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Study of mitochondrial function in thawed bull spermatozoa using selective electron transfer chain inhibitors

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ABSTRACT

Bull spermatozoa depend equally on glycolysis and oxidative phosphorylation for the maintenance of the energy necessary for their proper functioning. The aim of the present work was to delineate the mitochondrial activity of bull spermatozoa after incubation with specific inhibitors of the different mitochondrial complexes and evaluate their ROS production. Thawed bull sperm cells (30×10^6 mL^{-1} in Tyrode's extender) were incubated 1 and 3h at 37 °C with rotenone 5 μM (ROT), complex I inhibitor; dimethyl-malonate 10 mM (DMM), complex II inhibitor; carbonyl cyanide m-chlorophenyl hydrazine 5 μM (CCCP), uncoupling agent; antimycin A 1 μg/mL (ANTI), complex III inhibitor; oligomycin 5 μM (OLIGO), ATP synthase inhibitor, and 0.5% DMSO, vehicle (CTR). Sperm motility and kinematics were assessed by Hamilton Thorn IVOS 12.0. Mitochondrial membrane potential, mitochondrial $O_2^{\bullet-}$ production and H_2O_2 intracellular content were evaluated by BD FACSCalibur flow cytometer, and sperm viability (SYBR-14/PI) and mitochondrial activity (JC-1/SYBR-14/PI) were assessed by epifluorescence microscopy. A multivariate analysis was performed on the results. In addition, sperm kinematic features, registered for each motile spermatozoon, were studied by cluster analysis. The incubation during 1 or 3 h in presence of the inhibitors of mitochondrial functionality only had a minor effect on motility parameters, decreasing the proportion of the SP1 (fast progressive) subpopulation after 3 h of incubation with ROT, ANTI or OLIGO. The percentage of live spermatozoa with active mitochondria was reduced under the effect of ANTI and CCCP both at 1 and 3 h. In conclusion, mitochondrial function is somehow impaired in frozen thawed bull sperm as not all live cells showed active mitochondria. These results support the findings that bull spermatozoa can alternatively rely on oxidative phosphorylation or glycolysis for energy obtainment and that their mitochondria are less affected by ETC inhibitors.

1. Introduction

Artificial insemination (AI) is a very important tool in animal production systems to allow advancement of genetic selection, disease control and maximization of sire's performance and gene distribution. Semen preservation allows the spread of AI, and particularly, cryopreservation is the best technique to preserve semen for long time and to ease distribution of semen doses into different countries; the species in which this technique is most widely and successfully used is bovine [1,2].

Cryopreservation induces many damages and changes in different cells' districts, as reported by several research articles and well resumed in Ref. [1]. Among the changes in cell physiology, a status called "Cryocapacitation" is well known and described [2–4]: sperm cells after thawing acquire hyperactive motility and other inner cell's pathways (resembling those involved in capacitation process) are activated; as a results, sperm cells are precociously activated in absence of the oocyte to fertilize, and therefore may lose their fertilizing ability [1,2]. Not only this capacitation-like status, but also other alterations, that could be defined as damages, negatively affect the sperm function: membrane disruption, mitochondrial damage, excessive ROS production, DNA damage (disulphide bonds brake, DNA and RNA fragmentation); this whole situation may hesitate into genetic defects and altered motility [1,5].

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In the last years, research focused on mitochondrial integrity after cryopreservation as the activity of these organelles is crucial for maintaining cellular functions and energetic balance [6–10].

Indeed, the mitochondria is a key organelle for sperm function, sustaining not only flagellar motility but also critical for events such as capacitation and apoptosis [11]. The mitochondrial electron transport chain (ETC) plays a pivotal role in sperm function. The ETC not only provides much of the energy for motility, by sustaining oxidative phosphorylation, but it also has a role in the generation and balance of reactive oxygen species (ROS) [12]. ROS, principally produced by complex I and III of the ETC [7], are a major factor in the cryoinjuries in bull sperm [13], whereas being also critical for intracellular signaling [14].

Nevertheless, there are evident differences on the role of mitochondria even among phylogenetically related species. Bull sperm metabolism has been studied in the past decades (see Storey [6] for a thorough revision) and there is evidence that, in presence of oxygen, bull spermatozoa preferentially use oxidative phosphorylation to obtain energy from sugar substrates; on the contrary, under anaerobic condition, they preferentially use glycolysis and the ATP produced under this condition is enough to sustain motility. Other species do not demonstrate this metabolic adaptability (as, for example, horse, as reported by Giaretta et al. [15], Davila et al. [16]), while there is still debate on the role of different metabolic pathways in boar [17,18].

