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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

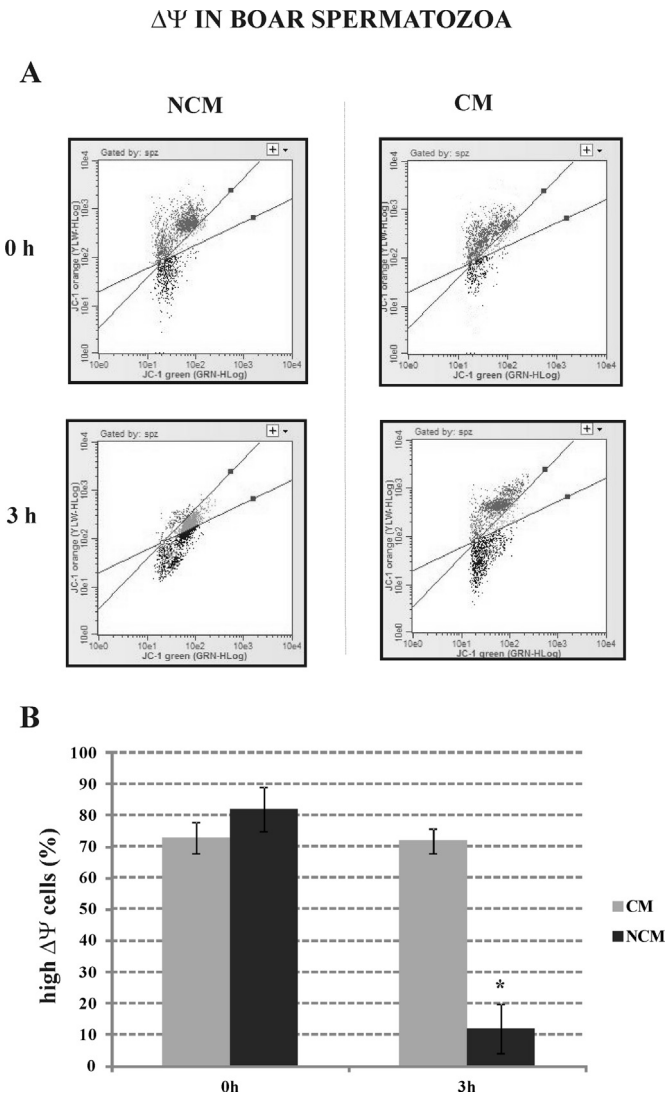

Fig. 1. Flow cytometry determination of mitochondrial ΔΨ in boar sperm capacitation. (A) Boar spermatozoa (35 × 10^6 cell) incubated for few seconds (0 h) or for three hours (3 h) in either capacitation (CM) or non capacitation (NCM) medium were assessed by flow cytometry by using JC-1 as a fluorimetric 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