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In boar sperm capacitation L-lactate and succinate, but not pyruvate and citrate, contribute to the mitochondrial membrane potential increase as monitored via safranine O fluorescence

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ABSTRACT

Having ascertained using JC-1 as a probe that, in distinction with the controls, during capacitation boar sperm maintains high mitochondrial membrane potential ($\Delta\Psi$), to gain some insight into the role of mitochondria in capacitation, we monitored $\Delta\Psi$ generation due to externally added metabolites either in hypotonically-treated spermatozoa (HTS) or in intact cells by using safranine O as a probe. During capacitation, the addition to HTS of L-lactate and succinate but not those of pyruvate, citrate and ascorbate + TMPD resulted in increase of $\Delta\Psi$ generation. Accordingly, the addition of L-lactate and succinate, but not that of citrate, to intact sperm resulted in $\Delta\Psi$ generation increased in capacitation.

1. Introduction

One of the outstanding questions in animal reproduction concerns the mechanism by which mammalian spermatozoa manage their energy levels and the role of mitochondria as regulators of sperm functions, including capacitation [1–3], the multifaceted maturation process rendering spermatozoa competent to fertilize [4], where an increase of the mitochondrial membrane potential ($\Delta\Psi$) occurs [5–7]. In sperm ATP synthesis can occur in mitochondria, via oxidative phosphorylation [see 8], but also in glycolysis; accordingly multiple glycolytic enzymes are present along the mouse sperm flagellum, likely to support sperm motility [9]. Importantly, the ATP source appears to be species-specific in spermatozoa due the conditions in the oviduct of the conspecific female [8]. In particular in pig, even if citrate, which inhibits phosphofructokinase I [10], is abundant in seminal plasma, ATP production was reported to depend on glycolytic flux [11]. In this regard, the existence of a mitochondrial L-lactate dehydrogenase (m-L-LDH) was definitively confirmed in mammalian, plant and yeast mitochondria [12–14], being its existence finally recognized by inclusion of m-L-LDH in the Mitocarta (http://www.broadinstitute.org/pubs/MitoCarta/index.html), but despite the occurrence also in sperm of an m-L-LDH [see for Ref. [12]], whether and how L-lactate and other metabolites present in seminal plasma play a role in sperm energy production, especially in capacitation, remains to be fully established [15].

We investigate this issue by monitoring whether and how $\Delta\Psi$ generation, used as an indicator of the mitochondrial function, changes in capacitation as a result of the addition of a variety of metabolites to either cell homogenate containing intact mitochondria or intact cells.

2. Materials and methods

2.1. Chemicals and culture media

All chemicals from Sigma Chemical Co (St. Luis, Mo) were of purest grade available and were used as Tris salts at pH 7.0–7.4 adjusted with Tris or HCl.

The non capacitating medium (NCM) was composed of 2.7 mM KCl, 1.2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 95 mM NaCl, 5.55 mM glucose, and 2 mM pyruvate (pH 7.4). In the capacitating medium (CM) 25 mM $NaHCO_3$, 5 mM $CaCl_2$, 0.4% BSA (type V, free of fatty acids) were also present.

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2.2. Sperm preparation

Sperm preparation was as in Ref. [16]. Briefly, freshly ejaculated semen was collected from fertile boars and transported to the laboratory at 16°C–18 °C within 30 min. The semen was divided into 2 equal fractions and centrifuged (10 min, 270 × g, 25 °C). The first fraction was suspended in CM (40 × 10^6 sperm/ml) to induce capacitation, and the second portion in NCM (40 × 10^6 sperm/ml) was used as a control. Sperm were then incubated at 38.5 °C in a humidified 5% CO_2 atmosphere.

2.3. Hypotonic treatment of sperm

Hypotonically-treated spermatozoa (HTS) was prepared as in Ref. [17] from fresh samples, incubated either in CM or NCM; briefly, samples were washed three times (800 g for 10 min at room temperature) in isotonic salt medium A (0.2% BSA, 113 mM KCl, 12.5 mM KH_2PO_4, 2.5 mM K_2HPO_4, 3 mM $MgCl_2$, 0.4 mM EDTA and 20 mM Tris–HCl pH 7.4). Spermatozoa were then subjected to hypotonic treatment by keeping cells in ice-chilled hypotonic medium (0.2% BSA, 7.74 mM K_2HPO_4, 2.24 mM KH_2PO_4 pH 7.4) for 1.5 h. Sperm were then washed three times using medium A, as above. L-LDH activity was checked by assaying photometrically, by means of Varioscan spectrofluorimeter, NADH oxidation ($\varepsilon_{334} = 6.22\ mM^{-1} \times cm^{-1}$) due to pyruvate addition to either intact sperm or HTS.

