Oxidation of Exogenous Lactate by Lactate Dehydrogenase C in the Midpiece of Rat Epididymal Sperm is Essential for Motility and Oxidative Activity

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Abstract: Problem statement: To identify the metabolic reaction-glycolysis or oxidative phosphorylation that is mainly involved in the production of energy required for rat sperm mobilization. **Approach:** Epididymal sperm were collected from Wistar rats and extended in lactate-containing or lactate-free raffinose-modified Krebs-Ringer Bicarbonate solution (mKRB)-egg yolk medium supplemented with 0, 1, 2, or 3 mM 2-Deoxy-D-Glucose (2 DG) and 1, 2, or 3 mM sodium Oxamate (OX). Sperm motility, straight-line velocity (VSL) and oxygen consumption were evaluated. Further, immunofluorescent localization of Lactate Dehydrogenase C (LDH-C) in sperm was also performed. **Results:** Low concentrations of 2DG (1 and 2 mM) did not significantly affect motility, VSL and oxygen consumption of sperm extended in the lactate-containing medium. While sperm motility and oxygen consumption were significantly inhibited by even 1mM 2DG in sperm extended in lactate-free medium. Sperm motility significantly inhibited in the case of sperm extended in lactate-containing and free-medium with 1 mM OX. We also found that sperm motility was not maintained in the absence of lactate throughout the 3 h incubation period. Immunofluorescence study revealed that mainly LDH-C was may be localized in the intramitochondrial region of the sperm. **Conclusion:** These results suggest that exogenous lactate enhances lactate oxidation by LDH-C, thereby promoting mitochondrial oxidative reactions in the midpiece and maintaining the mobilization of rat epididymal sperm.

Key words: Lactate, LDH-C, mitochondrial oxidative reaction, mobilization, rat epididymal sperm

INTRODUCTION

Under anaerobic conditions, lactate is the main end product of energy metabolism in sperm. It appears that lactate produced in the distal part of the flagellum is transferred to the mitochondria, where pyruvate and Nicotinamide Adenine Dinucleotide (NADH) are formed and then oxidized[1]. Lactate Dehydrogenase C (LDH-C) has been found in the mitochondria, cytosol and plasma membranes of sperm of several mammals[2-4]. While LDH-C is not localized in the mitochondria in mammalian somatic cells; intramitochondrial localization appears to be an exclusive property of the sperm-specific isoform of LDH[5].

We have previously reported that raffinose-modified Krebs Ringer Bicarbonate (mKRB)-egg yolk solution containing lactate, pyruvate and glucose improves the cryosurvival ability of rat epididymal sperm[6]. This improvement may be due to the enhanced metabolic activity of sperm extended in lactate-containing mKRB solution before freezing[7,8]. Here, we aim to identify the metabolic reaction-glycolysis or oxidative phosphorylation-that is mainly involved in the production of energy required for rat sperm mobilization. For this purpose, we examined rat epididymal sperm motility, straight-line velocity (VSL) and oxygen consumption by treatment with 2-deoxy-D-Glucose (2DG) and sodium Oxamate (OX), which are inhibitors of glycolysis[9] and LDH-C[10]. Further, immunofluorescent localization of LDH-C in sperm was also performed.

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MATERIALS AND METHODS

Extender: The basic extender used in this study was the raffinose-mKRB-egg yolk solution as defined by Yamashiro *et al.*[6,7]; it comprised 0.1 M raffinose (Sigma, St. Louis, MO, USA), 94.6 mM NaCl (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 4.78 mM KCl (Wako), 1.71 mM $CaCl_2 \cdot 2H_2O$ (Wako), 1.19 mM $MgSO_4 \cdot 7H_2O$ (Wako), 1.19 mM KH_2PO_4 (Wako), 25.07 mM $NaHCO_3$ (Wako), 32.37 mM sodium DL-lactate (Sigma), 0.5 mM sodium pyruvate (Wako), 5.56 mM glucose (Wako), 50 µg mL^{-1} streptomycin (Sigma) and 75 µg mL^{-1} penicillin (Sigma). The egg yolk was separated from the albumin and 20% (v:v.) egg yolk was added to the raffinose-mKRB solution. Egg yolk lipids were solubilized by adding 0.04% (w:v) Sodium Dodecyl Sulphate (SDS; Wako) to the solution. The solution was then centrifuged twice at 6,000 rpm for 30 min. The pH of the solution was adjusted to 7.3 with HCl and its osmotic pressure, to 400 mOsm. The supernatant was aspirated and filtered through a 0.45 µm thickness membrane filter (Sartorius, Goettingen, Germany).

