Sperm function and mitochondrial activity: An insight on boar sperm metabolism

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Abstract

In this study boar sperm mitochondrial activity was studied and deepened in order to delineate the main metabolic strategies used by boar sperm to obtain energy and to link them to sperm function. Boar spermatozoa were collected, diluted at $30x10^6$ spz/mL and incubated for 1 h with: Rotenone (ROT), complex I inhibitor, Dimethyl-malonate (DMM), complex II inhibitor, antimycin A (ANTI), complex III inhibitor, oligomycin (OLIGO), ATP synthase inhibitor, Carbonyl cyanide m-chlorophenyl hydrazone (CCCP), uncoupling agent, 2-deoxy-glucose (2DG), glucose agonist, and Dimethyl sulphoxide (DMSO) as control vehicle. Viability and mitochondrial membrane potential (Sybr14/PI/JC1 staining) and sperm motility (using CASA system) were assayed after incubation.

ROT, ANTI, OLIGO and CCCP significantly reduced total and progressive motility as well as cell velocities; ANTI and CCCP depressed mitochondrial membrane potential but did not affect cell viability. Cluster analysis of kinematic parameters showed some interesting features of sperm subpopulations: ANTI and CCCP caused a shift in sperm subpopulation towards "slow non progressive" cells, OLIGO and ROT caused a shift towards "average" and "slow non progressive" cells, while DMM and 2DG increased the "fast progressive" cells subpopulation.

Sperm mitochondrial respiration and substrate oxidation, assayed polographically and spectrofluorimetrically, respectively pointed out a high ATP turnover and a low spare respiratory capacity, mainly linked to the NADH-O_2 oxidase activity. Therefore, boar spermatozoa heavily rely on mitochondrial oxidative phosphorylation, and especially on Complex I activity, to produce ATP and fuel motility.

1. Introduction

Mammalian sperm cells have the central role to deliver the haploid male genome to the egg. To do this, sperm cells should go along the female genital tract and be integer from a morphological and a functional point of view [1].

It is well-known that sperm cells use different metabolic substrates to generate energy for the different cell functions such as motility, capacitation and sperm-oocyte interactions [2–4]. Various studies focused on the metabolic strategy of sperm cells and on the involvement of mitochondria in energy supplying system [5–7]. Some mammalian species, such as humans, mice and pigs, are reported to preferentially use glycolysis, while equine mainly uses mitochondrial oxidative phosphorylation (OXPHOS); others, namely bovine, exploit an intermediate strategy to fuel energy pathways [1,3,4,8–10].

Interestingly, mitochondria are known to rule different sperm functions [4,11–14] and to physiologically produce reactive oxygen species (ROS), which address cell signaling pathways [15]. However, any impairment of the mitochondrial electron transport chain increases ROS level, causing oxidative damage to biomolecules [16] and detrimental effects on sperm quality (DNA integrity, membrane stability) and functions (energy production, motility, capacitation).

To explore the relevance of mitochondria in sperm bioenergetics, previous studies aimed at identifying the specific function of each respiratory complex both by testing selective inhibitors of each complex or by fueling the respiratory chain by substrates entering specific steps [5,7,17].

To date, the most complete studies were performed in boar and stallion spermatozoa as representatives of the most diversified metabolic strategies in mammalian spermatozoa. Accordingly, boar spermatozoa have been suggested to use almost 95% glycolysis to fulfil energy requirements [2,3,18], while horse spermatozoa are more strictly dependent on mitochondrial oxidative phosphorylation [5,6,9].

Studies on mitochondrial functions in stallion and boar spermatozoa also demonstrated oxygen consumption coupled to ATP synthesis [5,17]. Mitochondrial respiration coupled to OXPHOS is the main ATP production system [19] and, under aerobic conditions, it is preferred to cellular acidification due to glycolysis. Sperm motility and fertilizing ability rely on the availability of ATP, the energy currency of the cell, in turn dependent on the mitochondrial status [20]. Finally, mitochondrial activity was associated to sperm capacitation in "*in vitro*" studies [5,17,21–23].

However, mitochondrial activities are not the unique bioenergetic source for sperm motility in many species and glycolysis can also be exploited to maintain sperm functions.

Whether boar spermatozoa are specifically dependent on glycolysis for their energy supply still remain to be defined; the same is true for the role of mitochondria in boar sperm function. The present study, which focuses on boar sperm mitochondria, combines two distinct approaches, namely functional investigations (motility and mitochondrial membrane potential linked to cell viability) and bioenergetic analyses, addressed to define how energy production is obtained. The aim is to understand if boar spermatozoa depend exclusively on glycolysis for their functionality and to improve the knowledge on the still controversial role of mitochondria in boar sperm function.