2.4. ΔΨ measurements

2.4.1. Flow cytometry

Sperm parameters of intact sperm incubated in either CM or NCM were assayed via flow cytometry using JC-1 by means of the Guava EasyCyte Cytometry System (IMV International Technologies, Maple Grove, MN). Briefly, boar spermatozoa (35 × 10^6 cells/ml) incubated for either few seconds or 3 h, in either CM or NCM were stained with JC-1 (Cat. No. 4500-0250, MitoPotential Kit, IMV), and measured using manufacturer's settings. 2000 events for each sample in triplicate were analyzed by the Guava® Mitopotential software (Billerica, MA). Debris was excluded from acquired events.

2.4.2. Fluorimetric safranine O assay

ΔΨ generation by mitochondria in HTS was monitored at 25 °C essentially as in Ref. [18], by measuring safranine O fluorescence changes (λex/em 520/570 nm) using a Varioscan spectrofluorimeter. 150 × 10^6 HTS were incubated in 2 ml of isotonic medium in the presence of 1.5 μM safranine O, at a ratio of 20 nmol/10^9 cells.

2.4.3. Statistical analysis

Statistical analysis was performed according to the Student's t test.

3. Results

In our experimental protocol freshly ejaculated boar spermatozoa, incubated as in Ref. [19] for 3 h either in NCM or CM, were checked for the occurrence of *in vitro* capacitation. This was confirmed by using the chlortetracycline (CTC) assay, by tyrosine phosphoprotein complex p32 assay [16,19] and by computer-assisted sperm analysis (CASA) motility measurements as in Ref. [20].

To confirm that capacitation results in a cell population retaining high ΔΨ we incubated cells in either CM or NCM for few seconds (0 time) or 3 h (time at which capacitation peaks) and their energy status was assessed by flow cytometry using JC-1 as a probe [21] (Fig. 1A). At 0 time about 70% of all the cell population