Collection of rat epididymal sperm: Both caudae epididymides were excised from Wistar rats aged >15 weeks. Sperm were collected as described previously[6,7].

Sperm motility evaluation: Effect of various concentrations of 2DG (Sigma) or OX (Sigma) in lactate-containing or lactate-free raffinose-mKRB-egg yolk solution on the motility characteristics of after collected sperm. Sperm samples were obtained from both caudae epididymides of 6 rats and suspended in 3.5 mL lactate-containing (3 rats) and lactate-free (3 rats) raffinose-mKRB-egg yolk solution at 37°C. The suspensions were divided into 7 aliquots and equal volumes of extender solution supplemented with 2DG (0, 2, 4, or 6 mM) or OX (2, 4, or 6 mM) were added to the suspensions, respectively. The final concentrations of 2DG and OX were halved. Sperm motility (%) and straight-line velocity (VSL; µm sec^{-1}) after incubation for 1, 2 and 3 h were assessed using a sperm motility analysis system (Kashimura, Tokyo, Japan)[7].

Measurement of oxygen consumption in sperm: Sperm from both caudae epididymides of 10 rats were used in this experiment. We assessed the effect of 2DG (0, 1, 2, or 3 mM) or OX (1, 2, or 3 mM) in lactate-containing (5 rats), or lactate-free (5 rats) raffinose-mKRB-egg yolk medium on the mitochondrial activity of sperm. The procedure for sample preparation was as described above. The oxygen-consumption rates of the sperm in all media were measured using Clark-type oxygen electrodes (Rank Brothers Ltd., Cambridge, UK) that were maintained at 37°C for 5 min and calibrated with air-saturated water at 37°C. Air-saturated water was assumed to contain 406 nmol oxygen mL^{-1}. Sperm samples (1 mL each) were suspended in a reaction chamber and carefully stirred, taking care to avoid the introduction of any external air into the chamber. The final sperm concentration in the incubation chamber was approximately 10×10^6 sperm mL^{-1}. Data were acquired using LabChart version 5.2 (AD Instruments, Castle Hill, Australia). The oxygen-consumption rates [nmol $min^{-1}/10 \times 10^6$ sperm] were expressed as described previously[8].

Immunofluorescent localization of LDH-C in rat sperm: Epididymal sperm samples were collected in lactate-containing raffinose-mKRB medium and labeled mitochondria with 25 nM MitoTracker Red CMXRos (Molecular Probes, Eugene, OR, USA) for 10 min at 37°C, modified staining method from a previous reported by Kim *et al.*[11]. Sperm were washed twice by centrifuging with Phosphate-Buffered Saline (PBS) for 2 min at 12,000 rpm and fixed with 4% formaldehyde (Wako) for 15 min at room temperature. After the samples were washed thrice in PBS, they were permeabilized with 0.2% Triton X-100 for 30 min. They were then washed thrice with 3% PBS-bovine serum albumin (BSA) and incubated overnight with anti-LDH-C antibody (dilution, 1:100; Santa Cruz Biotechnology Inc., USA) or with PBS-BSA solution (control) at room temperature. The sperm samples were then washed thrice in PBS-BSA and the secondary antibody Fluorescein Isothiocyanate (FITC)-conjugated donkey anti-goat Immunoglobulin (Ig) G (dilution, 1:200; Invitrogen, Carlsbad, CA, USA) was added to 3% PBS-BSA. The sperm samples were incubated in this solution for 1 h at room temperature. After the samples were washed thrice in PBS-BSA, the chromosomes in the sperm were stained with 4′,6′-Diamino-2′-Phenylindole (DAPI; Invitrogen) for 15 min at room temperature. Sperm were washed thrice with PBS-BSA and then smeared on glass slides. Immunofluorescent localization of LDH-C in rat sperm was performed using confocal scanning laser microscopy (Fluoview-FV1000; Olympus, Tokyo, Japan).