2. Materials and methods

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (Saint-Louis, MO, USA).

Boar semen was purchased from a commercial company (Inseme S.P.A., Modena, Italy). Sperm samples were sent to the laboratory within 2 h from collection, washed and resuspended in Androhep [24] at a final concentration of $30x10^6$ spz/mL unless otherwise specified. Minimum requirement for inclusion of sperm sample in the study was viability above 80% at the arrival at the laboratory.

Experiment 1. Assessment of functional activity of spermatozoa using different specific inhibitors.
Twenty-four ejaculates from 10 boars were used in this experiment. Sperm cells were incubated for 1 h at 37° C with Rotenone 5µM (ROT) complex I inhibitor, Dimethyl-malonate 10mM (DMM), complex II inhibitor, Carbonyl cyanide m-chlorophenyl hydrazone 5µM (CCCP), uncoupling agent, 2-deoxy-glucose 10mM (2DG) glucose agonist, antimycin A 1 µg/mL (ANTI), complex III inhibitor, oligomycin 4 µg/mL (OLIGO), ATP synthase inhibitor, and 2µL Dimethyl sulphoxide (DMSO), control vehicle. At the end of the incubation period mitochondrial respiration, sperm motility, sperm viability and mitochondrial activity were evaluated as described below.

Experiment 2. Influence of complex I and II activity on sperm parameters.
Eight ejaculates from 5 boars were used in this experiment. This experiment was performed to focus on complex I and II activity and to evaluate the effects of ROT, DMM and of their combination incubating sperm cells for 1 h at 37° C. Sperm motility, viability and mitochondrial activity were assayed.

Experiment 3. Mitochondrial respiration.
Five ejaculates from 3 different boars were used in this experiment. The experiment was conducted to focus on sperm ability to produce energy by mitochondrial oxidative phosphorylation by means of the methods described in section 2.1.3

Experiment 4. Mitochondrial substrates oxidation.

Seven ejaculates from 3 different boars were used in this experiment. The experiment was conducted to focus on activity of complex I and II and their capability to oxidate different substrates, as reported in section 2.1.4

2.1.1 Motility assessment

Sperm motility was measured by a computer-assisted sperm analysis system, using the open source Image J BGM plugin as described by Giaretta et al. [25]. Briefly, a Leitz diaplan microscope (Wild Leitz GmbH, D6330, Wetzlar, Germany) with a 10x plan objective with positive phase-contrast was used. The microscope was equipped with a Z31A Ascon technologic heated stage (Ascon technologic, PV – IT). The video camera, 3.1-megapixel CMOS USB 2.0 Infinity1-3 Camera (Lumenera corporation, Ottawa, ON, Canada), was coupled to the microscope by a c-mount adapter and videos were registered for three seconds at a resolution of 800x600 pixel and 60 frames/sec (fps). Images were recorded on a hard drive using the Infinity analyzing and capture software 6.4 (Lumenera corporation) and converted to avi format for subsequent analysis using the BGM Image J plugin.

Prior to any observation, spermatozoa ($30x10^6$ sperm/mL) were loaded onto a fixed height Leja Chamber SC 20-01-04-B (Leja, CIUDAD; The Netherlands). Five videos of separate fields and lasting three seconds each were recorded per sperm sample. Sperm motility endpoints assessed were: percent of total motile spermatozoa (TM), percent of progressive spermatozoa (PM), curvilinear velocity (VCL) and mean velocity (VAP), straight-line velocity (VSL), straightness (STR), linearity (LIN), beat cross frequency (BCF), lateral head displacement (ALH) and wobble (WOB). The setting parameters of the program were the followings: frames per second 60, number of frames 45, threshold path minimum VSL for motile sperm 10μm/sec; min VAP for motile 15 μm/sec; min VCL for motile 25 μm/sec; VAP cutoff for Progressive cells 25 μm/sec; STR cutoff for Progressive cells 75%.

2.1.2 Sperm viability and mitochondrial membrane potential

For each sample, an aliquot (30 μL) of semen (30 x 10^6 spz/mL) was incubated with 2 μL of a 300 μM propidium iodide (PI) stock solution (final concentration 16.7 μM), 2 μL of a 10 μM SYBR green-14 stock solution (final concentration 0.56 μM), both obtained from the live/dead sperm viability kit (Molecular Probes, Inc.) and 2 μL of a 150 μM JC-1 solution (final concentration 8.3 μM) for 20 min at 37°C in the dark. Ten μL of the sperm suspension were then placed on a slide and at least 200 spermatozoa per sample were scored using the above described fluorescence microscope. Spermatozoa stained with SYBR-14 but not with PI were considered as viable (SYBR-14+/PI-), whereas SYBR-14+/PI+ and SYBR-14-/PI+ spermatozoa were considered as non-viable. JC-1 monomers emit green fluorescence in mitochondria with low membrane potential (JC-1-) and form aggregates in mitochondria with high membrane potential (JC-1+), then emitting a bright red-orange fluorescence. Therefore, those viable sperm showing orange-red fluorescence in the mid piece (JC-1+) were considered as viable sperm with high mitochondrial membrane potential (SYBR-14+/PI-/JC-1+).