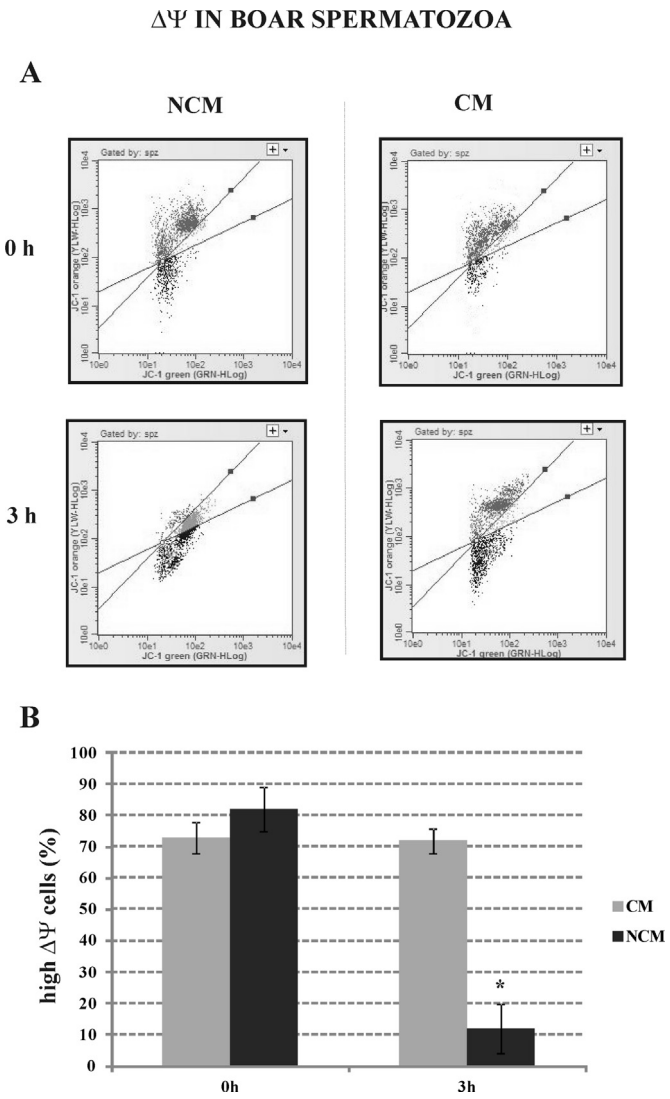


Fig. 1. Flow cytometry determination of mitochondrial ΔΨ in boar sperm capacitation. (A) Boar spermatozoa (35 × 10^6 cell) incubated for few seconds (0 h) or for three hours (3 h) in either capacitation (CM) or non capacitation (NCM) medium were assessed by flow cytometry by using JC-1 as a fluorimetric probe (for details see Section 2.4). Events in region upper/left represent spermatozoa with high mitochondrial ΔΨ; events in region lower/right represent spermatozoa with low ΔΨ; and events in region upper/right represent spermatozoa with depolarizing mitochondria. (B) Mean value (±SD) of % of cells showing high ΔΨ obtained in three different experiments carried out as in A. * significant difference according to Student's t test ($P < 0.05$).

exhibited high ΔΨ mitochondria as indicated by JC-1 response; at 3 h time, capacitated sample maintained their high ΔΨ population in distinction with not capacitated cells. Such a distinction proved to be significantly different (Fig. 1B).

In a series of control experiments, not shown in detail, spermatozoa proved to be intact and free of seminal plasma contamination since no L-LDH activity (present in seminal plasma [22]) were released as shown by the lack of NADH oxidation due to the addition of pyruvate to sperm cell incubated in the presence of NADH. As a result of hypotonic treatment, an homogenate was obtained containing intact mitochondria, as shown by the lack of NADH oxidation which occurred only after the addition of Triton X-100 which dissolves mitochondria.

Thus, as done in mammalian [23], yeast [24] and plant [18] mitochondria, use was made of safranine O, as a fluorimetric probe, to check whether ΔΨ generation could be continuously

monitored in HTS, which mirrors isolated functional mitochondria [25] (Fig. 2). For a validation experiment, mitochondrial function was checked in HTS incubated for few seconds in either CM (Fig. 2 trace a) or NCM (Fig. 2 trace b) under conditions in which capacitation is still lacking: in both cases the addition of succinate (5 mM) induced a rapid ΔΨ increase [18], as shown by the decrease in safranine O fluorescence, occurring essentially at the same rate, but with minor variation likely due to the different medium composition. As expected, the further addition of ADP (1 mM) caused ΔΨ decrease since ΔΨ is used to drive both ATP synthesis and ADP/ATP exchange via the adenine nucleotide translocator in an electrophoretic manner. The sequential addition of oligomycin (2 μg), used to block proton influx across ATP synthase, completely restored ΔΨ. Finally, ΔΨ was rapidly abolished by the addition of the uncoupler FCCP (1 μM).

Since glycolysis occurs in boar sperm [11], since L-lactate is present in the seminal plasma [26] and can be metabolized by boar spermatozoa [27] and in the light of [28,29] in which L-lactate mitochondrial metabolism is not considered, we compared pyruvate and L-lactate with respect to their capability to generate ΔΨ in capacitation. HTS were prepared from sperm incubated in CM for either few seconds (0 h-HTS), used as a control, or 3 h (3 h-HTS) (Fig. 3A); Externally added pyruvate (1 mM) caused a little ΔΨ generation both in 0 h-HTS and in 3 h-HTS; L-lactate addition (5 mM) resulted in ΔΨ generation at a rate much higher with respect to that measured for pyruvate, already at the beginning of capacitation time. In capacitation the rate of generation and the extent of ΔΨ due to L-lactate were about 150% of those of the controls. Increase of ΔΨ generation during capacitation was also found in the presence of malate (5 mM) either in the absence or presence of pyruvate (1 mM) and of glutamate plus malate (5 mM each, not shown), which can reduce the intramitochondrial $NAD(P)^+$. In parallel either citrate or succinate (5 mM each), both present in the seminal plasma [26,30,31], were also investigated. A negligible ΔΨ generation was found as a result of citrate addition to either 0 h- or 3 h-HTS, whereas the addition of succinate resulted in ΔΨ generation with a rate increased in capacitation up to about 160%. Contrarily, when ascorbate (5 mM) plus TMPD (1 mM) were used as a substrate pair of cytochrome c oxidase, no change of ΔΨ generation was found during capacitation. ΔΨ generation due to ascorbate plus TMPD proved to be insensitive to externally added cytochrome c (150 μM), this confirming that in both 0 h- and 3 h-HTS the outer mitochondrial membrane remains intact. The mean increase values of both rate and extent occurring in capacitation were reported in Fig. 3B.