Statistical analysis: The data were analysed with Analysis Of Variance (ANOVA) and Fisher's Protected Least-Significant Difference (PLSD) post hoc test performed using StatView (Abacus Concepts, Berkeley, CA, USA). All data are expressed as mean ± SEM. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Lactate is utilized by sperm of several species, including rats, as a suitable substrate for maintaining energy production and consumption as well as oxygen consumption[2-4,12]. However, the major biochemical reaction-glycolysis or oxidative phosphorylation-that generates the energy required for sperm mobilization in various species remains unknown because the energy-production pathway is species specific[13]. Here, we examined the effects of various concentrations of 2DG and OX in lactate-containing and lactate-free raffinose-mKRB-egg yolk medium on sperm motility characteristics and oxygen consumption. Results shown that low concentrations of 2DG (1 and 2 mM) did not significantly affect motility, straight-line velocity and oxygen consumption of after collected sperm extended

in the lactate-containing medium (Fig. 1a, c and e). While sperm motility and oxygen consumption were significantly ($p<0.05$) inhibited by even 1mM 2DG in sperm extended in lactate-free medium (Fig. 1b and f). Sperm motility significantly ($p<0.05$) inhibited in the case of sperm extended in lactate-containing and free-medium with 1 mM OX (Fig. 2a and b). As shown in Fig. 2d, dilution of the sperm suspension with lactate-free medium supplemented with 3 mM OX resulted in a significant ($p<0.05$) increased VSL as compared to the dilution of the sperm suspension without OX. Figure 2e shows that high concentrations of OX (3 mM) significantly ($p<0.05$) inhibited oxygen consumption after collected sperm extended in the lactate-containing medium. Low concentrations of OX (1 mM) caused similar inhibition of oxygen consumption of sperm extended in the absence of lactate medium (Fig. 2f).
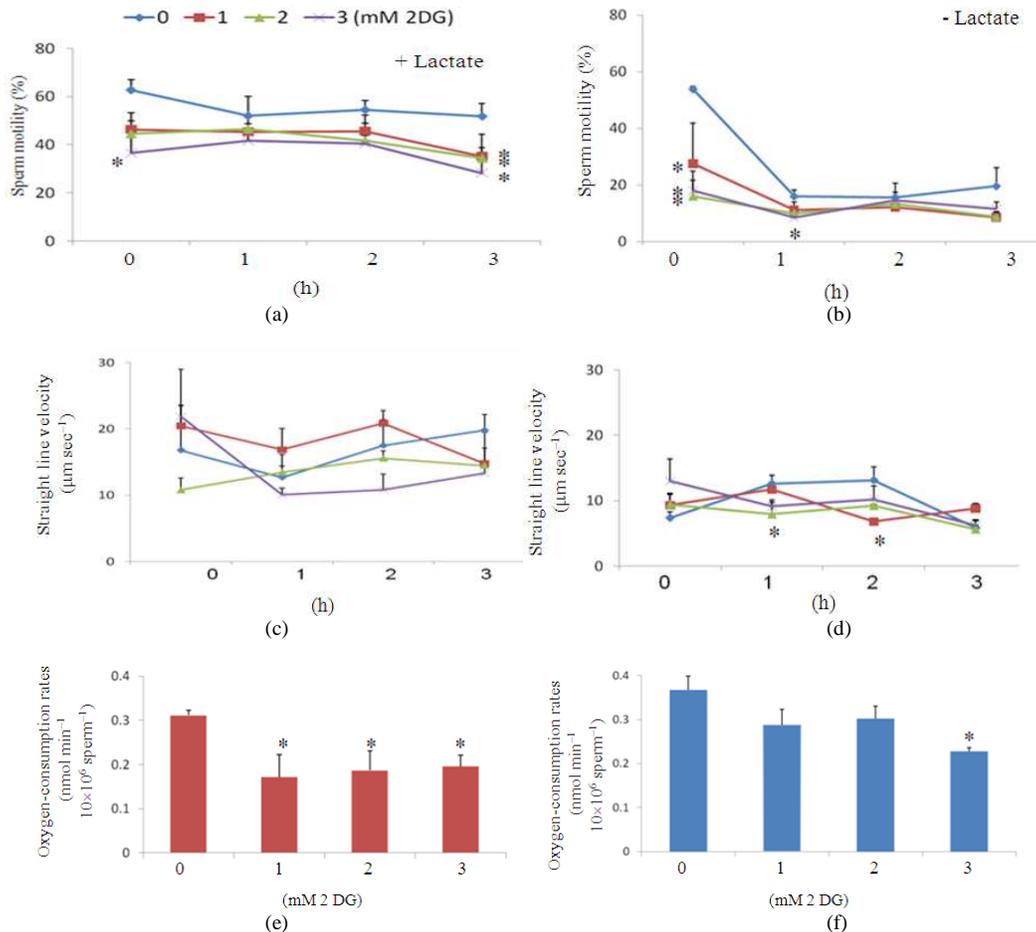

