conventional EM, e.g. dehydration, staining, and metal coating, can introduce artefacts and certainly removes the cell from physiological conditions supporting viability. This treatment thus compromises the interpretation of physiological changes which involves fragile structures of the specimen. X-ray microscopy offers the opportunity of obtaining high resolution images of cells in a liquid medium with the simple preparation techniques used for light microscopy (for a detailed overview of the biological applications of X-ray microscopy see Kirz *et al*., 1995). In previous studies, spermatozoa from mammalian species have been examined by combining X-ray absorption measurements with X-ray microscopy with 50 nm resolution in experiments aimed at analysing the DNA to protein ratios (Zhang *et al*., 1996). Recent developments in X-ray microscopy allow the visualization of structures down to 30 nm and have improved sample contrast in a liquid medium. By use of this equipment ultrastructural studies on human spermatozoa have been carried out showing details of the surface membranes (Abraham-Peskir *et al*., 1998).

In the present study we report on two morphological states of human sperm mitochondria, one apparently associated with fresh spermatozoa and the other with a treatment for in-vitro capacitation.

Materials and methods

Preparation of spermatozoa

Semen samples were obtained from four healthy donors. All samples were normal with regard to viscosity and sperm concentration ($>20\times10^6$ spermatozoa/ml) according to World Health Organization guidelines (WHO, 1992). One part of the spermatozoa sample used for studying fresh spermatozoa was kept in seminal plasma at 37°C until microscopy which was completed within 4 h after sample collection. The spermatozoa in another part of the ejaculate were studied after *in vitro* capacitation carried out essentially according to the WHO protocol (WHO, 1992): after incubating at 37°C for 1 h, motile spermatozoa were harvested from the semen by the swim-up technique into Tyrode's buffer (pH 7.4) supplemented with 1% bovine serum albumin (Sigma A-7638), sedimented by centrifugation for 10 min at 600 *g* and washed once in the same medium before being resuspended at a concentration of 15×10^6 spermatozoa/ml and incubated for 16 h at 37°C in humidified air with 5% CO_2. In order to evaluate to what extent the sperm samples had been capacitated, calcium ionophore (A23187)-induced acrosome reaction was studied by fluorescein isothiocyanate (FITC)-conjugated *Pisum sativum* agglutinin staining, carried out as originally described by Cross and Overstreet (1987) but using a high concentration of calcium ionophore (500 μM) to obtain maximal stimulation. The sample from donor no. 3 was split and analysed not only as fresh spermatozoa in seminal plasma and after in-vitro capacitation but also as untreated, i.e. in seminal plasma while incubating at 37°C according to the capacitation protocol (designated 'time-decay control'). Some of the swim-up harvested spermatozoa from donor no. 2 were left outside the incubator at room temperature (designated 'incubation control'). Fixation medium consisted of 0.35% (v/v) formalin in a 5% (w/v) $NaHCO_3$ buffer as described elsewhere (WHO, 1992).

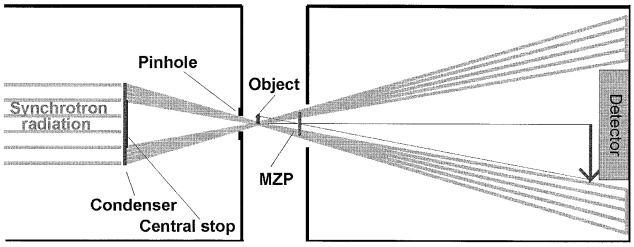

Figure 1. Ray diagram of the X-ray microscopy (not to scale). Radiation from the synchrotron is focused by a zone plate corresponding to condenser lens onto the sample object. A microzone plate (MZP) acting as an objective lens behind the sample forms a transmission image on a charge-coupled device (CCD) detector. See text for further details.

X-ray microscopy

After preliminary studies on human spermatozoa carried out with the Gottingen transmission X-ray microscope at BESSY (Berlin, Germany) (Schmahl *et al*., 1995, 1996), the full field imaging X-ray microscope located at ISA (Aarhus, Denmark) (Medenwaldt and Uggerhøj, 1998), was used to obtain the images presented in this study. Both microscopes are equipped with circular diffraction gratings, so called zone plates, as optical elements, providing a resolution of around 30 nm, which is an order of magnitude better than achieved by visible light microscopy. The high resolution was combined with high contrast in the micrographs by choosing an X-ray wavelength of 2.4 nm, where the absorption in organic material is much higher than in water. The optical set-up of the X-ray microscope (Figure 1) is equivalent to visible light transmission microscopes. Radiation from a source, in our case the synchrotron, is focused by a zone plate corresponding to a condenser lens onto the sample. A microzone plate (MZP) acting as an objective lens behind the sample forms a transmission image on a charge-coupled device (CCD) detector. The condenser has a large central stop which makes the transmitted X-rays form a hollow cone. Thereby, the too intense direct radiation from the storage ring is prevented from reaching the object and the detector.

