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ORIGINAL ARTICLE

# Oxygen uptake by mitochondria in demembranated human spermatozoa: a reliable tool for the evaluation of sperm respiratory efficiency

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## Summary

In this work we report a relatively simple and fast method for analysing oxygen consumption and therefore mitochondrial functionality, in individual human ejaculates. This oxygraphic method requires a low number of cells, is highly reproducible and linearly correlates with sperm concentration. Our results have shown that oxygen uptake by mitochondria of demembranated sperm cells from normozoospermic subjects is significantly stimulated by a large set of respiratory substrates and ADP. The respiratory control ratio (RCR) values indicate a good coupling between respiration and phosphorylation by sperm mitochondria and thus a well preserved integrity of the mitochondria themselves. Interestingly, whereas the rates of oxygen uptake, as expected, changed with different sperm concentrations, the RCR values remained constant, thus demonstrating a linear response of the assay. In asthenozoospermic subjects, however, a significant decrease in the sperm respiratory efficiency was found. The results obtained suggest that this method, besides its potential clinical application, could be useful for a deeper understanding of the biochemical properties of sperm mitochondria and their role in ATP production in human spermatozoa.

## Introduction

Mammalian reproduction is characterized by internal fertilization and usually the site of sperm–egg encounter is quite distant from the site of semen deposition (Hunter, 2005). As a consequence, sperm motility is fundamental for reproductive success. Indeed, mammalian sperm do display at least two different types of motility; one, in freshly ejaculated sperm and the second in sperm collected at the site of fertilization (hyperactivation) (Suarez & Ho, 2003). In humans, asthenozoospermia (poor sperm motility) is probably the most significant cause of male infertility but its aetiology is still unknown, although a large contribution from genetic factors is clearly established (Jansen & Burton, 2004; Turner, 2006). However, in this context controversial findings have been reported on the contribution of mitochondrial respiration to flagellar motility of human spermatozoa. On the one hand, it has been proposed that glycolysis plays a fundamental role in supplying ATP for sperm motility (Turner, 2006). On the other hand, an increasing amount of evidence suggests the involvement of mitochondria not only in motility, but also in sperm differentiation and maturation (Ruiz-Pesini *et al.*, 1998; Ford, 2006; Nakada *et al.*, 2006). A better clarification of the role of mitochondria in the function and in the overall metabolism of spermatozoa (Brooks & Mann, 1972) is therefore desirable.

The main function of mitochondria is the production of ATP by oxidative phosphorylation. These organelles continuously oxidize different substrates reducing, at the same time, oxygen to water (Chance & Williams, 1956). A useful indicator of mitochondrial functionality is, therefore, the oxygen consumption over time which is generally measured polarographically. However, although this relatively simple assay has been carefully standardized in particular tissues of model animals, as far as we

know, a standard and routine test does not exist in the case of human semen samples. Furthermore, mitochondria from spermatozoa show some peculiar characteristics which differentiate these organelles from those present in somatic tissues. For instance, their morphology and arrangement in the sperm cells are quite different because they form a mitochondrial sheath in the midpiece of the flagellum (Turner, 2006). In addition, while several differences have been discovered in the functionality of somatic and sperm mitochondria, such as the stimulatory role of malate on pyruvate oxidation (Storey & Kayne, 1977, 1978; Piasecka *et al.*, 2001), particular aspects of sperm mitochondria functionality still require further clarification.

We therefore undertook a study with the aim of defining accurate and reproducible experimental conditions in which a standard measurement of oxygen consumption by human sperm is possible. The results obtained open up new possibilities in clinical and biochemical studies aimed at clarifying the involvement of the mitochondria function in human sperm quality and performance.

## Materials and methods

### Sperm preparation procedure

Human ejaculates were obtained from 18 healthy males of proven fertility and four selected patients with ashtenozoospermia. After liquefaction at room temperature for about 30 min, semen analysis was performed according to World Health Organization (1999) guidelines. Spermatozoa were separated from seminal plasma by centrifugation at 800 ***g*** for 10 min and immediately used for oxygraphic studies or processed for hypotonic swelling.

As a control, animal studies were performed with sexually mature male Wistar rats (100–150 g). The caudal epididymal spermatozoa of the rats were flushed from the excised epididymis with isotonic salt medium (2 g/L BSA, 113 mmol/L KCl, 12.5 mmol/L $KH_2PO_4$, 2.5 mmol/L $K_2HPO_4$, 3 mmol/L $MgCl_2$, 0.4 mmol/L EDTA and 20 mmol/L Tris adjusted to pH 7.4 with HCl) and processed for oxygraphic studies after hypotonic treatment as described below. Experiments were carried out in accordance with local and national guidelines regarding animal experiments.

