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Original Research Article

Pyruvate enhances stallion sperm function in high glucose media improving overall metabolic efficiency

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ABSTRACT

If a mechanism of more efficient glycolysis depending on pyruvate is present in stallion spermatozoa, detrimental effects of higher glucose concentrations that are common in current commercial extenders could be counteracted. To test this hypothesis, spermatozoa were incubated in a 67 mM Glucose modified Tyrode's media in the presence of 1- or 10-mM pyruvate and in the Tyrode's basal media which contains 5 mM glucose. Spermatozoa incubated for 3 h at 37 °C in 67 mM Tyrode's media with 10 mM pyruvate showed increased motility in comparison with aliquots incubated in Tyrode's 5 mM glucose and Tyrode's 67 mM glucose (57.1 ± 3.5 and 58.1 ± 1.9 to 73.0 ± 1.1 %; $P < 0.01$). Spermatozoa incubated in Tyrode's with 67 mM glucose 10 mM pyruvate maintained the viability along the incubation (64.03 ± 15.4 vs 61.3 ± 10.2), while spermatozoa incubated in 67 mM Glucose-Tyrode's showed a decrease in viability (38.01 ± 11.2, $P < 0.01$). 40 mM oxamate, an inhibitor of the lactate dehydrogenase LDH, reduced sperm viability ($P < 0.05$, from 76 ± 5 in 67 mM Glucose/10 mM pyruvate to 68.0 ± 4.3 %, $P < 0.05$). Apoptotic markers increased in the presence of oxamate. ($P < 0.01$). UHPLC/MS/MS showed that 10 mM pyruvate increased pyruvate, lactate, ATP and NAD^+ while phosphoenolpyruvate decreased. The mechanisms that explain the improvement of in presence of 10 mM pyruvate involve the conversion of lactate to pyruvate and increased NAD^+ enhancing the efficiency of the glycolysis.

1. Introduction

The energetic metabolism comprises the oxidation of high-energy compounds, i.e. glucose to CO_2. The electrons released in these reactions are accepted by molecular oxygen, generating H_2O. Metabolism is then coupled to reactions of oxidation-reduction, in which the NAD(P)/NAD(P)H redox pairs play a major role [1]. Moreover, dysregulation of metabolism may disrupt the redox homeostasis [2].

The stallion spermatozoa are highly dependent on oxidative phosphorylation in the mitochondria [3,4]. Despite this, most current commercial extenders contain high concentrations of glucose. Excess of this hexose may increase the amounts of 2-oxoaldehydes such as glyoxal (G) and methylglyoxal (MG). Glycolysis is not a perfect process and includes a series of steps for elimination of phosphates from the trioses phosphates glyceraldehyde 3-phosphate and dihydroxyacetone phosphate [5]. During this process, glyoxal (G) and methylglyoxal (MG) are produced continuously, and their production is proportional to the concentration of glucose present [6–8]. These compounds due to their adjacent oxoaldehyde groups are electrophilic compounds able to oxidize lipids, DNA, and proteins [9,10]. Glucose is metabolized to pyruvate in the glycolysis, then pyruvate is incorporated into the mitochondrial matrix where is transformed into Acetyl- CoA entering the Krebs Cycle. Alternatively, after phosphorylation to glucose 6-phosphate, glucose may enter the pentose phosphate pathway producing reducing power in the form of NADPH. In the glycolysis for each mole of glucose, the enzyme glyceraldehyde 3 phosphate dehydrogenase (GAPDH) reduces 2 mol of NAD^+ to NADH, then NAD^+ is recycled back to NAD^+ in the mitochondria [11]. However, under some circumstances, mitochondrial respiration may not be sufficient to maintain enough NAD^+ for efficient glycolysis, and alternative mechanisms may be

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present. In this case, NAD^+ is regenerated by the reduction of pyruvate to lactate, by the enzyme lactate dehydrogenase (LDH). This reaction may occur under high oxygen concentration and is termed aerobic glycolysis or Warburg effect [12].