Fig. 1: Effect of 2-deoxy-D-Glucose (2DG) in lactate-containing or lactate-free medium on sperm motility (a, b) and straight-line velocity (c, d) after incubation for 3 h at 37°C. Oxygen consumption of sperm during incubation at 37°C for 5 min (e, f). Values are represented as mean ± SEM (n = 3 or 5). The asterisk (*) indicates significant differences in comparison to the control.
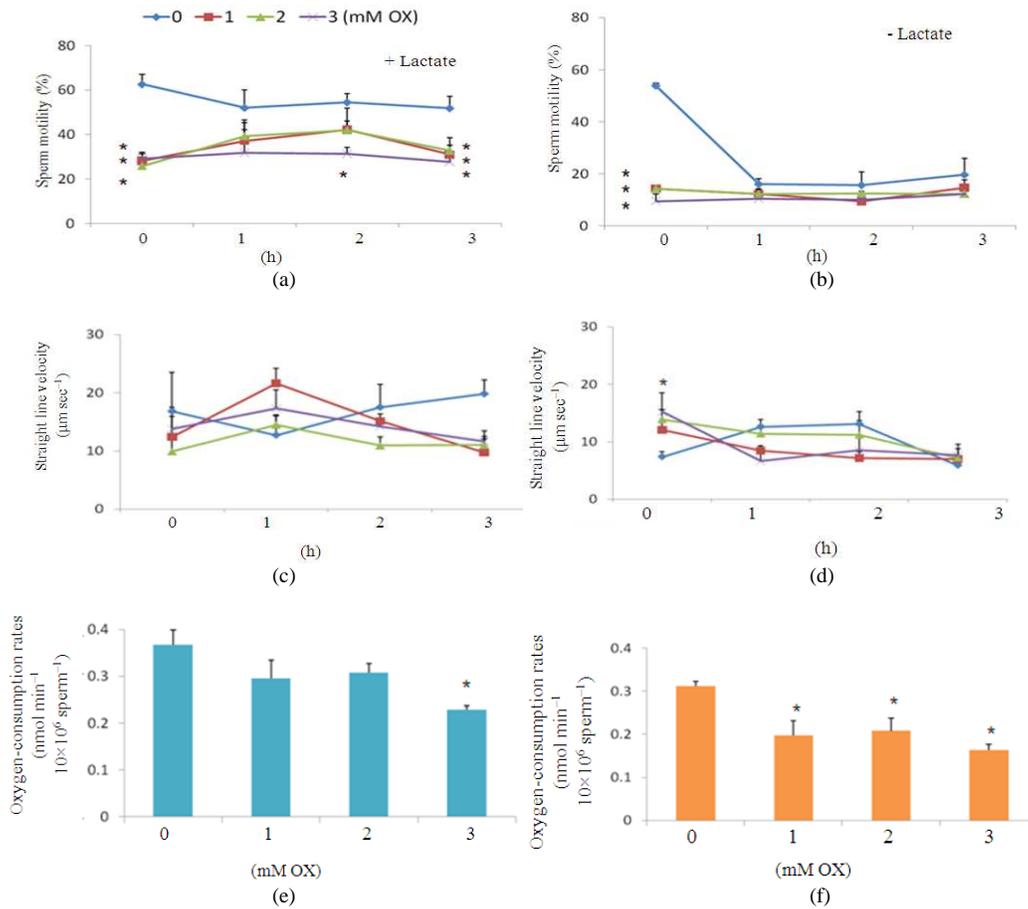
Fig. 2: Effect of sodium Oxamate (OX) in lactate-containing or lactate-free medium on sperm motility (a, b) and straight-line velocity (c, d) after incubation for 3 h at 37°C. Oxygen consumption of sperm during incubation at 37°C for 5 min (e, f). Values are represented as mean ± SEM (n = 3 or 5). The asterisk (*) indicates significant differences in comparison to the control.