Given this background, the aim of the present study is to better delineate the role and relevance for sperm functionality of mitochondria in post-thawed bull spermatozoa. We aimed at a mechanistic approach by using inhibitors of the different mitochondrial ETC complexes and the oxidative phosphorylation, and observing the effect on relevant physiological parameters, such as motility, viability, mitochondrial activity and ROS production (both from cytoplasmic and mitochondrial sources).

2. Material and methods

2.1. Experimental design

Twelve semen samples (3 biological replicates from 4 different bulls) were thawed in a water bath at 37C for 30 s and washed twice in Tyrode's medium at 2000×*g* for 10 min. The resultant pellet was resuspended in Tyrode's medium to obtain a final concentration of 30×10^6 mL^{-1}.

Semen samples were divided (aliquots of 500 μL) in the following experimental groups and incubated at 37 °C up to 3 h in presence of different inhibitors mitochondrial activity, To assess the effect of different ETC inhibitors on bull frozen sperm mitochondrial function, the following groups were analyzed at 1 and 3 h incubation at 37 °C, as reported in Nesci et al., [18]:

CTR: no inhibitors, 2 μL DMSO as vehicle.

ROT: 5 μM rotenone (complex I inhibitor);

DMM: 10 mM dimethyl-malonate (complex II inhibitor);

ANTI: 1.8 μM antimycin A (complex III inhibitor);

OLIGO: 5 μM oligomycin A (ATP-synthase inhibitor);

CCCP: 5 μM carbonyl cyanide m-chlorophenyl hydrazone (uncoupling agent).

Sperm motility, viability, mitochondrial activity, and production of intracellular and mitochondrial ROS were evaluated.

2.2. Chemicals and semen doses

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). Frozen bull semen was purchased from the National Institute for Artificial Insemination (INFA, Italy) in 500 μl straws a at a concentration of 60×10^6 sperm/mL.

2.3. Motility assessment

Sperm motility was assessed with a computer-assisted sperm analysis system (CASA, Hamilton Thorne, IVOS Ver. 12). 5 μL of each sample was placed onto a prewarmed (38C) Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and ~1000 spermatozoa per sample were evaluated. The CASA system was set at the following conditions: 60 frames per second; minimum contrast at 49; minimum cell size 6 pixels; progressive sperm motility: VAP 20 μm/s; percentage of straightness: 75; static cell cut-off: VAP 20 μm/s and VSL 5 μm/s. Sperm motility endpoints were: percentage of total motile spermatozoa (TM), percentage of progressive spermatozoa (PM), curvilinear velocity (VCL), average-path velocity (VAP), straight-line velocity (VSL), linearity (LIN), straightness (STR), wobble (WOB), lateral head displacement (ALH), beat cross frequency (BCF).

The CASA provided kinematic parameters both as sample means and for each sperm track. The individual observations were used to study the distribution of sperm subpopulations (See statistical analysis – cluster analysis for sperm motion).

2.4. Viability and mitochondrial activity assessment by epifluorescence microscopy (SYBR-14/PI/JC-1)

Due to the complexity for separating the fluorescence signals in the SYBR-14/PI/JC-1 combination, samples were assessed by epifluorescence microscopy. Methods were described thoroughly in Nesci et al., 2019. Briefly, for each sample, an aliquot (30 μL) of semen (30×10^6 spz/mL) was centrifuged and washed in Tyrode's medium at 800×*g* for 2 min, and then resuspended in Tyrode's medium as a final concentration of 30×10^6 mL^{-1}. Samples were then incubated with 2 μL of a 300 μM propidium iodide (PI) stock solution (final concentration 16.7 μM), 2 μL of a 10 mM SYBR-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 mM JC-1 solution (final concentration 8.3 mM) 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 Nikon Eclipse E600 epifluorescence microscope (Nikon Europe BV, Badhoeverdop, The Netherlands) at 1000x magnification and separated in 3 different subpopulations of live spermatozoa (SYBR-14$^+$PI$^-$): active mitochondria (all/mostly orange midpiece), partially active mitochondria (green midpiece with many orange spots) and inactive mitochondria (green midpiece with no or few orange spots).

