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THE MICROCALORIMETRIC APPROACH TO HUMAN SPERM PHYSIOLOGY

M.L. ANTONELLI and M. LUCIANI

Department of Chemistry, University of Rome "La Sapienza", Rome (Italy)

L. SILVESTRONI

Institute of V Clinica Medica, Reproductive Physiopathology Unit, University of Rome "La Sapienza", Rome (Italy)

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ABSTRACT

Heat conduction microcalorimetry has been adopted to evaluate overall basal metabolism in human spermatozoa. Heat output has also been measured in the presence of some metabolic inhibitors in an attempt to relate it to different cellular energetic pathways. Owing to the presence in spermatozoa of mechanochemical events of motility, microcalorimetric data have also been compared with those derived from cell motility analysis. When basally evoked heat was recorded in a rotating batch calorimeter, a mean value of 127.7 mcal h^{-1} per 10^8 spermatozoa was obtained, which was greatly different from that measured in a static calorimeter. On this basis, the adoption of microcalorimetric instruments in (human) sperm heat production studies is critically analysed. Dose–response experiments showed that metabolic poisons, azide, fluoride and rotenone, induced both heat output and motility reduction. Our first data indicate that, given appropriate experimental conditions, batch calorimetry could represent a promising, powerful tool in the still largely obscure, challenging field of human male gamete physio(patho)logy.

INTRODUCTION

Cell life is the result of a dramatically complex balance between metabolic demand, nutrient availability and energy exchanges. The correct operating of this functional network, i.e. the cell capacity to withstand or adapt to both intra- and extracellular metabolic needs, depends on a machinery which takes exogenous and/or endogenous (stored) substrates and transforms them 1 to provide "high energy" compounds (ATP), 2 to build, renew and modify cell structures (growth, reproduction and movement, respectively) and 3 to make available nutrients for intracellular stores.

Microcalorimetry has long been used to evaluate heat production rate as an indirect index of overall metabolism in several cell types [1,2] including spermatozoa [3,4]. In somatic cells, the high complexity of the metabolic

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pathways requires the adoption of sophisticated experimental designs for their relationships with power output to be identified and quantified. When working with spermatozoa (non-dividing, non-synthesising cells), microcalorimetric data are fairly readily related to cell metabolism: in fact, for these terminal, highly differentiated cells, the main energy-requiring process is represented by cell motility [5]. In aerobic conditions and in the presence of glycolysable substrates, this energy demand is satisfied by the production of ATP from oxidative and glycolytic pathways (the ratio of glucose utilisation to ATP yield is 1 : 38 and 1 : 2, respectively). However, due to the hydrodynamic energy exchanges (propulsion thrust and fluid movement) during sperm swimming, and sperm swimming energetics being directly dependent on ATP production and catabolism, some overlapping and interconversion may occur: consequently, difficulties may arise in calculating separately the metabolic and mechanochemical-related heat fractions.

MATERIALS AND METHODS

Materials

Sodium fluoride, sodium azide, rotenone and dimethylsulphoxide (DMSO) were purchased from Sigma (St. Louis, Montana). All other chemicals were of the best analytical grade available. For sperm preparation and incubation, a buffered salt solution was used (working buffer) consisting of 130 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.33 mM Na_2HPO_4, 3.5 mM $NaHCO_3$, 0.81 mM $MgSO_4 \cdot 7H_2O$ and 11.1 mM D-glucose.

In order to inhibit microbial growth, 0.003% (w/v) streptomycin sulphate and 0.005% (w/v) penicillin-G were added to the working buffer.

Sperm preparation

Semen samples from healthy, fertile volunteers were collected into sterile plastic containers and allowed to liquefy at room temperature (20–22 °C). Mechanical damage induced by cell washing with repeated centrifugations was avoided by isolating spermatozoa as follows. Aliquots (0.25 ml) of semen were transferred into plastic RIA tubes and carefully overlaid with 0.5 ml of working buffer. The preparations were then incubated in a vertical position at 30 °C for 20 min, during which time motile spermatozoa migrated into the buffer phase. At the end of the incubation period, the buffer phases containing motile spermatozoa were gently collected, pooled and centrifuged at $200 \times g$ for 10 minutes at room temperature. The sperm pellet was resuspended in the same buffer and the suspension was maintained at 18–20 °C until the start of the incubation in the calorimeter (less than 30 min). Both sperm suspensions and buffers were gently flushed with air (3

min) just before measurements were started. For inhibition experiments, each inhibitor was added at the selected concentration from a freshly prepared stock solution in DMSO (final DMSO concentration $< 0.3\%$); a preincubation of 15–20 min preceded the heat output measurements.