In the light of result of Fig. 3A, in a first series of experiments we confirmed that ΔΨ generation can also occur in intact sperm from either L-lactate or succinate taken up from the extracellular phase which mirrors the extracellular fluid (Fig. 3C). Externally added L-Lactate (5 mM) proved to cause ΔΨ increase when added to intact boar sperm incubated for few seconds in CM (0 h). Negligible ΔΨ generation was found due to the addition of citrate (5 mM), contrarily, succinate (5 mM) resulted in ΔΨ generation (Fig. 3C). About 70 and 90% increase of ΔΨ rate of generation was found in capacitation (3 h) due to L-lactate and succinate addition, respectively.

4. Discussion

In distinction with a variety of capacitation events including hyperactivation, changes in the membrane fluidity, protein tyrosine phosphorylation [see for Ref. [32]], as remarked in Ref. [3] the mitochondrial function in capacitation was poorly investigated; in particular the metabolites which drive the mitochondrial energy production remained unknown. Thus, the goal of this paper was to gain a first insight into the mechanism by which mitochondrial ΔΨ increases in boar sperm capacitation [5,6] and in particular to ascertain whether L-lactate, present in the seminal plasma with citrate, succinate, glucose and fructose [26,31,33], can contribute to mitochondrial extra energy production. We used ΔΨ since it mirrors the total mitochondrial potential energy to be used in the cell function including ATP synthesis, metabolite traffic across the mitochondrial membranes and thermogenesis. In this regard, we first resorted to a safranine O based method which, in distinction with JC-1, has the added dimension to allow for monitoring continuously ΔΨ generation. Indeed, use of JC-1 and similar probes does not allow for investigation of the mechanism by which ΔΨ is generated given that these probes cannot monitor the fast changes

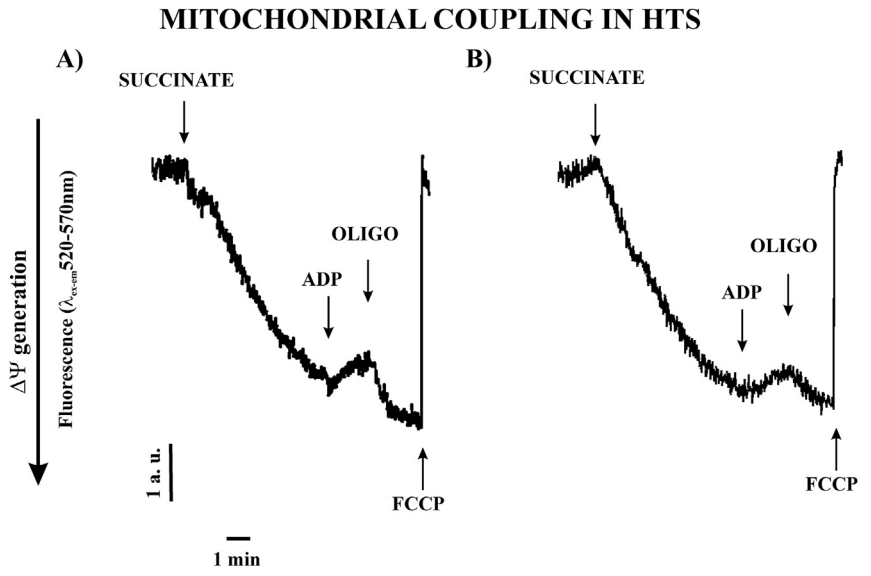


Fig. 2. Measurements of ΔΨ generation by coupled mitochondria in HTS via safranine O fluorescence. HTS (150×10^6 cells) from samples incubated for 5 s in either CM (A) or NCM (B) (see Section 2.4) were incubated in medium A added with 1.5 μM safranine O and fluorescence was continuously monitored ($\lambda_{ex}/\lambda_{em}$ 520/570 nm). At the arrows 5 mM succinate, 1 mM ADP, 2 μg oligomycin, 1 μM FCCP were added. a.u.: arbitrary units.