2.5. Flow cytometry analyses

Flow cytometry analyses were conducted to evaluate sperm viability associated with mitochondrial activity and mitochondrial/cellular ROS production or mitochondrial function. Reagents for flow cytometry were obtained from Thermo Fisher Scientific (Waltham, MA, USA) unless otherwise specified. Analyses were performed taking into account the recommendations of the International Society for Advancement of Cytometry [19]. In each assay, sperm concentration was adjusted to 1×10^6 mL^{-1} in a final volume of 0.5 mL Tyrode's medium, and spermatozoa were stained with the appropriate combinations of fluorochromes (described below). Once stained, samples were run through a FACSCalibur flow cytometer (Becton Dickinson, Milan, Italy) equipped with a 488 nm argon-ion laser and a 635 nm red diode laser. Emission of each fluorochrome was detected by using filters: 530/30 band-pass (green/FL1), 585/42 band-pass (orange/FL2), >670 long pass (far-red/FL3) and 661/16 band-pass (orange for red laser/FL4). Data were acquired using the BD CellQuest Pro software (Becton

Dickinson).

Signals were logarithmically amplified, and photomultiplier settings were adjusted to each particular staining method. FL1 was used to detect green fluorescence from SYBR-14, YO-PRO-1, CM-H_2DCFDA; FL3 was used to detect the red fluorescence from propidium iodide (PI) and MitoSOX; FL4 was used to detect the red fluorescence from MitoTracker deep red.

Side scatter height (SSC-H) and forward scatter height (FSC-H) were recorded in linear mode (in FSC vs. SSC dot plots), and the sperm population was positively gated based on FSC and SSC while other events were gated out. A minimum of 10,000 gated sperm events was evaluated per replicate.

In H_2DCFDA and MitoSOX flow cytometric assessments, percentages of non-DNA containing particles (debris), were determined to avoid an overestimation of sperm particles in the lower-left quadrant (LL, full negative) using information from the SYBR-14/PI analysis, as described by Petrunkina et al. [20] with some modifications.

2.5.1. Viability (SYBR-14/PI)

Sperm viability was determined by assessing the membrane integrity the SYBR-14 and PI fluorochromes (LIVE/DEAD Sperm Viability Kit; Molecular Probes, Invitrogen, Milan, Italy). SYBR-14 is a membrane-permeable dye, which stains the head of viable spermatozoa in green, while PI is a membrane impermeable dye that only penetrates through disrupted plasma membrane, staining the sperm heads of non-viable cells in red. Aliquots of sperm samples of 500 μL were stained with 5 μL SYBR-14 working solution (final concentration: 100 nM) and with 2.5 μL of PI (final concentration: 12 mM) for 10 min at 37 °C in the dark. Viable spermatozoa exhibited a positive staining for SYBR-14 and negative staining for PI (SYBR-14+/PI-).

2.5.2. Mitochondrial ROS production (YO-PRO-1/MitoSOX/Mitotracker deep red)

MitoSOX Red is a lipid-soluble, cell-permeable cation that selectively targets the mitochondrial matrix and thus can detect superoxide radicals ($O_2^{\bullet-}$) generation in this organelle. MitoSOX Red (MX) emits red fluorescence upon oxidation, detected by the FL3 photomultiplier. It was coupled with YO-PRO-1 as a viability counterstaining. YO-PRO-1 stains apoptotic/dead cells and emits green fluorescence upon binding to DNA, detected by the FL1 photomultiplier. The MitoTracker deep red probe (MT) was included to assess simultaneously mitochondrial integrity. MT accumulates in intact mitochondria and emits red fluorescence. MT is excited by the red diode laser, and the FL4 photomultiplier detects its fluorescence.

Same sperm samples were stained with YO-PRO-1 (in DMSO, 100 nM final concentration), 1 μL MitoSOX (in DMSO, 1 μM final concentration), and MT (in DMSO, 100 nM final concentration). Samples were incubated at 37 °C for 30 min in the dark.

The mitochondrial production of ROS by viable cells with intact or damaged mitochondria was recorded in this analysis. In this study, we evaluated the population of live spermatozoa (gating out YO-PRO-1$^+$ events), distinguishing cells with high and low $O_2^{\bullet-}$ generation within the subpopulations with intact or damaged mitochondria: MX^+MT^+, MX^-MT^+, MX^+MT^-, MX^-MT^-.

2.5.3. Cytoplasmic ROS production (CM-H_2DCFDA; propidium iodide; MitoTracker deep red)

CM-H_2DCFDA is a non-fluorescent agent that accumulates in the cell cytoplasm due to deacetylation and emits green fluorescence upon oxidation by H_2O_2, detected by FL1 photomultiplier, being converted to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). This staining was coupled with PI that stains spermatozoa with disrupted plasmalemma (dead spermatozoa) emitting red/orange fluorescence detected by the FL3 photomultiplier [21]. MitoTracker deep red (MT) was included to assess the mitochondrial integrity as described in methods below, being excited by the red diode laser and detecting the fluorescence with the FL-4 photomultiplier. Sperm samples were diluted in 500 mL of Tyrode's medium and stained with 2.5 mL CM-H_2DCFDA (in DMSO, 50 mM final concentration), 2.5 mL PI (in water, 2.4 mM final concentration), and 2.5 mL MT (in DMSO, 100 nM final concentration). Samples were incubated at 37 °C for 30 min in the dark.