During imaging, spermatozoa, either as semen or as harvested cells in Tyrode's buffer, were kept in a sealed chamber between two thin silicon foils at room temperature and atmospheric pressure. In this environment spermatozoa could survive for several hours. Washed polymer beads (Dynospheres) with a diameter of 5 μm (Plano, Marburg, Germany) were added to prevent collapse of the foils and maintain a liquid layer sufficiently thick to allow motility within the sealed chamber. Within the time it took to acquire an image, typically 5–20 s, the cell of interest had to remain stable in the X-ray microscope. The motile spermatozoa were therefore immobilized by a short pulse of radiation immediately before the image was acquired.

Statistical analysis

The proportions of tightly wrapped mitochondria and mitochondrial sheaths with circular areas of high X-ray transmission were compared by a one-tailed Fisher's exact test for 2×2 tables, calculated by use of the SOLO statistical software package (BMDP Statistical Software, Los Angeles, CA, USA).

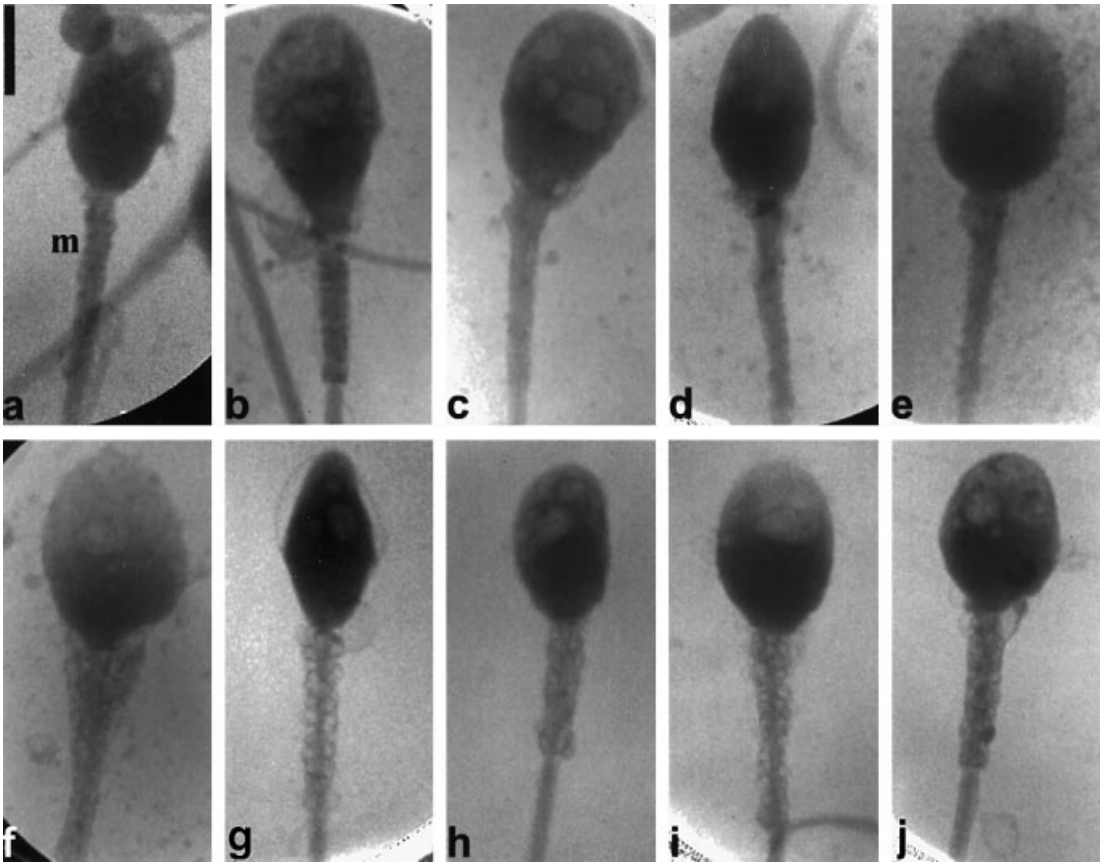

Figure 2. Images of human spermatozoa taken with the Aarhus X-ray microscope. Bar = 2 μm. Mitochondria (m). (**a**–**d**) Spermatozoa in seminal plasma, showing tightly wrapped mitochondria, (**a**) from donor 1, (**b**) from donor 2, (**c**) from donor 3, and (**d**) from donor 4. (**e**–**f**) Spermatozoa after 30–31 h in seminal plasma (from donor 3), (**e**) showing tightly wrapped mitochondria, (**f**) showing morphologically transformed mitochondria (dimensions of head region appear abnormal). (**g**–**i**) Spermatozoa after capacitation showing morphologically transformed mitochondria (**g**) from donor 1, (**h**) from donor 2, (**i**) from donor 3. (**j**) Spermatozoon fixed in formalin (from donor 2).

Results

With X-ray microscopy we were able to obtain high resolution transmission images of whole cells in solution without the need to fix, dehydrate or stain. We saw two distinct morphological states of the human sperm mitochondria, either tightly wrapped around the axoneme (Figure 2a–e), appearing compact and homogeneously X-ray absorbent, or having circular areas of high X-ray transmission (Figure 2f–j). The latter morphological state could be interpreted in two ways. Either the mitochondria were more loosely wrapped around the axoneme (the circular areas of high X-ray transmission corresponding to gaps between the mitochondria as they looped around the axonemal complex) or the mitochondria were distended, the areas of high X-ray transmission corresponding to translucent matrix material.