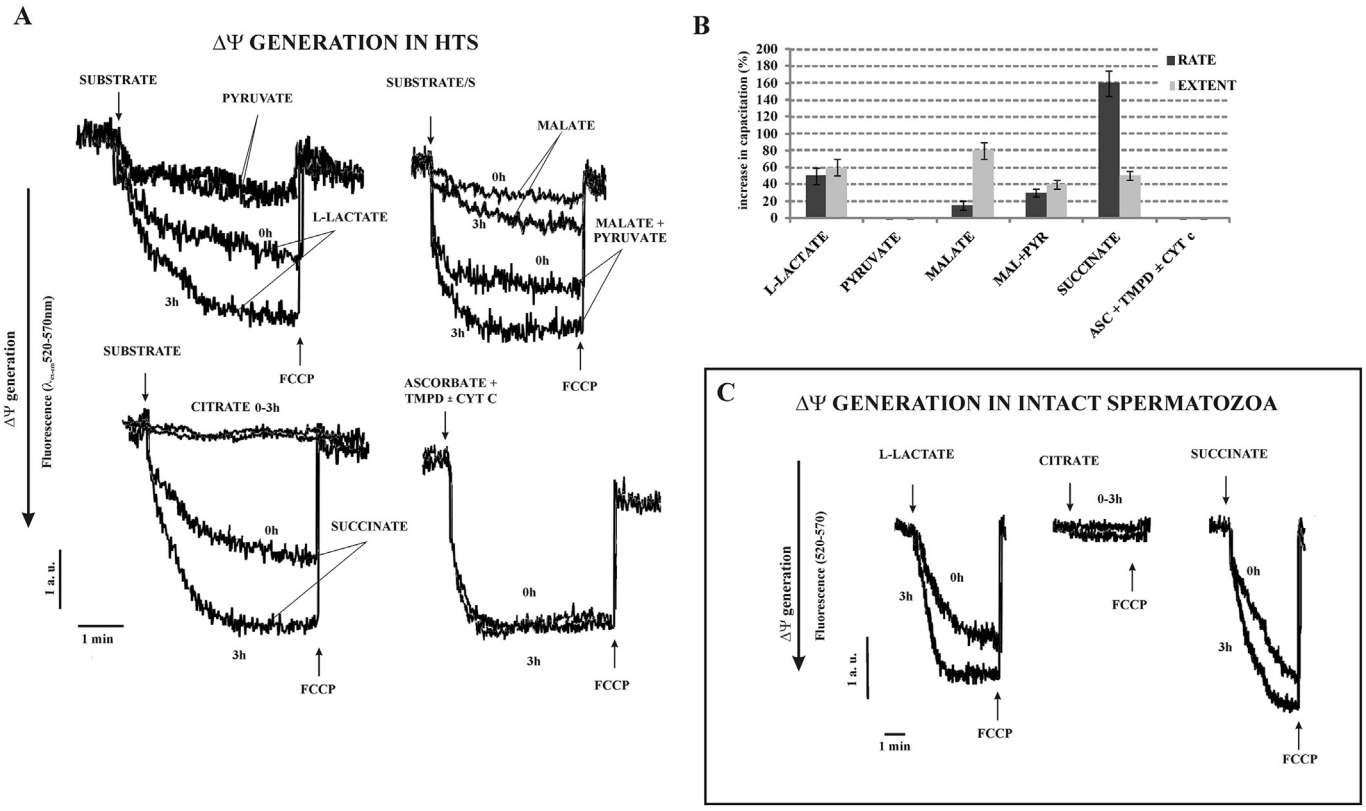
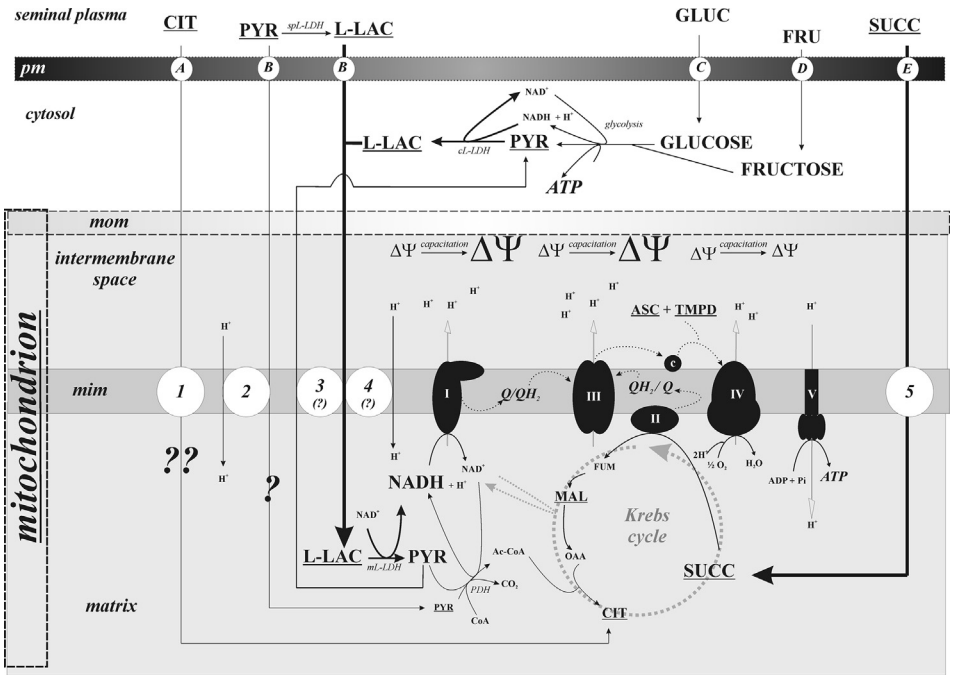