H_2O_2 production was assessed by H_2CDFDA oxidation in viable cells with intact or not mitochondria; we distinguished the population of live spermatozoa (ignoring PI + events), thus individuating cells with high and low cytoplasmic H_2O_2 generation in the subpopulations with high and low mitochondrial activity: DCF + MT+, DCF−MT+, DCF + MT−, DCF−MT−.

2.6. Statistical analysis

Data were analyzed using the R statistical environment v. 3.6.2 (The R Foundation for Statistical Computing, Vienna, Austria). Results are presented as the mean ± SEM.

2.6.1. Cluster analysis of motility data

Sperm kinematic parameters were recorded for each motile sperm cell for the three ejaculates of each of the four bulls, analyzed at 1 and 3 h incubation. VCL, VAP, VSL, LIN, STR, WOB, ALH, BCF, were used for cluster analysis. In total, 125646 cells were included in the analysis.

As described by Bucci et al. [22], data were first normalized. Then a principal component analysis was performed to reduce the number of variables to a few principal components (PC). These PC were used for carrying out a hierarchical clustering using Ward's method with Euclidean distances, identifying 4 clusters. The difference in the 4 clusters is a combination of different velocities, “vigor” of the movements and linearity, thus permitting to determine these subpopulations: SP1 − fast and progressively moving cells; SP2 - average velocity, mean linearity, vigorous movement; SP3 − slow, non linear low vigorous movement; SP4: mean-low velocity, non-linear, vigorous movements. Differences in the composition of the different clusters concerning the treatments were tested with Chi-square analysis. These clusters were expressed as proportions and used as additional variables.

2.6.2. Multivariate data analysis

Results were analyzed by linear mixed-effects models where treatment (levels CTL, ROT, DMM, CCCP, ANTI, and OLIGO) and time (1 h and 3 h of incubation) were considered as fixed effects (6 × 2 factorial arrangement). Bull and the ejaculate within the bull were included in the model as random effects. For each model, the residues were checked for variance homogeneity and normal distribution.

3. Results

3.1. CASA analysis (motility parameters)

Table 1 displays the effect of the mitochondria inhibitors on the motility parameters as defined by CASA (TM, PM, VAP, VSL, VCL, ALH, BCF, STR, LIN). Overall, the treatments did not affect the total motile sperm proportion (TM). Interestingly, progressive sperm increased at 3h under the effect of DMM respect to the control ($P < 0.05$). At 1h, velocities (VCL and VAP) decreased only with CCCP ($P < 0.05$), but linearity parameters decreased with DMM and ANTI, whereas BCF increased. At 3h, the three velocities VCL, VAP, and VSL

Table 1
Results from flow cytometric study of ROS production.

	1 h			3 h		
	Live	Intact mitochondria MitoSOX positive	Intact mitochondria H_2CDFDA $^+$ positive	Live	Intact mitochondria MitoSOX positive	Intact mitochondria H_2CDFDA positive
CTR	39.6 ± 11.6^{ab}	30.2 ± 16.1	1.4 ± 1.0	45.6 ± 8.8^{a}	24.1 ± 10.0	1.0 ± 0.9
ROT	39.8 ± 14.4^{a}	31.4 ± 16.1	1.7 ± 1.5	37.8 ± 11.7^{ab}	16.4 ± 6.7	0.7 ± 0.5
DMM	46.3 ± 15.9^{a}	28.5 ± 15.1	1.4 ± 1.3	37.3 ± 10.9^{ab}	12.4 ± 9.2*	0.7 ± 0.4
ANTI	39.3 ± 15.2^{a}	19.6 ± 11.1	2.0 ± 2.0	31.4 ± 8.7^{b}	18.8 ± 14.5	0.8 ± 0.6*
OLIGO	36.1 ± 15.1^{b}	28.7 ± 14.2	2.0 ± 1.8	36.8 ± 12.9^{ab}	18.7 ± 8.9	0.6 ± 0.3*
CCCP	48.4 ± 15.0^{a}	27.1 ± 15.8	1.9 ± 1.2	43.3 ± 10.6^{ab}	20.9 ± 5.3	0.9 ± 0.5*

Different letters indicate significative differences (p value < 0.05) in presence of different modulators at same time of incubation. The asterisk indicates significative differences (p value < 0.05) in presence of the same modulator between 1 h and 3 ours of incubation. Live (PI- cells); Intact mitochondria (PI-; MT+).

were differently affected, with CCCP tending to lower VCL, and ANTI lowering VAP and VSL. This effect of CCCP also resulted in an increase in LIN, STR, and WOB ($P < 0.05$ respect to CTR). However, it was ROT which decreased ALH at 3h, with no effect on BCF.