Cell concentration was determined with a Neubauer counting chamber after blocking sperm motility with diluted acetic acid. Cell viability was assessed before and after microcalorimetric measurement by supravital staining (0.3% Trypan blue, 2 min.) exclusion. Sperm motility was analysed by a computerised motion analysis system (CELL SOFT®).

Calorimeters

Early experiments were carried out in a BAM (Bioactivity Monitor) microcalorimeter (LKB 2277), equipped with two static steel ampoules of 5 ml volume [6].

Subsequent experiments were performed in a rotating batch microcalorimeter [7] of the heat conduction type (LKB 2017), equipped with gold vessels (total volume about 7 ml), a Multitemp cooling circulator (LKB 2209), a Control Unit (LKB 2107-350) and a potentiometric recorder (LKB 2210). Each vessel consists of a chamber divided by an interior wall into two compartments of 2.5 (small compartment) and 4.5 ml (large compartment). All instrumentation was housed in a thermostated room. Measurements were carried out at $37.00 \pm 0.01°$ C. The calorimetric accuracy was checked by measuring the heat of sucrose dilution [7]; our results were in agreement with the literature data to within 0.5%.

Calorimetric measurements

The instrument baseline was recorded before loading the calorimetric vessels. To measure the basal heat production, the measuring vessel was filled with 1 ml of aerated sperm suspension (large compartment) and 1 ml of working buffer (small compartment). The two compartments of the reference vessel received 1 ml of working buffer each. After 30 minutes thermal equilibration, the calorimetric unit was rotated and the heat output was recorded and monitored for at least 30 minutes. During the trace recording, the calorimeter drum was rotated at 10 minute intervals to minimise cell sedimentation inside the calorimetric vessel. Figure 1 shows a typical recording of power output (thermopile voltage versus time). The sperm-derived heat production has been calculated by considering that the instrument output is directly related to the heat evolved inside the calorimeter

$$V = K\delta q/\delta t \qquad (1)$$

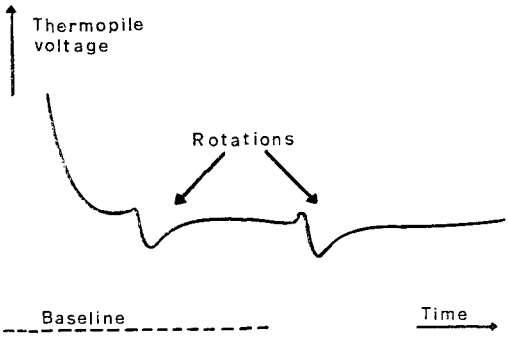

Fig. 1. A representative voltage–time curve obtained from a suspension of sperm cells in basal conditions. Arrows heads indicate drum revolutions.

where V is the thermopile voltage, K is the calibration constant of the instrument and q represents the heat evolved during the period of time t. By integrating the experimental curve (V versus t), it is possible to calculate the overall heat quantity, Q_{sp}, which is directly proportional to the area (A_{sp}) subtended by the curve

$$Q_{sp} = \epsilon A_{sp} \tag{2}$$

where ϵ is an experimental constant obtained under the same conditions as the measurements. Because

$$-\delta H_{sp} = Q_{sp}/\text{No. spermatozoa} \tag{3}$$

it is possible to evaluate the heat associated with the overall sperm metabolism during one hour. The value of A_{sp} was calculated by measuring the area subtended by the experimental curve (V versus t) in 10 minutes and then extrapolating to 1 h.

Inhibition experiments

For inhibition experiments, the two vessels were loaded as before and rotated without delay. After a steady state was obtained and recorded, aliquots of the inhibiting substance were rapidly added into the two vessels and the calorimetric unit was rotated again. Within about 15 minutes, a new steady state, relative to the heat production in the presence of inhibitor, was reached and recorded. The inhibition experiments gave both the dose–heat output and the dose–motility curves, from which the maximal inhibiting concentration (MIC) and the half-maximal inhibiting concentration (IC50) were calculated for each inhibitor. Each experiment was repeated at least three times and the data represent the mean $\pm$ SD.

RESULTS

Supravital staining and motility measurements indicated that more than 90% of the spermatozoa in the final suspension were intact and regularly motile. Preliminary experiments showed a good positive correlation ($R = 0.99$) between sperm number ($10–35 \times 10^6$/ml) and heat production (Fig. 2); therefore, the cell concentration was maintained in the above range. In our experimental conditions, it may be assumed that sufficient oxygen was available for the cells to sustain aerobic metabolism. Only the results obtained from the rotating batch microcalorimeter are reported; the reliability of the heat-ouput measurements derived from the static BAM microcalorimeter was, in fact, low due to cell sedimentation into the measuring vessel which led to a continuous downward increasing variation of the sperm–substrate ratio, independent of and interfering with the metabolic traffic.