### Hypotonic treatment of spermatozoa

Rat and human spermatozoa were washed three times (800 ***g*** for 10 min at room temperature) in isotonic salt medium. Spermatozoa were then subjected to hypotonic treatment essentially as described in Piasecka *et al.* (2001). Briefly, sperm cells were kept in ice-chilled hypotonic medium (potassium phosphate 10 mmol/L, pH 7.4, with 2 g/L BSA) for 1.5 h. Sperm were then washed three times using isotonic salt medium, pH 7.4, as previously described. An aliquot was processed for transmission electron microscopy (see below). Where indicated, sperm cells were treated with the impermeable fluorescent dye propidium iodide (final concentration 1 $\mu$g/mL) to check cell membrane removal. For oxygraphic studies the cell number was determined in a Bürker chamber.

### Oxygraphic studies

Oxygen uptake by spermatozoa was measured by using a Clark-type oxygen probe (Hansatech oxygraph; Hansatech Pentney, King's Lynn, UK) immersed in a magnetically stirred, 1 mL sample chamber in a water bath. Samples (intact or demembranated human sperm and demembranated rat sperm) were stirred vigorously in the reaction chamber (1 mL) at 36 °C in isotonic salt medium without EDTA. In each experiment, cells were temperature-equilibrated at 36 °C for 15 min prior to substrates, ADP or inhibitor addition. The rate of oxygen uptake by spermatozoa ($V$) was expressed as nmol $O_2 \cdot mL^{-1} \cdot min^{-1}/10^8$ cells. The respiratory control ratio (RCR) was calculated by dividing $V_3$ (rate of oxygen uptake measured in the presence of substrates + ADP, i.e. state 3 of respiration) by $V_4$ (rate of oxygen uptake measured with substrates alone, i.e. state 4 of respiration), whereas RCR* was obtained dividing FCCP-uncoupled by oligomycin-inhibited respiration rates. In these experiments we used the following substrates, inhibitors and artificial acceptors of electrons: L-malate, pyruvate, succinate, L-glycerol-3-phosphate, L-ascorbate, TMPD (tetramethyl-p-phenylenediamine), L-lactate, palmitoyl-carnitine, palmitoyl-CoA, oligomycin (inhibitor of the FoF1 ATP-synthase), FCCP (carbonylcyanide p-trifluoromethoxyphenylhydrazone, uncoupler of oxidative phosphorylation), antimycin A (inhibitor for complex III), rotenone (inhibitor for complex I), malonate (competitive inhibitor for succinate oxidation by complex II), mersalyl (a thiol reagent inhibiting the succinate transport into mitochondria) and KCN (inhibitor for complex IV).

### Transmission electron microscopy

Hypotonically treated human sperm were fixed in 4% glutaraldehyde, 5% paraformaldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4 for 2 h at 4 °C (Karnovsky, 1965) and post-fixed for 1 h at 4 °C in 0.1% osmium tetroxide in the same buffer. After dehydration, sperm were embedded in Epon-Araldite resin. Thin sections were stained with uranyl acetate and lead citrate and observed with a Philips CM10 electron microscope (Philips, Eindhoven, The Netherlands).

### Statistical analysis

Experimental and oxygraphic data were analysed statistically. All data are reported as mean ± SE.

## Results

The assay of oxygen consumption by sperm mitochondria is currently carried out in intact human gametes. In this study we used samples in which semen analysis confirmed that all ejaculates were normozoospermic (unless otherwise indicated) according to the parameters of the World Health Organization (1999) having a mean percentage of progressive motility of 58.0 ± 6.1% and a mean sperm concentration of $69.8 \pm 25.4 \cdot 10^6$ cells/mL, with less than one somatic cell per 10 optical fields (400×). After the addition of $10^8$ intact sperm cells to the oxygraph incubation mixture containing different respiratory substrates as previously reported (Peterson & Freund, 1970; Ford & Harrison, 1981) we found, at most, an oxygen uptake of 11.2 nmol $O_2 \cdot mL^{-1} \cdot min^{-1}/10^8$ cells. In this experimental system, however, it is difficult to determine an accurate and standardized oxygen uptake for several reasons, such as (i) the presence of unidentified and unquantified respiratory chain substrates and/or modulators in intact semen samples; (ii) the permeability barrier of the plasma membrane which may interfere, to a certain degree, with the availability of externally added substrates to mitochondria; (iii) the heterogeneity of the samples which may contain a mixed population of intact, broken and damaged cells. In order to overcome all these problems we made use of a different technique, originally employed in sperm of various mammals (Morton & Lardy, 1967; Keyhani & Storey, 1973a,b; Storey, 1975; Storey & Kayne, 1975; Calvin & Tubbs, 1978; Stewart & Forrester, 1979; Piasecka *et al.*, 2001), and consisting of the removal of the plasma membrane before carrying out the oxygen uptake assay, while preserving mitochondrial functionality and shape.