Substrates for the Tricarboxylic acid cycle, like lactate and pyruvate, are efficiently utilized by the stallion spermatozoa [13,14]; moreover, Gibb et al. [14] demonstrated that the optimal concentration of pyruvate in a modified Biggers, Whitten, and Whittingham (BWW) medium was 10 mM. In a recent study from our laboratory, 10 mM pyruvate in a media with only 1 mM glucose is more effective in maintaining stallion sperm function for 48 h than widely used commercial extenders [10]. Pyruvate entry into the mitochondrial matrix is a critical step for the efficient generation of reducing equivalents and ATP and for the biosynthesis of fatty acids and amino acids [15]. Moreover, is becoming clear that spermatozoa exhibit metabolic flexibility, defined as the use of alternative energy sources, and metabolic plasticity defined as the use of the same metabolite for different purposes [16].

Improvements in sperm function using metabolites readily metabolizable through the TCA have been attributed to their ability to readily feed the electron transport chain (ETC) in the mitochondria; 8 NADH and 2 $FADH_2$ are produced in the TCA cycle [17]. However, noncanonical mechanisms may also explain improvements in sperm functions. For example, the direct use of lactate in the TCA [18], or a more efficient glycolysis [19]; in both mechanisms lactate dehydrogenase (LDH) plays a major role. If a mechanism for more efficient glycolysis depending on pyruvate is present in stallion spermatozoa, detrimental effects of higher glucose concentrations, widely used in commercial extenders, could be counteracted by increasing pyruvate concentration in the media, and thus may substantially improve current sperm conservation procedures. To test this hypothesis stallion spermatozoa were incubated in high glucose concentration (67 mM) media (the concentration of a widely used commercial extender) [20] in the presence of 1- or 10-mM pyruvate also using Tyrode's basal media as control; if our hypothesis is correct improved functionality will be observed in high pyruvate media, furthermore, functional, and metabolic approaches were used to disclose the potential mechanisms behind this improvement.

2. Material and methods

2.1. Reagents and media

Oxamate, 6- aminonicotinamide, Monochlorobimane (MCB), and all other chemicals were purchased from Sigma Aldrich (Madrid, Spain). All other reagents for flow cytometry were purchased from Thermofisher (Carlsbad, Ca USA). ViaKrome 808 Fixable Viability Dye and DRAQ7 were purchased from Beckman Coulter (Indianapolis, IN USA). Ultra-pure deionized water (>18.2MΩ·cm) was produced from a Millipore Milli-Q Gradient system (Millipore, Bedford, MA, USA).

2.2. Semen collection and processing

Semen was collected from four fertile stallions maintained according to institutional and European animal care regulations (Law 6/2913 June 11th and European Directive 2010/63/EU). All procedures used in this study received approbation from the ethical committee of the University of Extremadura. Ejaculates were collected using a pre-warmed, lubricated Missouri model artificial vagina following standard veterinary practices. After collection, the ejaculate was immediately evaluated and processed in the adjacent laboratory. Colloidal centrifugation [21] was performed to remove dead spermatozoa and contaminating particles from the ejaculate. The pellet was re-extended to a final concentration of 20 $x10^6$ spermatozoa/ml in Tyrode's media (96 mM NaCl, 3.1 mM KCl, 2 mM $CaCl_2$.2H2O, 0.4 mM $MgSO_4.7H_2O$, 0.3 mM KH_2PO_4, 20 mM HEPES, 5 mM glucose, 21.7 mM NaLactate, 1 mM Na Pyruvate, 15 mM $NaHCO_3$, 3 mg/mL BSA, 0.05 g/L penicillin and 0.05 g/L dihydrostreptomycin sulphate) pH 7.4 [22]. Variations of this media with 67 mM glucose, or 67 mM glucose and 10 mM Pyruvate media were also used. All media maintained the same pH and osmolarity.