The mean value of basal heat production by seminal plasma-free spermatozoa was 127.7 ± 7 mcal h^{-1} per 10^8 cells. Once a steady state was reached, the calorimetric curve (thermophile V versus t) remained stable for at least one hour; the thermogram shape was comparable within different experiments performed in the same conditions, and successive drum rotations did not interfere with V versus t recording (see Fig. 1).

Sodium azide induced a drop in heat production (-70% of the control) with an MIC of 50 mM, and an IC50 of 6 mM (see Fig. 3). This inhibitor also induced a marked reduction of motile cells (-43% relative to the control) with an MIC of 5 mM and an IC50 of 2 mM; neither heat output nor cell motility varied further up to 20 mM inhibitor concentration. 2 mM Sodium fluoride induced an unexpected increase (25%) in heat production, followed by a 45% inhibition relative to the control at 5 mM NaF (IC50)

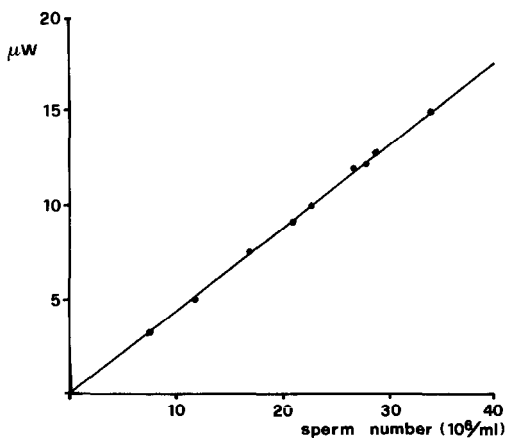

Fig. 2. Correlation of heat production and sperm concentration.

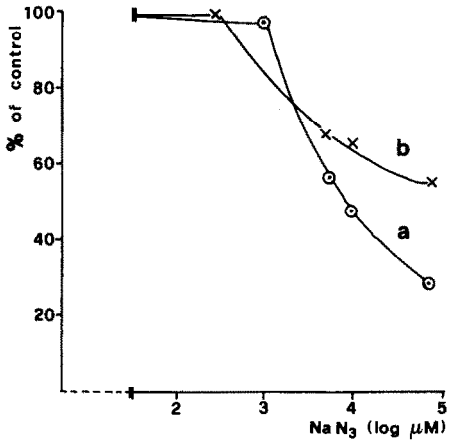

Fig. 3. Effects of sodium azide on sperm heat production (a) and motility (b). The data are expressed as % of control and each value represents the mean value ±S.D. of four measurements.

and by a further 15% reduction at 35–40 mM (MIC) (see Fig. 4). This inhibitor also induced (1–3 mM) an increase in the tail-beating amplitude before inhibiting motility (−50% relative to the control). In the presence of rotenone, a 50% reduction of heat output was induced by 0.125 mM inhibitor, with an IC50 of 0.05 mM; this inhibitor brought about a slight impairment of sperm motility, with maximal reduction (−10–15% relative to the control) attained at 0.04 mM (see Fig. 5).

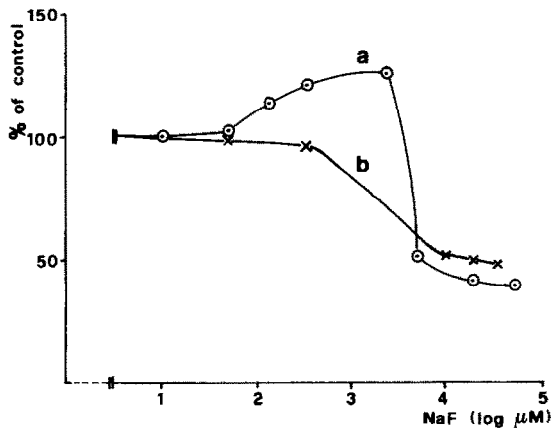

Fig. 4. Effect of sodium fluoride on sperm heat production (a) and motility (b). The data are expressed as % of control and each value represents the mean value ±S.D. of four measurements.

DISCUSSION

In the course of the calorimetric recordings, neither the viability not the motility of the control cells varied to a significant extent. Care was taken not to exceed 35×10^6 ml^{-1} sperm concentration in order to avoid the "crowding effect" (a negative correlation between cell number and heat output) which has been reported for various cell types [8].

The heat production by the spermatozoa may be assumed to be the sum of the power output of cell metabolism and motility, albeit the hydrodynamic contribution of sperm motility to total heat output is low, accounting for about 10% of free energy available from ATP hydrolysis [9].