2.1.3 Mitochondrial respiration.

The mitochondrial respiration was polarographically evaluated as oxygen consumption rate (OCR) was polarographically evaluated by Clark-type electrode using the Oxytherm System (Hansatech Instruments) at 38°C in a reaction medium (1mL) containing 10^6 cells in Androhep.

OCR values expressed as nmol O_2/min/10^6 cells were recorded after sequential addition of selective inhibitors. The ATP synthase inhibitor OLIGO was used to prevent mitochondrial ATP production by OXPHOS and address spermatozoa to glycolysis. The ionophore FCCP, which makes the mitochondrial membrane permeable to H^+, was employed to uncouple mitochondria and prevent ATP synthesis. Inhibition of the respiratory chain was obtained by the myxothiazol (Complex III inhibitor) plus ROT (Complex I inhibitor) mixture.

The OCR values before and after sequential addition of these inhibitors were used to calculate the bioenergetics parameters currently used to characterize mitochondrial respiration [26].

Basal respiration was detected as baseline OCR before OLIGO addition. Minimal respiration was measured as OCR in the presence of 4 μg/mL OLIGO, while maximal respiration was measured as OCR after addition of 0.5 μM FCCP. Non-mitochondrial respiration was evaluated as OCR in the presence of 1 μg/mL myxothiazol plus 4 μM ROT. The ATP turnover or oligomycin-sensitive respiration was obtained from the difference between the basal respiration and the minimal

respiration (OCR in presence of OLIGO). Finally, the difference between the maximal and the basal respiration provided the spare capacity, which represents the ability to respond to an increased energy demand and can be considered as a measure of the flexibility of the OXPHOS machinery [26].

2.1.4 Mitochondrial substrate oxidation

The capability of substrate oxidation was assayed in a reaction medium (2 mL) containing 10^6 cells in Androhep at 38°C. The oxidation of NADH and $FADH_2$, which transfer electrons to Complex I and II, respectively was detected by exploiting the autofluorescence of the reduced form of these coenzymes [27]. Autofluorescence intensity of NADH (excitation 360 nm, emission 455 nm) and $FADH_2$ (excitation 450 nm, emission 520 nm) were monitored by Shimadzu RF-6000 spectrofluorophotometer. NADH and $FADH_2$ oxidation were measured as decrease in the autofluorescence intensity after addition of the following inhibitors: 2 μM ROT, 5 mM DDM (only in $FADH_2$ autofluorescence) in order to inhibit complexes I and II, respectively; 1 μg/mL OLIGO, which blocks proton flux across the F1FO-ATP synthase and ATP generation, to verify the coupling between substrate oxidation and ATP synthesis and 0.5 μM FCCP, which makes the mitochondrial membrane permeable to protons, to maximize NADH or $FADH_2$ oxidation at minimal concentration of these substrates intensity, expressed as NADH or $FADH_2$ arbitrary units/min/10^6 cells, was evaluated by LabSolutions RF software.

3. Results

3.1. Sperm motility and mitochondrial activity (*Experiments 1 and 2*)

Sperm motility parameters and mitochondrial activity assessed by JC1 are reported in Table 1.

The total and progressive motility, as well as the three velocity parameters (VCL, VAP, VSL) and lateral head displacement (ALH) are negatively affected by the mitochondrial inhibitors ROT, ANTI, OLIGO and CCCP, that equally depress motility kinematics. Beat cross frequency (BCF) is significantly diminished (in comparison with control) only in CCCP group, while wobble (WOB) is negatively affected only by ROT.

Mitochondrial membrane potential significantly drops due to CCCP and ANTI treatment while sperm viability (JC1 positive and negative cells) is unaffected.

Table 2 shows the results obtained in sperm treated with complex I and II inhibitors namely ROT, DMM ad their combination. Apparently, the Complex I inhibitor ROT is the main responsible for the observed effects, since the treatment with a binary mixture of ROT plus the Complex II inhibitor DMM shows the same inhibition extent as the ROT treatment on all the parameters under study, apart from STR, LIN, BCF and mitochondrial activity, unaffected by all treatments.

Principal component analysis results used to determine clusters are reported in the supplementary file 1.