2.3. Experimental design

2.3.1. - Role of pyruvate improving sperm functionality in high glucose media

Ejaculates from 4 different fertile stallions routinely used as semen donors in our center were used. After collection and processing the ejaculate was split into three aliquots: Semen extended in Tyrode's basal media served as control, the second aliquot in modified Tyrode's containing 67 mM glucose (HG), and the third aliquot in modified Tyrode's containing 67 mM glucose and 10 mM pyruvate (HGHP). Sperm function was evaluated after 3 h of incubation at 37 °C using computer-assisted sperm analysis (CASA), flow cytometry, and metabolomics.

2.3.2. Role of glucose 6 phosphate dehydrogenase (G6PD) on the functionality of stallion spermatozoa

To determine the role of the pentose phosphate pathway in the functionality of spermatozoa incubated in high glucose-high pyruvate media. Aliquots were incubated up to 3 h at 37 °C in the presence of the glucose 6 phosphate dehydrogenase (6GDP) inhibitor, 6- aminonicotinamide using previously published minimal effective concentrations in mammalian spermatozoa (200 μM) [23]. Sperm function was evaluated after 3 h of incubation at 37 °C using computer-assisted sperm analysis (CASA) and flow cytometry.

2.3.3. Role of lactate dehydrogenase (LDH)on the functionality of stallion spermatozoa

To study the role of the lactate dehydrogenase (LDH) in stallion spermatozoa incubated under high glucose, and high pyruvate, aliquots were incubated for 3 h at 37 °C in the presence of the LDH inhibitor oxamate at previously used concentrations in mammalian spermatozoa (40 mM) [24]. Functional and metabolic studies were conducted. Sperm function was evaluated after 3 h of incubation at 37 °C using computer-assisted sperm analysis (CASA) and flow cytometry.

2.4. Computer-assisted Sperm analysis (CASA)

Sperm motility and velocity were assessed using a Computer-Assisted Sperm Analysis (CASA) system (ISAS Proiser, Valencia, Spain) according to standard protocols used at our center [25]. Semen samples were loaded in a Leja® chamber with a depth of 20 μm (Leja, Amsterdam, The Netherlands) and placed on a stage warmed at 37 °C. Analysis was based on an evaluation of 60 consecutive digitized images, frame rate 60Hz, obtained using a 10x negative phase-contrast objective (Olympus CX 41). At least 500 spermatozoa per sample were analyzed in random fields. Spermatozoa VAP >35 μm/s were considered motile. Spermatozoa deviating <45% from a straight line were classified as linearly motile. Other parameters studied included curvilinear velocity (VCL μm/s) defined as the timeline average velocity of a sperm head along its actual trajectory, the straight-line velocity (VSL μm/s), the velocity calculated along a straight line between the first and last points of the path and velocity along the average path (VAP μm/s) as the time-averaged velocity calculated along the average path.

2.5. Flow cytometry

Flow cytometry (FC) analyses were conducted using a Cytoflex ® LX equipped with ultraviolet, violet, blue, yellow, red, and infrared lasers. The instrument was calibrated daily using specific calibration beads provided by the manufacturer. A compensation overlap was performed before each experiment. Files were exported as FCS files and analyzed using Flowjo™ V10.8.1 Software (Ashland, OR, USA) and Cytobank™ (https://premium.cytobank.org/cytobank/login). Unstained, single-stained, and Fluorescence Minus One (FMO) controls, were used to