The stability of the recording after the V versus t curve reached a steady state does not unequivocally mean that metabolic interconversions did not take place during sperm incubation in the calorimeter. Some points should be made before discussing the experimental data. In contrast with somatic cells, in which the majority of the ATP is utilised for ion traffic at the membrane level, in spermatozoa the greatest fraction (70%) of the ATP available is used to sustain the mechanochemical events of microtubule sliding into the tail, i.e. cell motility [5]. These events allow the tail to beat, the beating being characterised by a sinusoidal shape of particular wavelength and amplitude. It has been reported that, when the viscosity of the suspension medium was taken as that of water (7×10^{-4} Pa sec^{-1}), the sperm power output ranged from 5 to 9 W per 10^{14} cells (depending on the

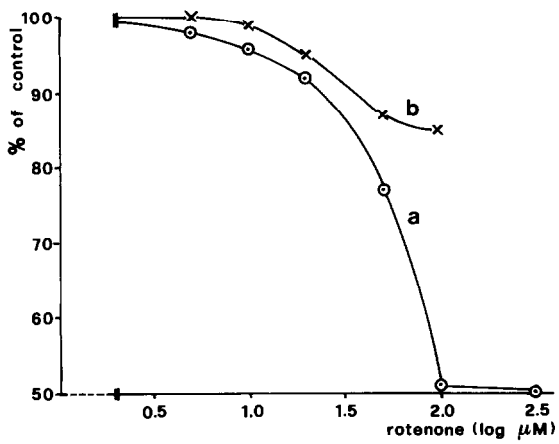

Fig. 5. Reduction of sperm heat production (a) and motility (b) induced by rotenone. The data are expressed as % of control and each value represents the mean value ±S.D. of four measurements.

metabolic state of the spermatozoa), with a positive correlation between the power output and the amplitude of the flagellar beat [10].

However, the wide interspecies differences between ATP hydrolysis rate and cell motility pattern [11–13] make it difficult to separate these two activities and to transpose animal data into the human field.

The reduction of the heat production rate in the presence of the selected inhibitors, NaN_3, NaF and rotenone, was expected, in accordance with their known poisoning effects on metabolism.

In particular, sodium azide, a blocker of sperm respiration and oxidative phosphorylation, is known to prevent ATP synthesis; as a consequence, both thermal output and ATP production are reduced, the latter accounting for the observed reduction of cell motility. However, the much less pronounced inhibition of sperm motility with respect to heat production could be due to a persistent production of ATP from the Krebs cycle through the β-oxidation of cellular (membrane) fatty acids.

The increase in heat output observed in the presence of 2 mM NaF is rather puzzling; it could perhaps be explained by considering that, at this concentration, fluoride causes an increase in the amplitude of tail beating (data not shown) and that the shape of the tail beating has been reported to be positively correlated with sperm power output [10]. However, the low contribution of tail beating to total heat flow in sperm speaks against the above interpretation; more likely, the enhancing effect of fluoride could be ascribed to its phospholipase-C-mediated stimulating action on phospholipid breakdown [14].

At first glance, the fluoride-induced reduction of heat flow and motility could be ascribed to its inhibitory effect on the glycolytic enzyme enolase [15], leading to a decreased ATP production from glucose catabolism; the low ATP yield of the glycolysis relative to that obtained from respiration, however, does not explain the strong inhibitory effect of fluoride on sperm motility in terms of ATP metabolism. This apparent discrepancy could be explained on the basis of the known inhibitory effect of fluoride on microtubule dynein ATPase [16] from which the chemical energy for microtubule sliding (i.e. for sperm motility) is derived. In fact, in the presence of 5 mM fluoride, the cells stop moving and the sperm tails stiffen, assuming a rod-like shape.

Rotenone, an uncoupler of oxidative phosphorylation which inhibits electron transfer acting at the level of the mitochondrial nicotinamide adenine dinucleotide dehydrogenase, does not interfere with the glycolytic chain: therefore, in the presence of rotenone, the cell becomes totally dependent on the glycolytic synthesis of ATP. In our experimental conditions, where sufficient extracellular glucose is available, the ATP demand for sperm motility is satisfied by the glycolysis; with this in mind, the reduction of heat output (due to mitochondrial inhibition) was expected to occur, as was, in addition, the negligible inhibitory effect of rotenone on motility.

CONCLUSION

Microcalorimetry, if properly applied, appears to be a suitable method for research into human sperm energetics. The analysis of power output data obtained in the presence of specific inhibitors and/or stimulators, could help to isolate and characterise physiologically entangled metabolic pathways. In addition to the cognitive value of the experimental data obtainable from calorimetric studies, the application of this methodology in pathological states (e.g. reduced sperm number and motility) could also give useful information concerning some still unexplained conditions of male infertility. Finally, if applied in the presence of biological stimulants (deriving from both the male and female genital tracts), microcalorimetry could also be a promising tool for understanding the challenging and still largely unknown chain of cytological events (i.e. the "capacitation" and the "acrosome reaction") that the male gamete undergoes in the female genital tract to enable it to reach and fertilise the ovum.

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