Cluster analysis reveals some important features of motile sperm subpopulations: four different clusters emerged from the analysis that, on the basis of mean kinematic parameters values, were named as: rapid progressive, rapid non progressive, average; slow non progressive. Data are presented in Table 3. Spermatozoa were assigned to the different clusters and, as reported in Table 3, significant differences in the percentages of spermatozoa belonging to each subpopulation were found between treatments. It is evident that DMM and 2DG induce a shift in sperm subpopulation and an increase in rapid progressive cell subpopulation. ANTI and CCCP determine an increase in slow non progressive cells subpopulation, while OLIGO and ROT determine an increase in both average and slow non progressive sperm subpopulations. Finally, ROT + DMM determine a strong shift towards slow non progressive and average sperm subpopulation.

3.2. Sperm mitochondrial respiration and substrate oxidation (*Experiments 3 and 4*)

The OCR evaluation in absence or presence of the mitochondrial inhibitors allows the bioenergetics characterization of boar sperm mitochondria. The basal respiration attains 65% of the maximal respiration detected in the presence of FCCP (0.167 nmol O_2/min/10^6 cells) and is inhibited by 90% with OLIGO. The calculated ATP turnover is 0.097 nmol O_2/min/10^6 cells. The strong inhibition of respiration by OLIGO is the result of a good coupling efficiency (0.914 A.U.), close to the unit, which is the maximum attainable. The mitochondrial ATP production rate (486.85 pmol ATP/min), indirectly obtained from ATP turnover, provides how fast cells work to produce ATP. Moreover, sperm mitochondria are characterized by a high phosphorylation efficiency as shown by the high respiratory control ratio (7.56 A.U.). The ratio of the FCCP-stimulated respiration to the respiration in the presence of OLIGO, that is the ability of the cell to convert into ATP the activities of respiratory complexes, and low spare respiratory capacity (0.083 nmol O_2/min/10^6 cells) is a good diagnostic tool in bioenergetics because, by indicating the ability of

mitochondria to respond to an increased energy demand, it can show how close to its bioenergetic limit a cell is working (Fig. 1A). These data show that sperm mitochondria work slightly below this limit.

The fluorimetric detection of the mitochondrial oxidation of NADH and $FADH_2$, which transfer electrons to Complex I and II, respectively, provides information on the respiratory complexes in sperm mitochondria preferentially used to feed the electron transport chain. ROT induces a striking decrease in NADH autofluorescence intensity (Fig. 1B), which suggests that NADH is oxidized through activation of mitochondrial respiration. The inhibition of ADP phosphorylation by OLIGO in coupled sperm mitochondria blocks the respiration and maintains the NADH level. Interesting, the FCCP-dependent NADH oxidation, which mirrors the maximal respiration, attains the same value as the basal respiration (control) (Fig. 1B). $FADH_2$ oxidation is inhibited by DMM, and by OLIGO, thus confirming that mitochondrial respiration is stimulated by complex II substrates and coupled to ATP synthesis. However, $FADH_2$ autofluorescence intensity does not increase in the presence of mitochondrial uncoupler FCCP (Fig. 1C). Thus, the NADH and $FADH_2$ autofluorescence intensity drop clearly shows that ROT, by blocking Complex I, prevents any transfer of reducing equivalents in the oxidation-reduction reactions during sperm mitochondrial respiration (Fig. 1B, C).

4. Discussion

In this study bioenergetic analyses clearly show that boar sperm mitochondria exploit OXPHOS and are susceptible to OLIGO, to the uncoupler FCCP and to the classical inhibitors of respiratory complexes. Interestingly, since the basal respiration is close to the FCCP-stimulated maximal respiration, the spare respiratory capacity, which indicates the capability of producing extra ATP by OXPHOS to respond to an increased energy demand, is low. Therefore, under physiological conditions, sperm mitochondria work at their maximal capacity to produce energy. Accordingly, the close-to-unit respiratory control ratio, which indicates a good coupling efficiency, shows that sperm mitochondria efficiently exploit the electrochemical gradient generated by mitochondrial respiration to phosphorylate ADP (Fig. 1A). Noteworthy, NADH oxidation is preferred to $FADH_2$ one. Since neither NADH nor $FADH_2$ autofluorescence is stimulated by FCCP, both oxidases (Complexes I and II) attain the maximal activities. Moreover, the high ROT inhibition suggests

that mitochondrial respiration mainly depends on complex I (Fig. 1B, C). Consistently, the data from the Experiment 2 (Table 2) show that sperm motility and mitochondrial potential are apparently unaffected by DMM treatment, while the inhibition of motility (total and progressive motility as well as the other main kinematic parameters) by ROT is maintained when spermatozoa are incubated with ROT and DMM in combination.