3.2. Cluster analysis and subpopulations from CASA data

After cluster analysis 4 different subpopulations were detected, named SP1-4. Details on the PCA and clustering, including the kinematics characteristics of sperm cells belonging to each subpopulation, are shown in the Supplementary file 1 (Tables S1 and S2).

Fig. 2 represents the percentage of spermatozoa belonging to each subpopulation at 1 h and 3 h incubation depending on the treatment. No differences were found at 1 h of incubation, similarly to the small changes found for the average kinematic parameters. After 3 h incubation, SP1 (comprising the fastest and most progressive spermatozoa) decreased by ROT and OLIGO, and especially in ANTI, with concomitant increases of the slowest populations SP3 (slow-progressive) and SP4 (slow-non progressive). Interestingly, ROT and OLIGO also increased SP2 (fast but with low linearity combined with high BCF and ALH, similar to hyperactivation).

3.3. Epifluorescence microscope analysis

Fig. 1 shows the effects of the different inhibitors of mitochondrial activity on the percentages of the different subpopulations in live spermatozoa, with detailed results in the Supplementary file 1. No significant differences in the subpopulation percentages were recorded within each treatment between 1 and 3 h of incubation. At both times, ANTI and CCCP caused dramatic changes ($P < 0.05$) in the composition of mitochondrial activity populations (considering the live sperm), abolishing fully active-mitochondria sperm. However, whereas ANTI maintained a relevant proportion of partially active mitochondria (~70% at 1h and ~50% at 3h), CCCP samples presented almost exclusively inactive-mitochondria sperm at both times.

3.4. Flow cytometry analysis

Table 2 reports the results of flow cytometric evaluations after 1 and 3 h incubation, focusing on ROS production in the live-mitochondrial active population. Whereas the treatments did not significantly affect mitochondrial or cytoplasmic ROS yield (considering sperm with intact mitochondria), there was some effect of the incubation time. Cytoplasmic ROS in mitochondria-active spermatozoa slightly decreased with time in the OLIGO, ANTI, and CCCP treatments. Mitochondrial superoxide yield showed more variability and a general trend to decreasing in most treatments, but this effect was only significant for DMM.

4. Discussion

Post-thawing bull sperm quality has been amply investigated, but there is relatively few information on sperm energetics. Therefore, this study focused on mitochondrial activity of thawed bull spermatozoa to determine the interrelationship (and possible effects) between mitochondrial function, motility and ROS production.

To study mitochondrial membrane potential, we decided to evaluate sperm cells stained with SYBR-14/PI/JC-1 by epifluorescence microscopy, as it permits to delineate in a clearer manner different subpopulations of live sperm cells with active/partially active/inactive mitochondria [23,24]. Interestingly, we found that only ANTI and CCCP affected MMP (indeed, only after 1 h of incubation). Considering the individual inhibition of the ETC complexes, ANTI showed a dramatic change comparing to ROT and MMP. This effect was also reported in fresh bull semen [25]. The lack of effect of ROT and MMP suggest some kind of compensation between complex I and II, indicating that the two complexes may work alternatively if one of them is blocked, thus permitting a cycling of $NADH/FADH_2$ for subsequent oxidative reactions. The effect of ANTI shows the relevance of complex III, and raises the question of the effect of the simultaneous blocking of I and II, which could be answered in a subsequent study. Nevertheless, in about half of the cells at least part of the ETC remained pumping H^+ and therefore maintaining locally high MMP in the mitochondria even if blocking complex III, as demonstrated by the proportion of the partially active mitochondria subpopulation.

Even though effects on mitochondrial activity seems to be immediate for ANTI and CCCP, there was a low impact of mitochondrial activity on sperm function after thawing, demonstrated by the results on ROS production: no alteration or increase in ROS production is evidenced in cells under different treatments with specific ECT inhibitors. Indeed, cytoplasmic ROS levels were low for almost all live cells in all treatments, with a moderate proportion of cells yielding high mitochondrial superoxide. This is relevant considering that we studied the subpopulation with high MMP, potentially producing $O_2^{\bullet -}$ through leaking active ETC. We may speculate that mitochondria of thawed spermatozoa have a higher level of uncoupling events [26], including an inability to increase respiration and oxygen consumption rate (OCR). This might maintain the ROS production low, due to a general defective function of mitochondria. This situation deserves further insight, since bull thawed sperm cells are fertile, despite being bioenergetically challenged [27].