Human spermatozoa were therefore subjected to hypotonic swelling and the effects of such treatment were subsequently analyzed by fluorescence, light and electron microscopy. As shown in Fig. 1, samples were devoid of contamination by somatic cells (a) and staining with propidium iodide demonstrated that sperm cells were demembranated by the hypotonic treatment (b). These observations were further confirmed by transmission electron microscopy. As expected, the plasma membrane of such a kind of spermatozoa appeared completely disrupted, whereas the mitochondria seemed unaltered in their morphology and in the helical configuration in the sperm midpiece (Fig. 2). The rupture of the plasma membrane, followed by the washing steps, caused the loss of the various metabolites contained inside the cells, thus exposing sperm mitochondria to an environment with a well-defined composition.

The respiration capacity of these demembranated cells was then tested polarographically, as reported in Fig. 3. The addition of 10 mmol/L of each of the two substrates, pyruvate and malate, to $2 \times 10^7$ demembranated sperm cells promoted a significant oxygen uptake by mitochondria (respiration state 4) which was further stimulated by 0.76 μmol/L ADP (respiration state 3) (Fig. 3a). Figure 3(b) shows a similar experiment in which the influence of specific inhibitors of the mitochondrial respiratory chain was tested. As expected, oligomycin significantly inhibited the pyruvate/malate-stimulated oxygen uptake, whereas FCCP maximally reversed this inhibition. Antimycin A, on the other hand, completely abolished oxygen consumption by sperm mitochondria. The addition of succinate to rotenone-treated sperm mitochondria caused a significant increase in oxygen

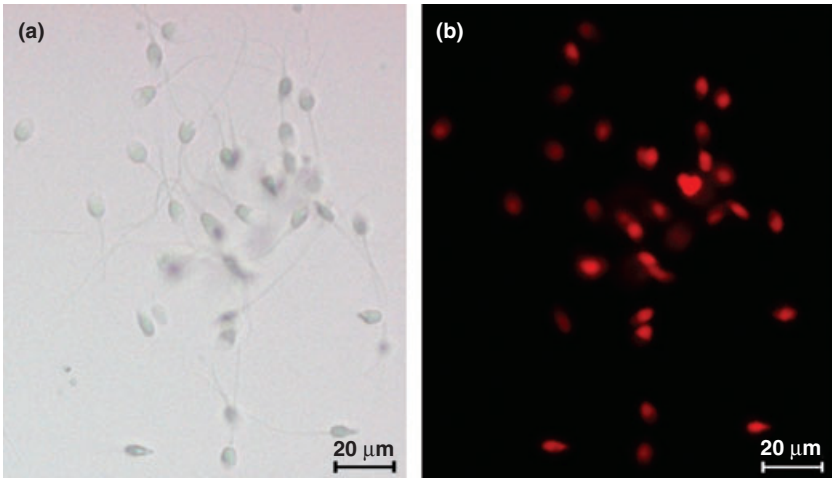


**Figure 1** Effect of hypotonic treatment on ejaculated spermatozoa. (a) Light micrograph of smeared human spermatozoa from a representative sample and (b) fluorescence micrograph of the same optical field after treatment with the non-permeable nuclear dye propidium iodide. All cells are strongly labelled.