In mammals the number of ATP molecules synthesized by OXPHOS for each electron pair transferred via the respiratory chain (known as P/O ratio) from NADH to O_2 is 2.7, while for $FADH_2$ oxidation is 1.6 [28]. Most likely, boar sperm mitochondria require an efficient OXPHOS and possess an adequate bioenergetics machinery to preferentially exploit Complex I, which is more advantageous in terms of ATP production, to supply the electron transport pathway.

Motility is the prominent ATP-consuming activity of sperm cells. Interestingly, motility (total and progressive) is reduced by OLIGO, which blocks the ATP synthase (Complex V), and by ROT, which inhibits Complex I. Similarly, ANTI, complex III inhibitor, prevents the inner mitochondrial membrane polarization required for ATP synthesis and, as a consequence, it depresses total and progressive motility like OLIGO, ROT and CCCP. On the contrary DMM, inhibitor of complex II, does not affect total and progressive motility, as well as other sperm kinematic parameters. To sum up, sperm motility is reduced under two conditions: insufficient ATP availability, if complex V cannot synthesize ATP, and inhibition of Complex I and III, which cannot energize the mitochondrial membrane. Indeed, CCCP, by dissipating the transmembrane electrochemical gradient, prevents ATP synthesis by complex V and decreases sperm motility.

Regarding other kinematic parameters, the three velocity parameters (VCL, VAP and VSL) are decreased by ROT, OLIGO, ANTI and CCCP; this fact is interesting as not only the molecules decrease the number of motile sperm, but they even depress their speed, thus making them move more slowly than controls. The same is registered for lateral head displacement; this confirms that the strength of spermatozoa movement is limited by the above-mentioned inhibitors and so they move slowly and with less displacement from the mean trajectory. Interestingly, straightness and linearity are not compromised by the inhibitors and maintain the same levels of the control group; this could mean that the trajectory of sperm cells is not that different when using the inhibitors, but they only move slowly.

The strict relation between motility and mitochondrial activity was demonstrated in in horse spermatozoa [7,29] and the activity of OLIGO is also confirmed in the study by Ramiò-Lluch [17] in boar sperm cells.

Noteworthy, the mitochondrial membrane potential persists in the presence of OLIGO and ROT, but it is decreased by ANTI and CCCP treatments. Probably other pathways, which do not involve the respiratory complexes I and II, can fuel the respiratory chain via ubiquinone by starting electron transfer from ubiquinol to O_2 directly from complex III (Table 1). Even if mitochondria do not produce ATP, a low ATP level, produced by glycolysis, can maintain a polarized inner mitochondrial membrane to ensure ionic homeostasis. This is confirmed by data from ANTI and CCCP treatments: even if the mitochondrial membrane potential is depressed, sperm cells do not die, since they can sustain membrane integrity.

The addition of 2GD to the incubation medium does not affect sperm motility or the mitochondrial membrane potential. Conversely Davila and colleagues [5], demonstrated that 2DG was able to depress some motility kinematic parameters as well as total motility. Most likely, under our experimental conditions, the presence of glucose in the medium may fuel the glycolytic pathway so as to produce sufficient ATP to avoid the 2DG negative effect on sperm motility.

Some interesting features of sperm subpopulation composition and change after incubation with the different inhibitors are revealed by cluster analysis. As stated above this statistical technique permitted to delineate four different sperm subpopulations. The average sperm subpopulation in control sample showed a high amount of rapid progressive cells (around 40%) and "average" cells, with the rapid non progressive cluster, [30] not highly represented. It is noteworthy that both 2DG and DMM induced an increase in rapid progressive subpopulation. Rapid progressive cells increase when the mitochondrial oxidation is preferred to the glycolytic pathway in presence of 2DG. Moreover, in mitochondria the NADH-O_2 oxidase produces more ATP than succinate-O_2 oxidase and the rapid progressive subpopulation needs a lot of ATP for motility. Interestingly, succinate oxidation produces ubiquinol (the reduced form of coenzyme Q_{10}) that, instead of transferring electrons to Complex III, in energized mitochondria can also drive the energy-dependent NAD^+ reduction by the complex I through the reverse electron transfer mechanism [31]. Conversely, in presence of DMM, which blocks Complex II, Complex I can only work in the forward mode and oxidize NADH. Moreover, "rapid progressive" spermatozoa subpopulation is deeply depressed by OLIGO, ROT, ANTI and CCCP, and an increase of the "slow non

progressive" spermatozoa subpopulation is concomitant when ATP synthesis and/ or NADH oxidation are inhibited. These data point out the important role of mitochondrial ATP production in boar sperm motility (Table 3).

These features of the different subpopulations were also visible in kinematics parameters resumed in Table 1, but the cluster analysis permitted to better delineate the motility features of these different subpopulation and it is interesting to note that this "unsupervised" statistical technique evidenced a biological feature of sperm cells.