Fig. 3. The addition of certain metabolites to either HTS (A,B) or intact boar spermatozoa (C) results in ΔΨ generation either in the absence or in the presence of capacitation. (A) HTS (150×10^6 cells each) obtained from samples incubated in CM for either few seconds (0 h) or 3 h were incubated in medium A (see Section 2.3) in the presence of 1.5 μM safranine O and fluorescence was continuously monitored ($\lambda_{ex}/\lambda_{em}$ 520/570 nm). At the arrows the following additions were made: either 1 mM pyruvate or 5 mM L-lactate, either 5 mM malate alone, or 1 mM malate plus 5 mM pyruvate, either 5 mM citrate or 5 mM succinate (added in the presence of 2 μg rotenone), 5 mM ascorbate plus 1 mM TMPD (added in the presence of 2 μg antimycin A) either in the presence or in the absence of 150 μM cytochrome c, and 1 μM FCCP. (B) Mean values (±SD) of ΔΨ generation increase occurring in HTS in capacitation, obtained in three different experiments carried out as in A, are reported. Controls were both rate and extent values obtained for each substrate/substrate pair at 0 h. (C) Intact boar sperm (150×10^6 cells), previously incubated in CM for few seconds (0 h) or for the time required for capacitation to occur (3 h), were incubated in medium A added with 1.5 μM safranine O and fluorescence was continuously monitored ($\lambda_{ex}/\lambda_{em}$ 520/570 nm). At the arrows additions were made at the indicated concentrations: 5 mM L-lactate, 5 mM citrate, 5 mM succinate and 1 μM FCCP. a.u.: arbitrary units.