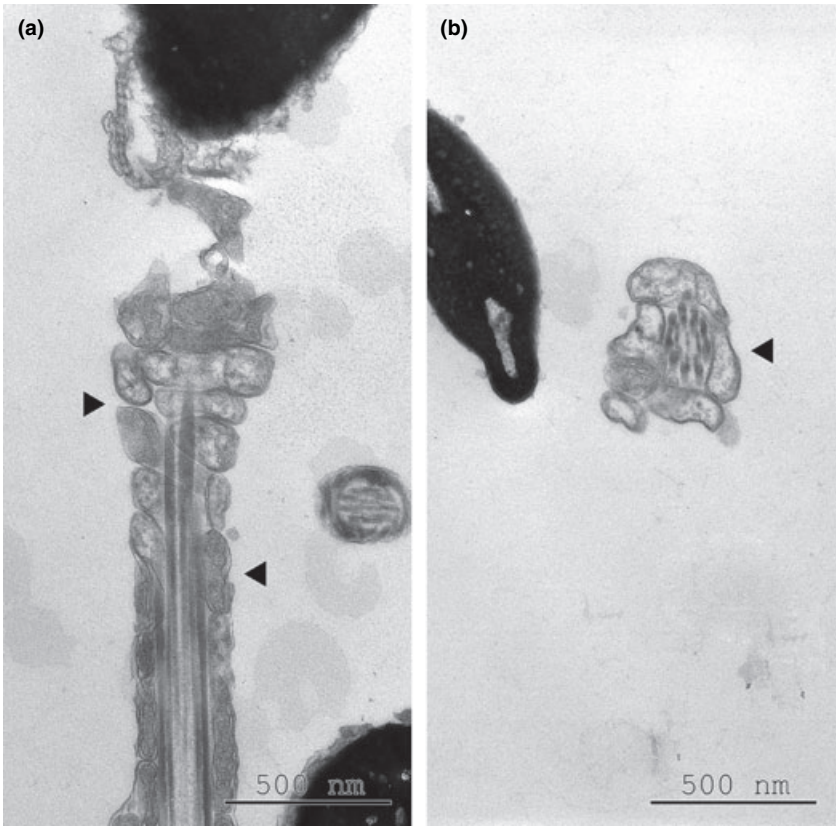


**Figure 2** Transmission electron micrograph of human spermatozoa after hypotonic treatment. Loss of plasma membrane (arrowheads) is clearly evident in both longitudinal (a) and cross sections (b). Note the good preservation of the shape and morphology of the mitochondria.

consumption (Fig. 3c). As expected, oxygen uptake was further stimulated by ADP and completely inhibited by malonate (Fig. 3c). Interestingly, the respiratory activity of this sample of sperm mitochondria started again, as previously reported (Carey *et al.*, 1981), with glycerol-3-phosphate and it was eventually inhibited by antimycin A (Fig. 3c). As shown in Fig. 3(d), the oxygen uptake by rotenone-treated and succinate-respiring human sperm mitochondria was blocked by the addition of mersalyl. All these findings, i.e. the insensitivity of succinate oxidation to rotenone and the strong inhibition of succinate oxidation by malonate and mersalyl, suggest an active complex II in human spermatozoa, as previously reported in rat sperm samples (Piasecka *et al.*, 2001). In the experiments reported in Fig. 3(e,f) the sperm suspension was first pre-incubated with rotenone and antimycin A, inhibitors of complex I and III, respectively, and then with a mixture of TMPD and ascorbate. In these conditions, a significant oxygen uptake, KCN-sensitive, was clearly registered (Fig. 3e,f). Interestingly, the addition of cytochrome *c*, before or after the addition of ascorbate and TMPD, did not influence the oxygen uptake by sperm mitochondria (compare Fig. 3e with Fig. 3f). These last results indicate the presence of an active complex IV in the intact mitochondria of hypotonically treated human sperm samples.

We then investigated the capability of human sperm samples to oxidize lactate, as previously reported in rabbit sperm mitochondria (Storey & Kayne, 1977, 1978). Figure 4(a) shows that the addition of lactate to sperm mitochondria, pre-incubated with malate, caused a significant oxygen consumption which, as expected, was further stimulated by ADP, and then inhibited by oligomycin. The subsequent FCCP-induced uncoupled rate of oxygen uptake was inhibited (35% decrease) by the addition of pyruvate (Fig. 4a). Figure 4(b) shows a similar experiment in which the oxygen uptake by sperm mitochondria was induced by pyruvate, instead of lactate, added at the beginning of the experiment. In agreement with previous findings (Storey & Kayne, 1978), the subsequent addition of lactate slightly stimulated the oxygen consumption induced by malate and pyruvate (Fig. 4b). We also explored the capability of the hypotonically treated human sperm samples to oxidize fatty acids (Carey *et al.*, 1981). Figure 4(c) shows the strong stimulation of oxygen uptake by sperm mitochondria subsequent to the addition of palmitoyl-carnitine. On the contrary, a lower oxygen uptake was detected in the presence of palmitoyl-CoA (Fig. 4d). Interestingly, a strong stimulation of mitochondrial respiration was seen after the addition of