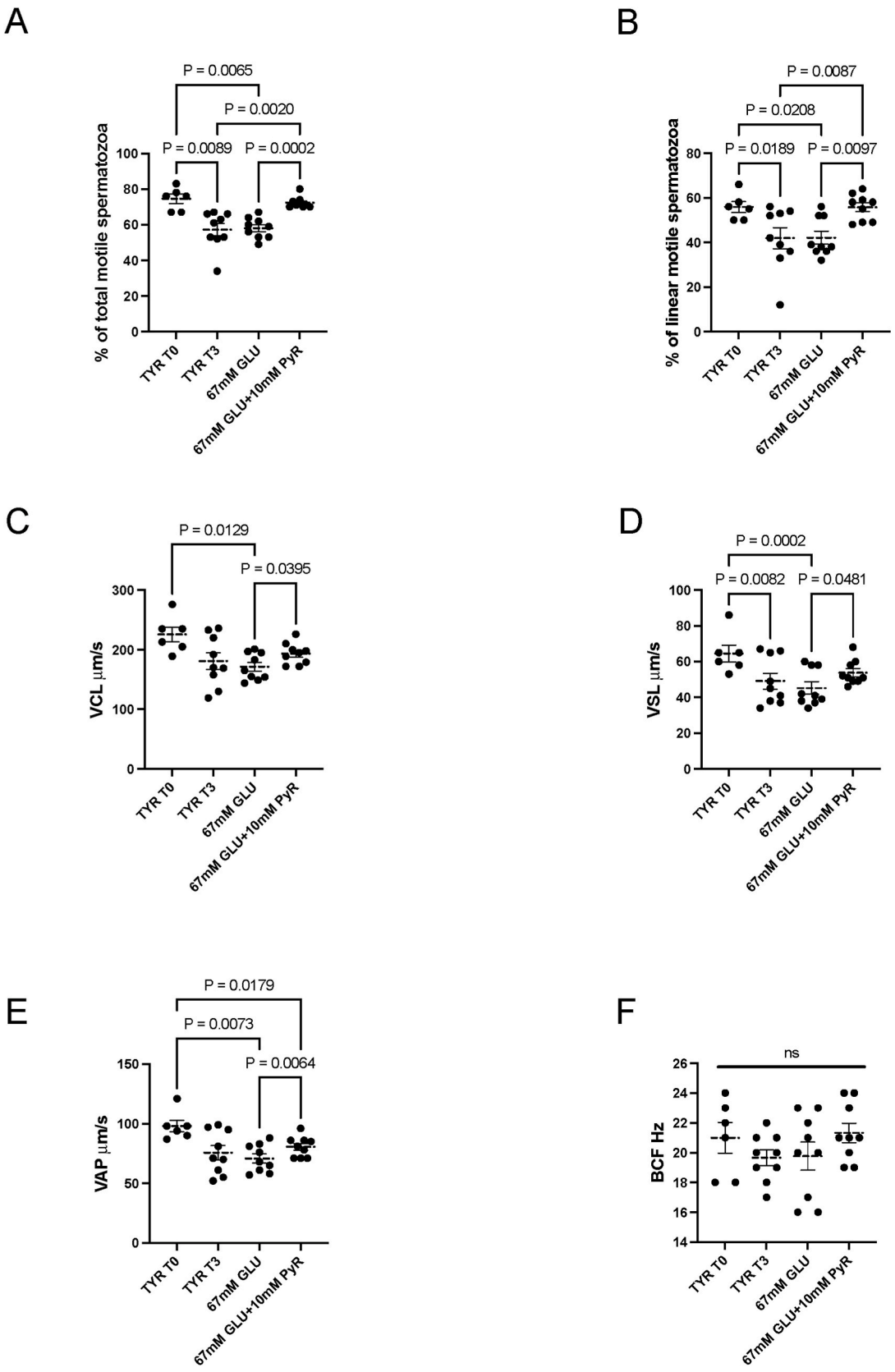

Fig. 1. Motility and velocities of stallion spermatozoa incubated for 3 h at 37 °C in different media. Ejaculates were processed as described in material and methods, and split in different media, basal Tyrode's, a 67 mM glucose Tyrode's and 67 mM Tyrode's, 10 mM pyruvate Tyrode's. Computer-assisted sperm analysis was conducted at the beginning and after 3 h of incubation at 37 °C. A) Percentage of total motile spermatozoa, B) Percentage of linear motile spermatozoa, C) Circular velocity (VCL; µm/s), D) Straight line velocity (VSL; µm/s), E) Average path velocity (VAP; µm/s) F) Beat cross frequency (BCF; Hz). Data are expressed as means ± SEM and are derived from 3 independent experiments (3 stallions 3 replicates each, n = 9).

determine compensations and positive and negative events, as well as to set regions of interest as described in previous publications by our laboratory [26,27].