In the present study, the mitochondrial activity characterization reveals that boar sperm total motility and the rate of rapid progressive sperm are markedly dependent on OXPHOS, namely mitochondrial ATP production. Indeed, mitochondria provide sperm with energy in form of ATP produced by the ATP synthase, while respiratory complexes (NADH-O_2 oxidase) indirectly impact ATP synthesis by producing the mitochondrial electrochemical gradient. Accordingly, 2DG has no effect on motility parameters. Consistently, a similar inhibitory effect on sperm motility by ROT and ANTI was observed in stallion sperm [7].

The overall data suggest that boar spermatozoa, even if commonly considered to preferentially exploit glycolysis to obtain energy, heavily rely on mitochondrial OXPHOS for ATP production to fuel motility. Therefore, an impaired mitochondrial activity should be considered as a possible cause of defective boar sperm functions.

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Figure

Figure 1. Sperm mitochondria activities. A) Oxygen consumption rates (OCR) before and after sequential additions of oligomycin (OLIGO), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), and a mixture of rotenone and Myxothiazol (Rot+Myxo), at the times indicated; B) NADH autofluorescence intensity in spermatozoa exposed to rotenone, oligomycin and FCCP; C) $FADH_2$ autofluorescence intensity in spermatozoa exposed to rotenone, dimethylmalonate (DMM), oligomycin and FCCP. The data are the mean values ±SD (n=7). Different letters indicate significantly different values within each treatment ($P \leq 0.05$).

Table 1. Motility parameters and mitochondrial activity of differently treated spermatozoa.

Treat	TM	PM	VCL	VAP	VSL	STR	LIN	ALH	BCF	WOB	JC1+live	JC1-live
CTR	52.32±13.49^{a}	28.23±8.04^{a}	153.98±36.12^{a}	73.23±17.02^{a}	56.28±13.84^{a}	75.87±4.43	39.52±6.74	6.03±1.36^{a}	25.22±2.18^{a}	48.43±7.72^{ab}	83.90±7.67^{a}	0.18±0.27^{a}
ROT	16.48±7.79^{b}	6.00±4.24^{b}	100.69±32.89^{b}	34.78±6.79^{b}	25.47±5.11^{b}	75.26±7.08	36.68±11.67	3.81±1.02^{b}	24.49±2.07^{ab}	38.11±9.31^{b}	81.24±8.77^{a}	0.52±1.35^{a}
DMM	55.15±13.96^{a}	28.54±10.55^{a}	166.36±30.08^{a}	81.96±15.72^{a}	60.69±13.71^{a}	74.05±6.86	39.16±7.88	6.54±1.00^{a}	24.27±2.17^{ab}	50.37±9.65^{a}	84.22±6.20^{a}	0.05±0.13^{a}
ANTI	10.46±8.94^{b}	3.92±3.91^{b}	97.42±58.13^{b}	36.14±14.08^{b}	26.72±9.16^{b}	75.79±11.02	44.43±16.54	4.02±2.13^{b}	22.69±2.86^{ab}	46.86±16.45^{ab}	0.10±0.30^{b}	81.87±5.81^{b}
OLIGO	12.56±9.16^{b}	4.76±4.13^{b}	109.46±54.83^{b}	37.35±12.20^{b}	26.79±8.46^{b}	73.79±6.93	39.57±15.71	4.26±1.98^{b}	24.44±3.61^{ab}	42.00±18.08^{ab}	83.62±4.75^{a}	0.07±0.19^{a}
CCCP	14.52±8.04^{b}	5.60±5.08^{b}	86.11±30.13^{b}	33.32±10.44^{b}	25.26±8.46^{b}	77.15±6.80	40.00±10.85	3.52±1.41^{b}	22.27±2.48^{b}	43.23±8.15^{ab}	0.03±0.11^{b}	78.15±7.48^{b}
2DG	54.25±10.71^{a}	28.47±6.48^{a}	163.70±30.70^{a}	75.81±14.82^{a}	56.96±12.60^{a}	74.18±4.92	37.64±5.75	6.36±1.14^{a}	25.31±2.43^{ab}	46.91±7.53^{ab}	82.66±9.69^{a}	0.08±0.19^{a}

Sperm motility parameters are: total motility (TM), progressive motility (PM), ALH amplitude of lateral head displacement (µM); BCF beat cross frequency (Hz); LIN linearity (%); STR straightness (%); VCL curvilinear velocity (µm/sec); VAP average path velocity (µm/sec); VSL straight line velocity (µm/sec), wobble (%) WOB; JC1+ live: living cells % with high mitochondrial membrane potential; JC1 - live: living cells % with low mitochondrial membrane potential. 24 ejaculates from 10 boars were used in this experiment; different superscripts within a column indicate a significant difference for $p<0.05$.