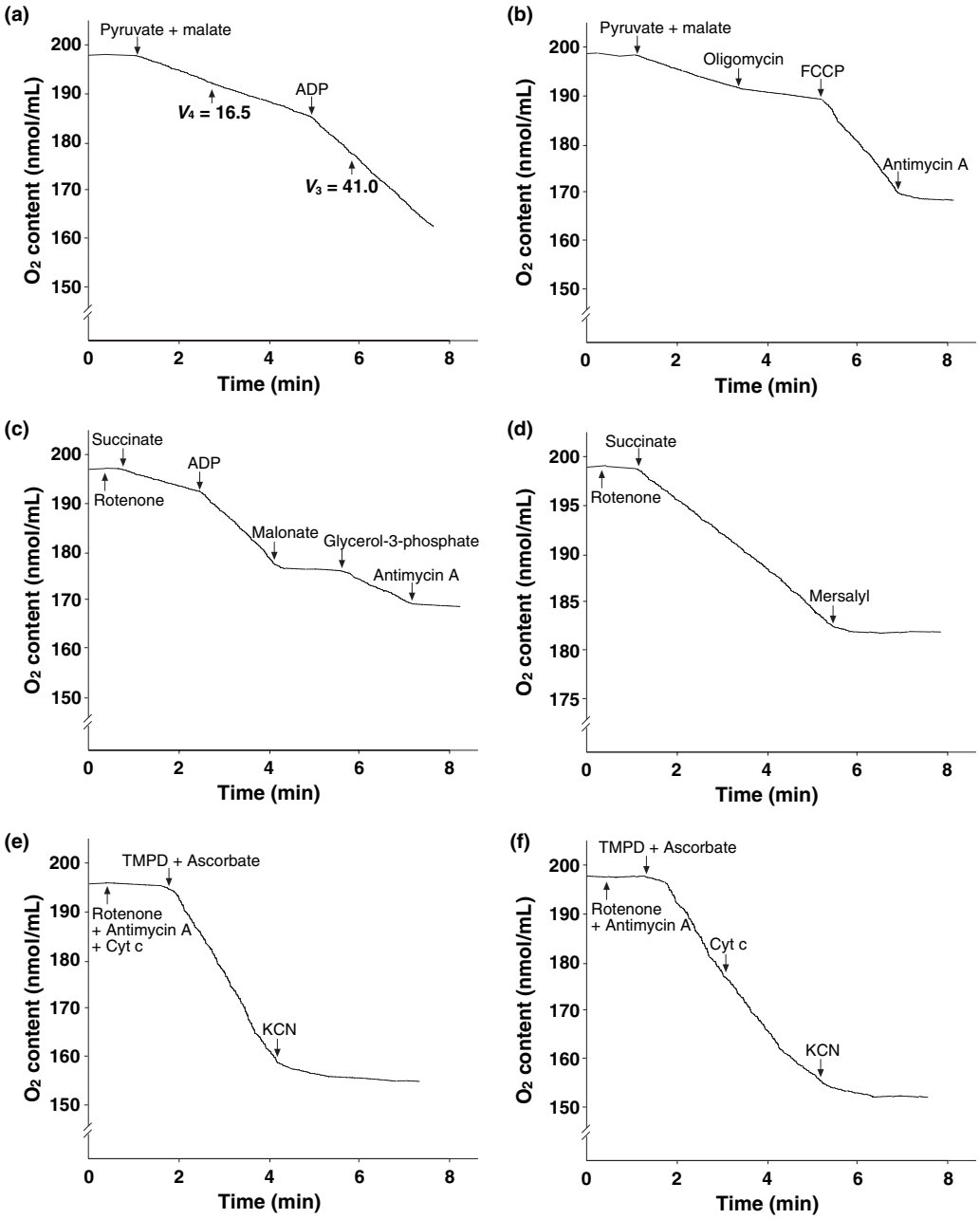


**Figure 3** Respiration capacity of demembranated sperm cells. Samples ($2 \times 10^7$ cells) were stirred vigorously in the oxygraph reaction chamber at 36 °C in isotonic salt medium (without EDTA) containing 15 mmol/L inorganic phosphate. Oxygen uptake by spermatozoa was measured polarographically in the presence of various substrates or inhibitors. (a) Oxygen uptake by spermatozoa in the presence of 10 mmol/L malate and 10 mmol/L pyruvate and 0.76 μmol/L ADP. The rate of oxygen uptake in respiration states 4 ($V_4$) and 3 ($V_3$) is expressed as nmol $O_2 \cdot mL^{-1} \cdot min^{-1}/10^8$ cells. (b) Oxygen consumption was measured in the presence of 10 mmol/L malate and 10 mmol/L pyruvate, followed by the addition of 0.88 μmol/L oligomycin, 1 μmol/L FCCP and 2 μmol/L antimycin A. (c) Oxygen uptake by rotenone (15 μmol/L)-pretreated sperm samples in the presence of succinate (10 mmol/L) and ADP (0.76 μmol/L). The subsequent additions were as indicated: 60 mmol/L malonate, 6 mmol/L glycerol-3-phosphate and 2 μmol/L antimycin A. (d) Oxygen uptake by rotenone (15 μmol/L)-pretreated sperm samples in the presence of succinate (10 mmol/L) followed by the addition of 0.8 mmol/L mersalyl. (e) Samples were incubated in the presence of 15 μmol/L rotenone, 2 μmol/L antimycin A and 13 mmol/L cytochrome c. Respiration was started by the addition of a mixture of 10 mmol/L ascorbate and 1 mmol/L TMPD and blocked by 18 mmol/L KCN. (f) Sperm samples were incubated in the presence of 15 μmol/L rotenone and 2 μmol/L antimycin A. Respiration was started by the addition of a mixture of 10 mmol/L ascorbate and 1 mmol/L TMPD, followed by the addition of 13 mmol/L cytochrome c and blocked by 18 mmol/L KCN.