We show too, that a brief incubation with treatments blocking the different elements of the ETC and oxidative phosphorylation have a small and even negligible effects on sperm motility, but they are evident after a longer period (especially considering the subpopulation composition of the sperm samples). Motility could

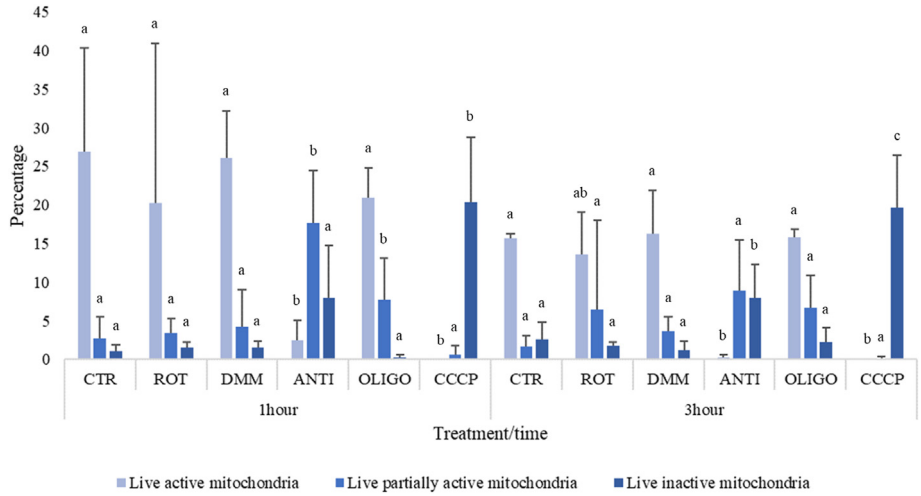

Fig. 1. Different distribution of live cells with active (blue bar), partially active (orange bar) and inactive (grey bar) mitochondria depending on treatment after 1 and 3 h incubation at 37 °C. CTR: 2 μL of DMSO, ROT: 5 μM rotenone, DMM: 10 mM dimethyl-malonate, ANTI: 1.8 μM antimycin A, OLIGO: 5 μM oligomycin A, CCCP: 5 μM carbonyl cyanide m-chlorophenyl hydrazone. Different letters indicate significant differences ($p < 0.05$) in the distribution of the subpopulation compared with the control group at the same time point. N = 12 (straws from 3 different ejaculates from 4 different bulls). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

sustain on glycolysis-derived ATP for some time, but ultimately mitochondrial activity might be crucial for maintaining it at optimal levels.

The inhibition of specific ECT complexes is an effective mechanistic approach already demonstrated in studying fresh bull spermatozoa [28–30]. The main finding raising from those studies is that bull sperm cells are not strictly dependent on the intactness of the ETC to support motility, which seems to be also true in thawed sperm according to our results. A possible explanation is that bull sperm cells are demonstrated to be able to rely both on oxidative metabolism and glycolysis (Storey, [31] for review), and our data furnish evidence to further support those findings. Other studies in different species, such as porcine [23,32–34], equine [15,16,35] and human [36] showed that, spermatozoa from those species are more susceptible to ECT inhibitors, in particular to ROT, ANTI, CCCP and OLIGO. The inhibition of complex I and III induces a decrease in proton transport, therefore a reduced mitochondrial membrane potential and, as a consequence, a reduced activity of the ATP synthase (directly inhibited in the case of OLIGO). This picture clearly shows that stallion, boar and human spermatozoa rely on mitochondrial ATP production to maintain motility.

To better define the impact of the ECT inhibition on thawed bull sperm motility, we performed a cluster analysis on motile sperm cells. This approach has already permitted to unveil some interesting characteristics that are not emerging from the overall motility study [15,33]. Interestingly, we found that ANTI altered the motile sperm subpopulation pattern after the 3-h incubation, reducing sperm velocity and partly altering the swimming pattern (cells shifting from the fast-progressive SP1 to the slow-progressive SP4 and slow-non progressive SP2). This shift demonstrates that inhibiting complex III results in a decrease in sperm velocity and swimming vigor. The exact mechanism of action could not be