The sample cells remained motile within the target chamber and the liquid layer stayed thick enough for motility because of the 5 μm dynospheres used as spacers. The spermatozoa were identified swimming into the field by low-resolution real-time imaging and subsequently immobilized by exposure to X-rays. After formalin-fixation following in-vitro capacitation the proportion of spermatozoa with morphologically transformed mitochondria as analysed with X-ray microscopy was similar to the non-fixed sample, indicating that damage due to X-ray radiation does not explain the transformation of the mitochondria. In the X-ray microscope, spermatozoa survived in the X-ray beam for a period of ~1 s, and during the acquisition of an image, which lasted several seconds, no obvious morphological changes were seen (calculations on radiation damage in X-ray microscopy can be found in Schneider, 1994 and Jacobsen *et al.*, 1998).

A vigorous motility pattern of in-vitro capacitated spermatozoa was observed although no formal characterization of hyperactivation was carried out with computer-assisted semen analysis (CASA). Addition of calcium ionophore induced acrosome-reaction in 80–90% of the spermatozoa (acrosome loss was observed in ~3% of the cells in control without ionophore added). Spermatozoa treated only by washing, i.e. not harvested by the swim-up procedure, showed no significant change in the staining pattern after incubation with ionophore.

Head dimensions corresponded in general with the morphological classification of human spermatozoa given in the WHO laboratory handbook (4.0–5.5 μm long, 2.5–3.5 μm wide), and the mitochondrial sheath contained 11–15 turns consistent with previous reports on human spermatozoa (Curry and Watson, 1995). The dimensions measured on the X-ray microscopic images are shown as a model of the loosely wrapped state (Figure 3) of a simplified mid-piece region, depicting a single helix.

The frequency of occurrence of the two mitochondrial states

in spermatozoa in seminal plasma and after capacitation are given for the four donors in Table I. For all four donors the occurrence of morphologically transformed mitochondria on spermatozoa in seminal plasma was low (13% of a total of 95 spermatozoa imaged). In contrast, after in-vitro capacitation there was a significantly higher proportion ($P < 10^{-5}$) of morphologically transformed mitochondria (96% of a total of 79 spermatozoa imaged). After spermatozoa were harvested by swim-up technique and left for 24 h at room temperature, without exposure to capacitating conditions, there was likewise an increase in the proportion of morphologically transformed mitochondria (Table I), but the proportion of 58% with morphologically transformed mitochondria was significantly lower ($P < 0.004$) than in a sample from the same donor (donor no. 2) incubated under capacitating conditions. There was no major increase in the occurrence of morphologically transformed mitochondria after 29–38 h in seminal plasma (Table I).