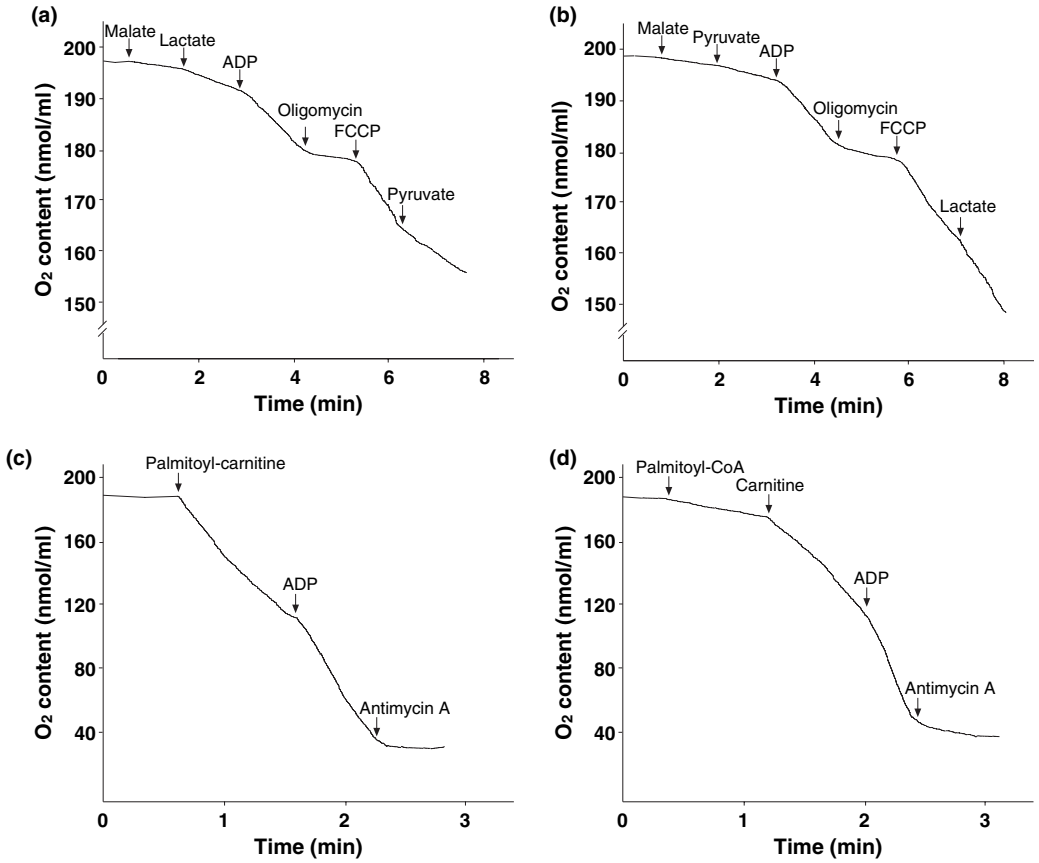


**Figure 4** Oxygen uptake by demembranated sperm cells. The experimental conditions were identical to those described in the legend to Fig. 3. (a) Effect of pyruvate on the oxygen uptake rate with lactate and malate as substrates. Oxygen consumption was measured in the presence of 10 mmol/L malate, followed by the addition of 10 mmol/L lactate, 0.76 $\mu$mol/L ADP, 0.88 $\mu$mol/L oligomycin, 1 $\mu$mol/L FCCP and 10 mmol/L pyruvate. (b) Effect of lactate on the oxygen uptake rate with pyruvate and malate as substrates. All additions were made as shown. (c) Oxygen uptake by spermatozoa was measured in the presence of 1 mmol/L palmitoyl-carnitine, followed by the addition of 0.76 $\mu$mol/L ADP and 2 $\mu$mol/L antimycin A. (d) 44 $\mu$mol/L palmitoyl-CoA was added to demembranated spermatozoa. All additions (1 mmol/L carnitine, 0.76 $\mu$mol/L ADP and 2 $\mu$mol/L antimycin A) were made as shown.

carnitine (Fig. 4d). These findings therefore suggest that an active carnitine palmitoyltransferase is operating in human sperm mitochondria which are also able to efficiently oxidize exogenous added fatty acids.

Table 1 reports the mean values of $V_3$ (oxygen uptake rate in state 3 of respiration), $V_4$ (oxygen uptake rate in state 4 of respiration), obtained from pyruvate/malate-respiring mitochondria as shown in Fig. 3(a), and their ratio, also known as RCR. In addition, we reported a different ratio, indicated as RCR*, calculated by dividing the oxygen uptake rate in FCCP-uncoupled mitochondria by that measured in oligomicyn-inhibited organelles. With $5 \times 10^7$ demembranated sperm cells a $V_3$ of $48.0 \pm 6.0$ nmol $O_2 \cdot mL^{-1} \cdot min^{-1}/10^8$ cells and an RCR of $2.5 \pm 0.2$ were found (Table 1). This last value represents an indicator of mitochondria integrity and suggests a good coupling between the respiration and phosphorylation