We also found that sperm motility and VSL were not maintained for 3 h in the absence of lactate (Fig. 1b and d and Fig. 2b and d). These results suggesting the exogenous lactate may be utilized as oxidative substrate in mitochondria for ATP production to support mobilization of rat epididymal sperm.

Interestingly, when sperm were extended into medium with lactate (containing glucose and pyruvate), motility patterns had a particularly rigid wave form (Fig. 3a). In contrast, when the sperm extended in lactate-omitted medium, some of the sperm in which waveform of flagellar was low amplitude curve (Fig. 3b). Moreover, when sperm were incubated at 37°C for 3 h in the lactate containing solution, the pattern and vigor of the sperm that the anterior region of the sperm formed a high amplitude curve, straightened and the high-

amplitude bend of the anterior portion of the flagellum were observed (Fig. 3c). Taken together, this thought is in concern with the different regulatory of systems operating the motility patterns by different energy-yielding substrates, suggesting new possibilities for energy utilizing and translocation mechanisms that regulate the movement patterns of rat sperm (Fig. 4). If the rat sperm can be controlled flagellar movement in a way that changed by energy-yielding substrates such as lactate, it would be possible to enhance their fertilizing ability of rat sperm for *in vitro* fertilization by using a both of the fresh and frozen-thawed sperm. However, there is a lack of information for physiological trigger regarding how rat sperm switches on their flagellar movement at the real-time of fertilization, such as "hyper-activation" or "ultra-activation".
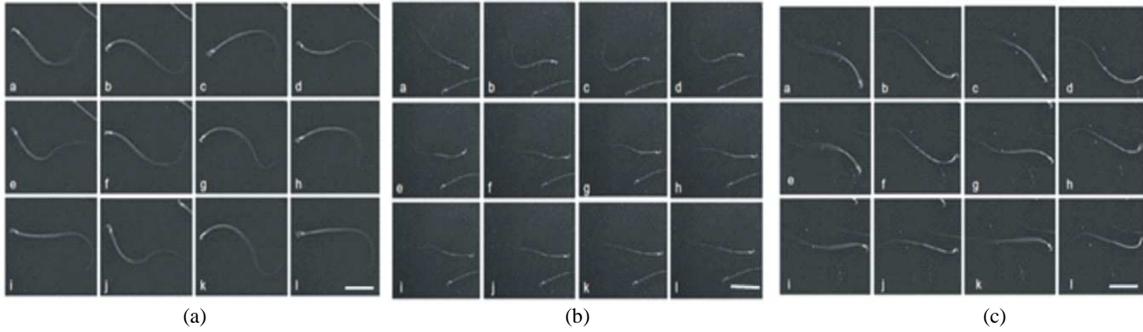
(a)　　　　　　　　　　　(b)　　　　　　　　　　　(c)