CTR: control; ROT: rotenone; DMM dimetil-malonate; ANTI: antimycin; OLIGO: oligomycin; CCCP: carbonyl cyanide m-chlorophenyl hydrazine; 2DG: 2-desoxy-glucose

Table 2. Mean values for motility parameters and mitochondrial activity in differently treated spermatozoa.

Treatment	TM	PM	VCL	VAP	VSL	STR	LIN	ALH	BCF	WOB	JC1+live	JC1-live
CTR	42.93±9.68^{a}	24.88±3.96^{a}	142.56±36.14^{a}	75.67±18.39^{a}	59.44±13.72^{a}	78.13±3.52	43.88±7.86	5.88±1.38^{a}	25.30±2.72^{ab}	54.00±8.4^{a}	85.89±3.65	0.00±0.00
ROT	16.65±8.77^{b}	7.08±4.61^{b}	86.18±25.65^{b}	34.23±6.01^{b}	26.40±5.45^{b}	78.25±7.65	41.63±13.88	3.46±0.83^{b}	24.96±1.71^{ab}	42.88±10.74^{b}	79.41±11.49	0.28±0.61
DMM	49.75±18.02^{a}	29.24±12.01^{a}	154.79±33.79^{a}	83.79±18.02^{a}	64.92±15.67^{a}	78.0±4.14	44.12±7.66	6.30±1.21^{a}	24.32±2.15^{a}	55.25±11.54^{a}	84.36±4.50	0.00±0.00
DMM+ROT	8.77±3.73^{b}	2.74±2.11^{b}	79.24±15.21^{b}	29.81±6.38^{b}	23.03±4.94^{b}	79.88±5.64	38.62±8.14	3.01±0.57^{b}	27.74±2.59^{b}	41.00±5.50^{b}	84.30±4.33	0.19±0.27

Sperm motility parameters are: TM total motility, PM progressive motility, VCL curvilinear velocity (µm/sec), VAP average path velocity (µm/sec), VSL straight line velocity (µm/sec), STR straightness (%), LIN linearity (%), ALH amplitude of lateral head displacement (µM); BCF beat cross frequency (Hz), WOB wobble (%). 8 ejaculates from 5 boars were used in this experiment. Different superscripts within a column indicate a significant difference for $p<0.05$.

CTR: control; ROT: rotenone; DMM Dimetil-malonate

Table 3. Cluster analysis results. Different distribution of spermatozoa subpopulation % depending on cluster and treatment.

Treatment	Rapid non progressive	Rapid progressive	Average	Slow non progressive
2DG	13.7[a]	47[c]	24.9[a]	14.4[c]
ANTI	20.7[b]	7.8[b]	25.4[a]	46.1[b]
CCCP	11.8[a]	9.3[b]	29.3[a]	49.6[b]
CTR	11.7[a]	38.1[a]	28.8[a]	21.4[a]
DMM	11.5[a]	47.2[c]	24.8[a]	16.5[c]
DMM+ROT	6.4[c]	2.9[b]	36.4[b]	54.3[b]
OLIGO	17.1[b]	8.3[b]	41.4b[b]	33.3[b]
ROT	11.6[a]	7.4[b]	36.3[b]	44.7[b]

Each row represents the percentage of cells belonging to the different cluster (considering 100% the sum of percentage for each treatment). Different superscripts within a column indicate significant differences between treatments (chi square test, $p<0.001$)

A total of 10658 sperm cells were analysed, respectively: CTR 3403 cells; 2DG 1499 cells; 3412 cells; DMM+ROT 140 cells; ANTI 421 cells; OLIGO 532 cells; ROT 768 cells.