2.5.1. Measurement of GSH, viability, and mitochondrial membrane potential in stallion spermatozoa

Intracellular GSH was measured by adapting previously published protocols optimized for GSH detection using flow cytometry [28] and adapted to equine spermatozoa in our laboratory [29]. This assay is based in the use of MCB for a short, 10 min incubation period, that allows the preferent staining of GSH without interference from other intracellular thiols. The mitochondrial membrane potential and sperm viability were also simultaneously assessed. In brief, sperm aliquots (1-5 x 10^6 sperm/mL) were stained with JC-1 1 μM, (30 min in the dark at r. t.), DRAQ7 3 μM, and monochlorobimane (MCB) 10 μM (10 min in the dark at r.t). After assessment of the quality of the flow, doublets and debris were gated out, MCB was detected at a peak excitation of 405 nm and emission of 450/45 nm BP, JC-1 was detected at a peak excitation of 511 nm and emission of 596 nm (aggregates) and DRAQ7, at a peak excitation of 640, and emission of 690 nm. JC-1 and MCB fluorescences were recorded and analyzed as relative fluorescence units (R.F.U.)

2.5.2. Assessment of caspase 3 activity and phosphatidylserine (PS) translocation

Annexin V 647 conjugated and CellEvent® Caspase 3/7 Green Detection Reagent were combined in a multiparametric test and evaluated by FC [30]. The caspase 3 assay has been previously validated in our laboratory through western blotting and after treatment with known apoptotic inhibitors, staurosporine, thapsigargin and betulinic acid [31]. This assay consists of a four amino acid peptide (DEVD) conjugated to a nucleic acid binding dye. The activation of caspase 3 and caspase 7 proteins enables them to cleave the caspase 3/7 recognition sequence that is encoded in the DEVD peptide. Cleavage of the recognition sequence and binding of the DNA by the reagent labels apoptotic cells [32]. Samples were loaded with Hoechst 33342 (0.3 μM) and CellEvent® (2 μM) and incubated at room temperature for 15 min. Following this, the samples were washed by a short centrifugation spin for 12" and suspended in 200 μl of annexin binding-buffer (solution in 10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Five μL of Annexin V were added to 200 μL of sample. After 15 min of incubation in the dark at room temperature, 400 μL of 1 × Annexin binding buffer was added before reading in the flow cytometer. To gate dead spermatozoa samples were stained with Viakrome 808 (2.5 μL of the reconstructed solution as indicated by the manufacturer). Samples were evaluated in a Cytoflex LX® flow cytometer, (Beckman Coulter). CellEvent® staining was validated as previously described [31].

2.5.3. Glucose incorporation assay

2-NBDG (2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-il)amino)-2-desoxiglucose), a fluorescent glucose analog, was used to monitor glucose incorporation in the spermatozoa under different conditions. Stallion spermatozoa were washed resuspended in PBS and loaded with 2-NBDG 20 μM and incubated at 37 °C for 30 min in the dark in PBS, spermatozoa were washed and stained with ethidium homodimer (Eth-1) to exclude dead cells from the analysis. 2-NDBG was monitored flow cytometrically at emission and excitation maxima of 465/540 nm. Cytochalasin B 20 μM inhibits glucose transport and is used as the negative control.

2.5.4. -Metabolomics

Samples were washed in PBS (600g x 10'), then the pellet formed by the spermatozoa was frozen immediately in liquid nitrogen and kept frozen at −80 °C until analysis. The sperm pellet was then re-extended in 300 mL of Milli-Q water and sonicated for 3 s. Immediately afterward, it was centrifuged at 6452 g at 4 °C for 3 min and the supernatant was injected into the UHPLC-MS/MS.