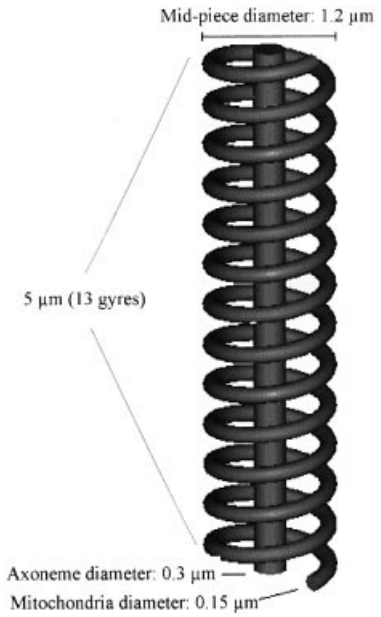

Figure 3. Approximate dimensions of loosely wrapped mitochondria.

Discussion

A striking change in mitochondria towards a more loosely wrapped or distended morphology was observed in spermatozoa subjected to the in-vitro capacitation protocol, compared with spermatozoa in seminal plasma with mitochondria ordered in a tight sheath around the axoneme. This is unlikely to be a result of decay over time or radiation damage as the controls included indicate that these factors alone cannot cause the change observed. Spermatozoa incubated in the capacitation buffer but at atmospheric CO_2 tension and at room temperature showed significantly fewer cells with morphologically transformed mitochondria than spermatozoa of the same sample incubated under standard in-vitro capacitation conditions, i.e. at 37°C and 5% CO_2. This excludes buffer properties such as osmolarity and ionic strength as sole causes of the change.

Evaluation of the maturation stage of spermatozoa by the ability to undergo calcium ionophore-induced acrosome reaction suffers from involving an agent which is unlikely to have any chemical counterpart in the intrauterine environment and consequently suffers from lack of good reference conditions. In the present study, the great majority of spermatozoa treated according to the in-vitro capacitation protocol were able to undergo acrosome reaction whereas washed spermatozoa lacked this ability. This indicates that the ability

Table I. Occurrence of tightly wrapped and morphologically transformed mitochondria in seminal plasma, after in-vitro capacitation, 24 h after harvesting by swim-up technique, fixed in formalin after in-vitro capacitation, and after 29–38 h in seminal plasma

	With tightly wrapped mitochondria	With morphologically transformed mitochondria	Percentage with morphologically transformed mitochondria
Spermatozoa in seminal plasma			
Donor no. 1	27	3	10
Donor no. 2	19	1	5
Donor no. 3	16	4	20
Donor no. 4	21	4	16
Total	83	12	13
Spermatozoa after in-vitro capacitation			
Donor no. 1	1	29	97
Donor no. 2	0	20	100
Donor no. 3	1	19	95
Donor no. 4	1	8	89
Total	3	76	96
Controls			
Incubation control:			
24 h at room temperature after harvesting			
Donor no. 2	5	7	58
Time-decay control:			
After 29–38 h at 37°C in seminal plasma			
Donor no. 3	13	3	19
Fixed in formalin after in-vitro capacitation			
Donor no. 2	0	15	100

to undergo acrosome reaction is caused by in-vitro capacitation and not by adverse effects such as toxicity of the ionophore. The high percentage of spermatozoa which underwent ionophore-induced acrosome reaction may be associated with the likewise high percentage of in-vitro capacitated spermatozoa with morphologically transformed mitochondria, thereby suggesting that the altered mitochondrial morphology is part of the process of capacitation.

Change in mitochondrial morphology is in line with the general concept that mitochondria are dynamic organelles which can change their size and shape depending on the metabolic state of a cell (Sadava, 1993) and during spermiogenesis (Woolley 1970; Otani *et al*., 1988). The model in Figure 3 depicts a single mitochondrial helix; however, it has been considered that mammalian sperm mitochondria wrap around the axonemal complex as a double (Otani *et al*., 1988), triple or quadruple helix (Phillips, 1977). In the present study we did not determine the number of helical strands from our X-ray micrographs. Based on studies of spermatozoa from over 200 mammalian species Cardullo and Baltz (1991) calculated the tail beat frequency to be increasing with the mitochondrial volume. If the morphological change seen after capacitation is interpreted as a distension of the mitochondria then our study does support an increase in mitochondrial volume as accompanying the capacitation process. It may thus be speculated that the observed change in mitochondrial morphology is associated with the change of motility of capacitated spermatozoa into the state of hyperactivitation.

The focus on changes in the sperm head and acrosomal regions as a part of the maturation process seems a natural choice when considering that the ultimate aim of the capacitation process is to allow for fusion between the spermatozoon and the oocyte. Our study, however, suggests that the changes in the mitochondrial region may also be of importance in the understanding of the complex phenomenon of capacitation.

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