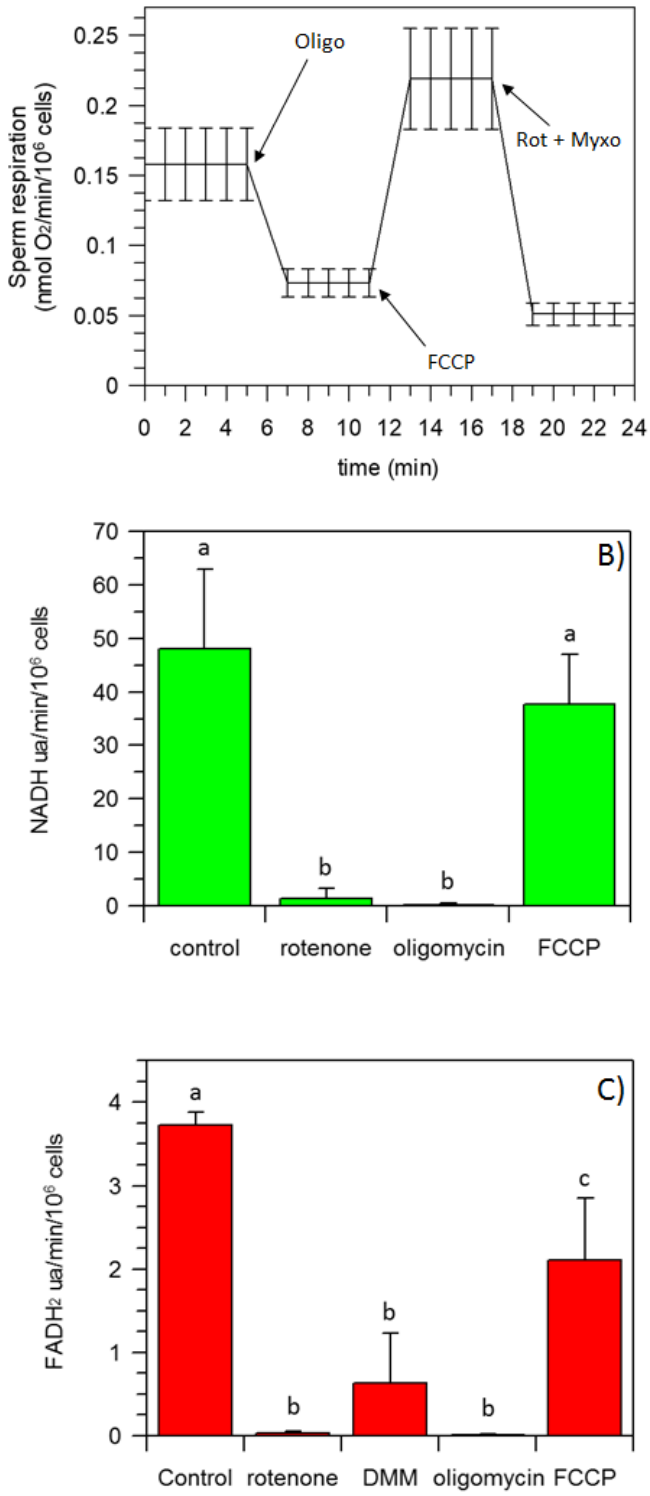
Sperm respiration (nmol O2/min/10^6 cells)
0.25
0.2
0.15
0.1
0.05
0
Oligo
Rot + Myxo
FCCP
0 2 4 6 8 10 12 14 16 18 20 22 24
time (min)
NADH ua/min/10^6 cells
70
60
50
40
30
20
10
0
B)
a
b
b
a
control
rotenone
oligomycin
FCCP
FADH2 ua/min/10^6 cells
4
3
2
1
0
C)
a
b
b
b
c
Control
rotenone
DMM
oligomycin
FCCP

Figure 1

Supplementary file 1. Principal components loadings and variance

	Comp.1	Comp.2	Comp.3	Comp.4	Comp.5	Comp.6	Comp.7	Comp.8
ALHs	0.46	0.23	0.23	0.23	0.29	0.68	0.29	0.01
BCFs	-0.20	0.06	-0.48	0.85	-0.03	0.02	-0.05	-0.01
LINs	0.12	-0.63	0.16	0.11	-0.34	0.41	-0.51	-0.09
STRs	0.06	-0.60	-0.17	-0.01	0.70	-0.12	0.08	0.30
VAPs	0.53	-0.03	-0.13	0.06	-0.44	-0.21	0.14	0.66
VCLs	0.44	0.35	-0.19	-0.05	0.31	-0.16	-0.72	-0.06
VSLs	0.50	-0.25	-0.19	0.04	-0.10	-0.29	0.31	-0.68
Variances	3.11	2.07	1.39	0.76	0.38	0.17	0.09	0.02
SD	1.76	1.44	1.18	0.87	0.62	0.42	0.31	0.15
Proportion of variance	0.39	0.26	0.17	0.10	0.05	0.02	0.01	0.00
Cumulative variance	0.39	0.65	0.82	0.92	0.96	0.99	1.00	1.00

Principal components analysis' report of component loadings, variances and description of the different impact of each single component (SD, proportion of variance and cumulative variance).

Authors contributions

All the Authors contributed equally to the writing of this article.

DB and SN performed experiments, effected the statistical analysis and organized the experimental design; all the other authors performed the experiments and contributed to experimental design adjustment.

Highlights

Boar sperm metabolism and function is not yet fully understood

Assessment of mitochondrial complex I, II, III and V function and membrane potential with specific inhibitors

Respiration study using polarographic method

Boar spermatozoa prefer using complex I and are dependant on mitochondrial intactness for motility

Different inhibitors modify sperm subpopulation kinematics