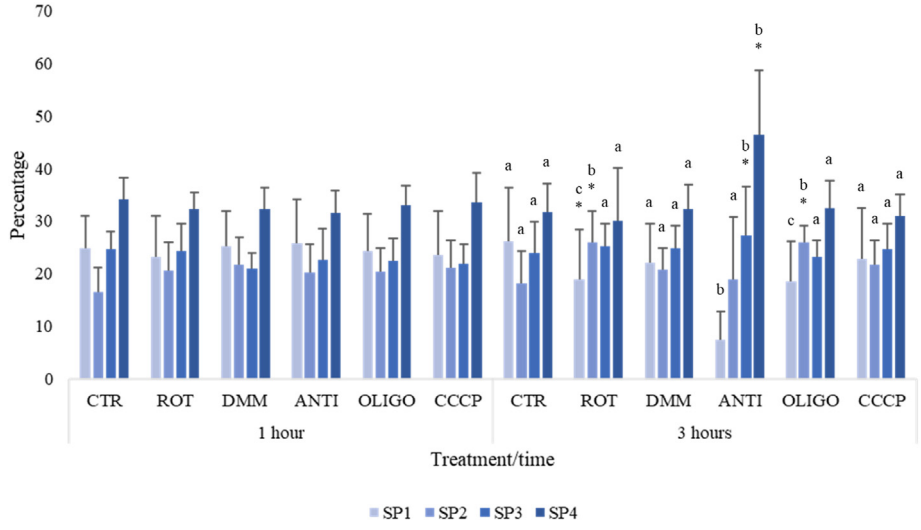

Fig. 2. Distribution of the four motility subpopulations (SP1-4) in motile sperm cells at 1 and 3h of incubation at 37 °C with different modulators of the mitochondrial activity.; CTR: 2 μL of DMSO, ROT: 5 μM rotenone, DMM: 10 mM dimethyl-malonate, ANTI: 1.8 μM antimycin A, OLIGO: 5 μM oligomycin A, CCCP: 5 μM carbonyl cyanide m-chlorophenyl hydrazone. Different letters indicate significant differences ($p < 0.05$) in the distribution of the subpopulation respect the control group. Asterisks indicate differences ($p < 0.05$) in the distribution of the subpopulation at 3h compared to 1h in the same treatment. N = 12 (straws from 3 different ejaculates from 4 different bulls).

Table 2
Motility parameters at 1 and 3 h of incubation with ECT inhibitors.

1 h incubation									
Treat	TM	PM	VCL μm/s	VAP μm/s	VSL μm/s	STR	LIN	ALH μm	BCF Hz
CTR	30.0 ± 14.0	16.2 ± 7.4	64.7 ± 32.4^{a}	38.2 ± 16.5^{a}	31.1 ± 15.2^{ab}	82 ± 18^{ab}	58 ± 30^{a}	2.7 ± 1.4	21.7 ± 5.6^{a}
ROT	34.4 ± 10.6	15.7 ± 6.4	66.2 ± 26.3^{a}	34.1 ± 17.3^{ab}	27.6 ± 16.7^{ab}	77 ± 18^{a}	50 ± 28^{a}	2.8 ± 1.0	23.9 ± 4.6^{abc}
DMM	35.6 ± 15.8	17.4 ± 8.9	75.4 ± 34.9^{a}	34.1 ± 18.2^{ab}	25.7 ± 15.2^{a}	76 ± 18^{a}	39 ± 29^{b}	2.9 ± 1.7	25.9 ± 6.2^{c}
ANTI	25.6 ± 9.9	13.7 ± 6.8	70.6 ± 35.2^{a}	34.5 ± 17.4^{ab}	27.0 ± 15.6^{ab}	79 ± 17^{a}	45 ± 24^{b}	2.9 ± 1.6	25.0 ± 4.9^{bc}
OLIGO	29.0 ± 10.3	15.7 ± 8.8	71.6 ± 35.9^{a}	39.0 ± 21.7^{a}	31.8 ± 20.8^{b}	81 ± 18^{ab}	52 ± 29^{a}	2.2 ± 1.8	23.5 ± 6.0^{ab}
CCCP	24.3 ± 13.2	12.00 ± 9.8	55.0 ± 28.5^{b}	31.8 ± 13.4^{b}	27.7 ± 12.0^{ab}	87 ± 15^{b}	59 ± 20^{a}	2.5 ± 1.4	23.3 ± 6.3^{abc}
3 h incubation									
Treat	TM	PM	VCL μm/s	VAP μm/s	VSL μm/s	STR	LIN	ALH μm	BCF Hz
CTR	30.3 ± 13.1	16.1 ± 9.2^{ab}	72.9 ± 34.8^{a}	37.2 ± 20.6^{ab}	28.6 ± 18.6^{ab}	77 ± 18^{a}*	44 ± 22^{a}*	2.9 ± 1.7^{a}	24.5 ± 6.0*
ROT	26.8 ± 8.7	16.0 ± 7.4^{ab}	71.5 ± 32.3^{a}*	35.6 ± 21.9^{ab}	27.5 ± 14.7^{ab}	81 ± 19^{ab}	45 ± 27^{a}	2.8 ± 1.2^{a}	23.1 ± 6.1
DMM	34.9 ± 12.9	22.8 ± 10.6^{b}	76.4 ± 32.9^{a}	37.9 ± 18.5^{ab}	31.1 ± 17.7^{ab}*	81 ± 18^{ab}	46 ± 28^{a}	2.9 ± 1.5^{a}	24.1 ± 6.7
ANTI	22.9 ± 13.1	12.3 ± 8.4^{a}	68.4 ± 35.1^{ab}	32.0 ± 13.4^{a}	24.5 ± 11.5^{a}	78 ± 16^{ab}	44 ± 24^{a}	2.7 ± 1.4^{a}	24.5 ± 5.5
OLIGO	25.9 ± 13.5	15.9 ± 5.9^{ab}	73.8 ± 31.8^{a}	41.4 ± 25.4^{b}	35.4 ± 26.2^{b}	81 ± 17^{ab}	51 ± 28^{a}	2.5 ± 2.0^{a}	23.6 ± 6.0
CCCP	22.3 ± 13.7	11.6 ± 9.3^{a}	51.7 ± 29.6^{b}	34.7 ± 19.2^{ab}	29.3 ± 16.2^{b}	86 ± 16^{b}	64 ± 24^{b}	2.1 ± 1.3^{a}	21.7 ± 7.2