processes. The RCR* value, which represents a more definitive measure of coupling, was $5.5 \pm 0.9$. It was very interesting to note that with a lesser amount ($2 \times 10^7$) of sperm cells we found similar values of RCR and RCR* (Table 1). These findings indicate that our experimental system responds in a linear fashion with a limited amount of sperm cells, thus suggesting the possibility of its use in oligozoospermic subjects. The limit of sensitivity of this experimental system was found to be $1.5 \times 10^7$ demembranated sperm cells (data not shown).

For comparison, we isolated sperm cells from rat cauda epididymis and treated them in the same way as the human samples. The $V_3$ and $V_4$ values obtained with the rat sperm cells are lower with respect to those measured with the human semen samples (Table 1), but similar to previously reported data (Piasecka *et al.*, 2001).

In order to test the possibility of a difference in mitochondrial function between progressively motile and less active sperm, we eventually measured the respiration

**Table 1** Oxygen consumption rates and RCR values

| | | nmol $O_2 \cdot mL^{-1} \cdot min^{-1}/10^8$ cells | | | |
|---|---|---|---|---|---|
| | Cell number | $V_3$ | $V_4$ | RCR | RCR* |
| Human sperm ($n$ = 6) | $5 \times 10^7$ | 48.0 ± 6.0 | 20.3 ± 3.0 | 2.5 ± 0.2 | 5.5 ± 0.9 |
| Human sperm ($n$ = 9) | $2 \times 10^7$ | 45.3 ± 5.6 | 18.5 ± 2.5 | 2.5 ± 0.1 | 5.8 ± 1.0 |
| Rat sperm ($n$ = 5) | $2 \times 10^7$ | 22.3 ± 1.3 | 7.9 ± 1.1 | 2.9 ± 0.3 | 5.7 ± 0.8 |

$V_3$ represents the rate of oxygen uptake by spermatozoa in the presence of substrates (15 mmol/L inorganic phosphate, 10 mmol/L malate and 10 mmol/L pyruvate) plus ADP (0.76 μmol/L), whereas $V_4$ is the rate measured with substrates alone. RCR was calculated by dividing $V_3$ by $V_4$. RCR* is the ratio of uncoupled (1 μmol/L FCCP) and oligomycin (0.88 μmol/L)-inhibited respiration rates.