Fig. 3: Representative patterns of rat sperm movements which were extended in the presence (a) or absence (b) of lactate in medium. In C, sperm was incubated at 37°C for 3 h in the lactate containing solution. Panels a to l indicate the turn. Scale bar = 50 μm
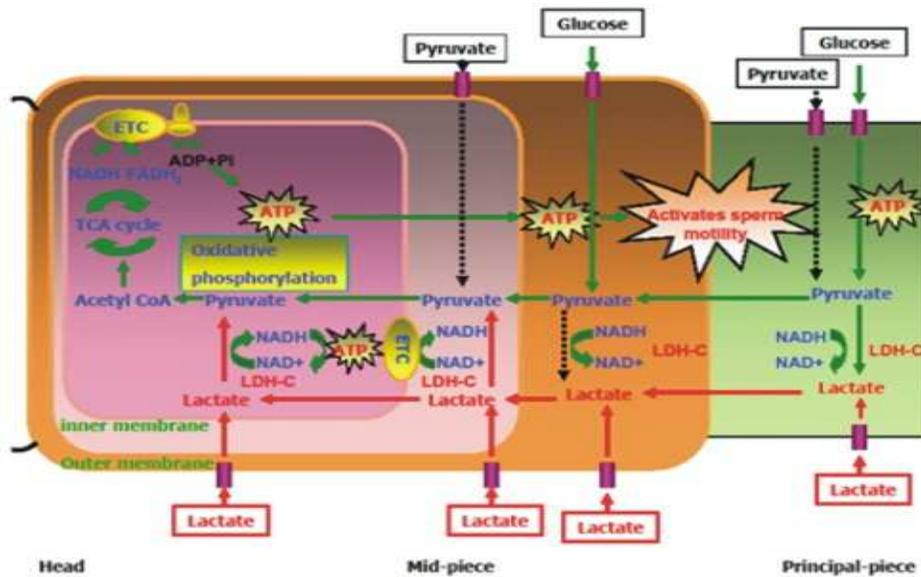

Fig. 4: Hypothesis of lactate-transport system for energy production and translocation in the rat sperm.
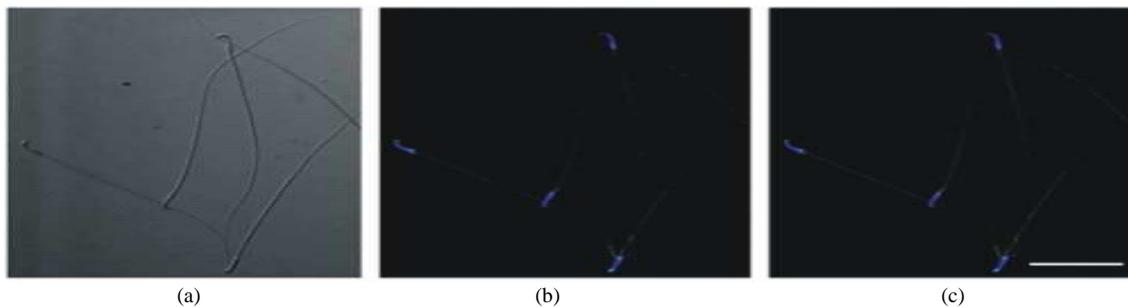

(a)　　　　　　　　　　　(b)　　　　　　　　　　　(c)

Fig. 5: Immunofluorescent localization of lactate dehydrogenase C (LDH-C). Representative micrographs of immunohistochemistry for LDH-C (green), MitoTracker (red) and chromosome (blue). (a) Phase-contrast image, (b) 4′,6′-diamino-2′-phenylindole (DAPI) + fluorescein isothiocyanate (FITC) and (c) DAPI + FITC + MitoTracker. Scale bar = 50 μm

Further, immunofluorescence study revealed that mainly LDH-C was may be localized in the intramitochondrial region of the midpiece of rat sperm (Fig. 5). Thus, exogenous lactate enhances lactate oxidation by LDH-C, thereby promoting mitochondrial oxidative reactions in the midpiece and maintaining the mobilization of rat epididymal sperm.

CONCLUSION

Both glycolysis and oxidative phosphorylation for the supply of energy required for the mobilization of rat sperm. Although exogenous lactate can be used by sperm as an essential substrate to maintain highly regulated metabolic capacities and contributes to the mobilization of rat epididymal sperm.

ACKNOWLEDGMENT

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