The different letters indicate significative differences (p value < 0.05) in presence of different modulators at same time of incubation. The asterisk indicates significative differences (p value < 0.05) in presence of the same modulator between 1 h and 3 h of incubation. TM total motility; PM progressive motility; VCL: Curvilinear velocity; VAP: Average-path velocity; VSL: Straight-line velocity; STR: Straightness; LIN: Linearity; ALH: Lateral head displacement; BCF: Beat cross frequency.

defined, as it is not possible to subsequently isolate cells from different SP and analyze their characteristics. We could only speculate about an altered redox balance (demonstrated in freshly ejaculated bull semen by Bulkeley et al. [9]) or a reduced ATP production via ATP synthase due to a lower mitochondrial membrane potential. In a recent study, specifically studying the bioenergetics of frozen-thawed bull sperm mitochondria [26], we showed a decrease in mitochondrial basal respiration (measured by Agilent Seahorse technology) when using ANTI and ROT, which support the idea that both complex I and III activity is necessary to establish the mitochondrial membrane potential needed to permit ATP synthase activity. In addition, we showed that the mitochondrial damage produced by the freezing-thawing process makes these cells' mitochondria uncoupled, namely unable to respond with increased oxygen consumption in the presence of FCCP (Algieri et al., 2022 [26]).

An effect on motile sperm subpopulation was also exerted by ROT and OLIGO after the 3-h incubation. Contrarily to ANTI, the SP1 cells shifted to SP2, suggesting a modulation rather than an impairment in motility (hyperactivated-like whereas maintaining VCL).

Again, evidence was given that bull thawed sperm cells could rely on glycolysis [31] and the "short" surviving period in the female genital tract (if compared with other species as dog, horse and pig) to meet the mature egg to fertilize, could justify this intriguing feature.

5. Conclusion

In conclusion it could be stated that in most cases bull thawed spermatozoa mitochondria are partially damaged or uncoupled and ATP support to motility would be alternatively driven from the glycolytic pathway.

In addition, regarding thawed bull semen mitochondrial functionality, we showed that complex III is essential for maintaining MMP and, consequently ATP synthesis, while complex I and II may be interchangeable. ROS production is very low, probably because the uncoupling effect of cryopreservation process.

CRediT authorship contribution statement

Olga Blanco-Prieto: performed the experiments and wrote the first draft of the manuscript. **Beatrice Mislei:** performed the experiments. **Felipe Martínez-Pastor:** performed the experiments, corrected the article draft and participate to experimental conceptualization. **Marcella Spinaci:** performed the final review of the manuscript and critically revised the results. **Gaetano Mari:** performed the final review of the manuscript and critically revised the results. **Diego Bucci:** is the corresponding author, performed the final review of the manuscript and experimental conceptualization.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2023.05.021.

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