capacity of human sperm mitochondria from some asthenozoospermic subjects (mean percentage of sperm motility 36.3 ± 1.7%; $n$ = 4). In these samples we found a significant reduction in the active state of respiration ($V_3$ =19.8 ± 1.5 nmol $O_2 \cdot mL^{-1} \cdot min^{-1}/10^8$ cells) when measured in the same experimental conditions used for normozoospermic subjects (Table 1, $V_3$ = 45.3 ± 5.6 nmol $O_2 \cdot mL^{-1} \cdot min^{-1}/10^8$ cells). On the contrary, the $V_4$ values were almost unaffected (18.0 ± 1.6 in astheno- vs. 18.5 ± 2.5 nmol $O_2 \cdot mL^{-1} \cdot min^{-1}/10^8$ cells in normozoospermic samples), thus leading to a significantly lower value of RCR (1.1 + 0.1) in asthenozoospermic subjects in comparison with normozoospermic ones (RCR = 2.5 ± 0.1).

## Discussion

The role of mitochondria in sperm quality and functionality is currently under investigation (Miki *et al.*, 2004; Ford, 2006; Nakada *et al.*, 2006; Turner, 2006). The reason is mainly due to the fact that the cause of several cases of asthenozoospermia is still unknown. A strong correlation between sperm motility and/or the overall quality of human sperm, on the one hand, and the mitochondrial functionality, on the other hand, has been previously reported by using different technical approaches (Ruiz-Pesini *et al.*, 1998, 2000; Gallon *et al.*, 2006; Nakada *et al.*, 2006). This in spite of the fact that other authors have underlined the key role of cytosolic glycolysis in supplying ATP for sperm motility, although its role seems to be highly species-specific (Miki *et al.*, 2004; Turner, 2006). In any case, the availability of a simple and reproducible assay for the measurement of oxygen uptake by sperm mitochondria represents an appealing goal in clinical and biochemical studies.

An overview of the available literature revealed that the measurements of oxygen uptake in human sperm mitochondria are currently carried out using intact sperm. This experimental system presents several problems, such as a lack of reproducibility in the assay due to the presence of unidentified internal substrates, the heterogeneity of the samples and/or the presence of sperm aggregates. These problems, at least theoretically, may be more significant in pathological samples. Indeed, it is well known from mitochondrial bioenergetics studies that such a kind of assay requires well defined and standardized conditions. With our experimental system we obtained several advantages. First, we measured the oxygen uptake in the presence of defined concentrations of substrates and ADP which have direct access to the mitochondrial respiratory chain, thus allowing a direct comparison of semen samples obtained from different subjects. Secondly, in these experimental conditions higher velocities of oxygen uptake by human sperm samples were found with respect to the values obtained with intact cells (48.0 nmol $O_2 \cdot mL^{-1} \cdot min^{-1}/10^8$ demembranated cells vs. 11.2 nmol $O_2 \cdot mL^{-1} \cdot min^{-1}/10^8$ intact sperm cells). Finally, taking into consideration that it is difficult, if not impossible, to purify sufficient amounts of mitochondria from individual human ejaculates for respiration studies, our model represents an interesting improvement with respect to intact sperm cells, being closer to the model currently used in bioenergetics experiments, i.e. purified rat liver mitochondria. Different from these latter results, however, sperm mitochondria are strongly resistant to the hypotonic treatment for the specific physicochemical properties of the outer mitochondrial membrane (Piasecka *et al.*, 2001; Turner, 2006).

The results showed a proper functionality of the various mitochondrial respiratory chain complexes and a tight coupling between respiration and phosphorylation. Mitochondria of hypotonically treated human sperm samples respired in the presence of a large set of substrates and in the case of malate, pyruvate and lactate showed the same characteristics previously described in rabbit (Storey & Kayne, 1978). Furthermore, a significant decrease in the mitochondrial respiratory efficiency was found in asthenozoospermic subjects. It has to be kept in mind, however, that a correlation between motility and oxygen uptake by sperm mitochondria does not necessarily mean that all the ATP utilized by the sperm axoneme

is coming from the mitochondrial oxidative phosphorylation. The great majority of the ATP utilized by the sperm flagellum could also come from anaerobic glycolysis. Oxygen uptake by mitochondria may also be correlated with other indicators of sperm quality, such as membrane and DNA integrity. Further studies are therefore necessary to clarify better all these aspects.

In our opinion, the implications of this study are the following. On the one hand, a careful and detailed investigation of the mitochondrial bioenergetics of sperm will provide more insight into the basic biology of the spermatozoon with the aim of obtaining more information on the role of sperm mitochondria in motility and on the overall quality of the gametes. On the other hand, we are confident that this relatively simple and fast assay, requiring a limited amount of sperm cells, can be used as a routine semen analysis in clinical investigation.

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