in mitochondrial ΔΨ occurring as a result of substrate addition [see also 34]. We confirm here our original finding [5] that capacitation results in the increased population retaining high ΔΨ in capacitated sperm (Fig. 1) and show that intact coupled mitochondria (Fig. 2) exhibit a tremendous increased capability to generate ΔΨ during capacitation, which counterbalances the ΔΨ decrease found in the control, by oxidizing L-lactate and succinate, but not citrate and pyruvate (Fig. 3). Our finding that pyruvate is a poor mitochondrial energy substrate is not unique: notice that pyruvate as the sole substrate was metabolized very slowly by bovine [35] and boar spermatozoa mitochondria [27]. On the other hand, in Ref. [3] it was proposed that the joined operation of the cytosolic and mitochondrial L-LDH and of the lactate carrier/s [12] could allow for both the progress of glycolysis by the production of NAD^+ and the progress of mitochondrial energy production by the transport of reducing equivalents from the cytosol into mitochondria. However here we show that L-lactate, but not pyruvate, is a major mitochondrial energy substrate in capacitation, perhaps as a final glycolysis product as proposed in neurons [36]. The failure of externally added citrate to cause ΔΨ generation both in HTS and in intact cells is apparently in contrast with [37] where it was shown that the incubation of boar spermatozoa with 10 mM citrate resulted in carbon dioxide production and L-lactate release in the extracellular space. On the other hand, the failure of citrate to sustain high ATP levels and sperm motility was also found [see 29], thus we are forced to propose that such a difference depends on different experimental conditions. Contrarily, L-lactate, as well as succinate which is present in seminal plasma and can enter both cell and mitochondria [30–38], cause ΔΨ generation in both HTS and intact cells, with an increase during capacitation. At present the mechanism by which the rate of ΔΨ increases in capacitation must remain a matter of speculation. ΔΨ generation due to the addition of respiratory substrates to coupled mitochondria derives from a process including a variety of steps: i. uptake of the substrates mostly in a carrier mediated manner, ii. their oxidation by specific dehydrogenases located inside mitochondria, iii. electron flow along the respiratory chain accompanied by proton pumping from the matrix to the intermembrane space to generate ΔΨ. In experiments of Fig. 3C the possible of modification of plasma membrane permeability should also be taken in consideration. Use of a variety of non-penetrant compounds (which can affect only transport processes) and/or of specific inhibitors of any step of capacitation is needed to gain some insight into this issue. Notice that since capacitation is related to the controlled generation of reactive oxygen species (ROS) [39–41], L-lactate might be also involved in ROS generation due to the existence of the putative L-lactate oxidase [42].

Interestingly, cytochrome c oxidase, which under certain conditions regulates reducing equivalent flux along respiratory chain [for refs see Ref. [43]], and whose stimulation by He–Ne laser was correlated with energy charge increase in spermatozoa [44,45], is not responsible for ΔΨ increase since no difference in the rate of ΔΨ generation due to ascorbate + TMPD was found.



Scheme 1. ΔΨ increase during boar sperm capacitation. Boar sperm cells convert glucose (GLUC) and/or fructose (FRU), entered the cell via their specific transporter (C and D respectively), to pyruvate (PYR) with ATP production. PYR, which poorly enters mitochondria via its own carrier (2), is reduced to L-lactate (L-LAC) due to the cytosolic L-LDH (cL-LDH). The newly synthesized L-lactate together with L-lactate taken up, via the monocarboxylate transporter (*B*), from seminal plasma where is formed via the seminal L-LDH (spL-LDH), enters mitochondria via a the putative carrier L-LAC/H^+ symporter (4). Succinate (SUCC), which enters spermatozoa in a carried mediated manner (*E*), is assumed to enter mitochondria via the dicarboxylate carrier (5). Sperm mitochondria can oxidize L-lactate (via the mL-LDH) and succinate (via succinate dehydrogenase - Complex II) thus reducing intramitochondrial NAD^+ and FAD respectively. The electron flow along the respiratory chain caused by the oxidation of NADH by complex I and of $FADH_2$ results is ΔΨ generation which increases in capacitation. Contrarily, no ΔΨ generation increase in capacitation occurs due to complex IV. Both in controls and in capacitated samples, PYR and citrate (CIT) are poorly transported in the mitochondria via (2) and tricarboxylate carrier (1), respectively. Moreover, L-LAC/PYR shuttle, due to the putative L-LAC/PYR antiporter (3) and to both c- and m-L-LDH, could occur in boar sperm. Abbreviations: pm: plasma membrane; mom: mitochondrial outer membrane; mim: mitochondrial inner membrane; ASC: ascorbate; TMPD: *N,N,N′,N′*-Tetramethyl-*p*-phenylenediamine; Q/QH_2: oxidized and reduced form of Coenzyme Q, respectively; c: cytochrome c; Ac-CoA: acetyl-coenzyme A; CoA: coenzyme A; FUM: fumarate; MAL: L-malate; OAA: oxaloacetate; PDH: pyruvate dehydrogenase complex. Substrates checked in this paper are reported underlined.

The possible scenario for energy metabolism in capacitation as derived from Refs. [11,38,46] and this work is summarized in Scheme 1.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Transparency document

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