2.5.4.1. Analysis of nucleosides, nucleotides, amino acids, sugars and organic acids by UHPLC-MS QqQ.

These metabolites were analyzed by UHPLC/MS system consisting of an Agilent 1290 Infinity II Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an automated multisampler module and a High-Speed Binary Pump, coupled to an Agilent 6470 QqQ Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) interface. The HPLC and QqQ detector were controlled using MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.08.00). The sample was injected onto an Agilent HILIC-Z HPLC column (4.6 mm, 100 mm 2.1 μm, Agilent Technologies), thermostatted at 35 °C, at a flow rate of 0.4 ml/min. The injection volume was 7 μL. For gradient elution, solvent A was: 10 mM ammonium acetate at a pH 9 in Milli Q water and solvent B was: 10 mM ammonium acetate at a pH 9 in Milli Q water: acetonitrile 10:90. To start with 98 % solvent B was maintained until 5 min. Solvent B was then decreased from 98 to 60 % from 5 to 10 min and held at 60% for an additional 2 min. Then solvent B returned to the initial conditions up to 15 min. The mass spectrometer was operated in negative mode and was run in MS/MS mode. Nitrogen was used as a nebulizing gas, drying gas, sheath gas and collision gas. The nebulizer gas pressure was set to 35 psi, whereas the drying gas flow was set to 12 l/min at a temperature of 250 °C, and the sheath gas flow was set to 15 l/min at a temperature of 350 °C. The capillary spray and fragmentor voltages were 3500 V and 100 V respectively. The MRM conditions were optimized by injecting a standard solution of each metabolite at different collision energy (CE). Data processing and analysis were performed using the MassHunter Quantitative Analysis Software (Rev B.07.00.201, Agilent Technologies, Santa Clara, CA, USA).

2.6. Statistical analysis

All experiments were repeated three times with independent samples. The normality of the data was assessed using the Kolmogorov-Smirnoff test. Paired t-tests and One-way ANOVA followed by Dunnett's multiple comparisons test and were performed using GraphPad Prism version 7.00 for Mac, La Jolla California USA, www.graphpad.com. Differences were considered significant when $P < 0.05$. Results are displayed as means ± SEM. FlowSOM clustering analysis was performed in Cytobank® (Beckman Coulter) as described in Ref. [33].

3. Results

3.1. High pyruvate improves sperm motility and velocities

Stallion spermatozoa incubated in 67 mM Tyrode's media supplemented with 10 mM pyruvate showed increased motility in comparison with aliquots of the same ejaculate incubated in Tyrodes 5 mM glucose and Tyrode's 67 mM glucose. From 57.1 ± 3.5 and 58.1 ± 1.9 to 73.0 ± 1.1 % ($P = 0.0020$ and $P < 0.0002$; Fig. 1A), this was also observed in the percentages of linear motile spermatozoa (Fig. 1B; $P = 0.0087$ and $P = 0.0097$). The addition of 10 mM Pyruvate to the media with 67 mM Glucose maintained the percentage of total and linear motile spermatozoa, while spermatozoa incubated in Tyrode's media and the media containing only 67 mM glucose experienced significant reductions in the percentages of total ($P = 0.0089$ and $P = 0.0065$) and linear motile spermatozoa ($P = 0.0189$ and $P = 0.0208$) after 3 h of incubation at 37 °C. The addition of pyruvate to the 67 mM glucose media resulted in significant improvements of all sperm velocities (Fig. 1C–E), VCL was 171.4 ± 14.2 μM/s in the 67 mM glucose media and 193.4 ± 17.5 μM/s in the 67 mM G 10 mM pyruvate ($P = 0.0395$; Fig. 1C), the VSL and VAP followed a similar trend (Fig. 1D–E), and no changes were observed in the BCF (Fig. 1F).

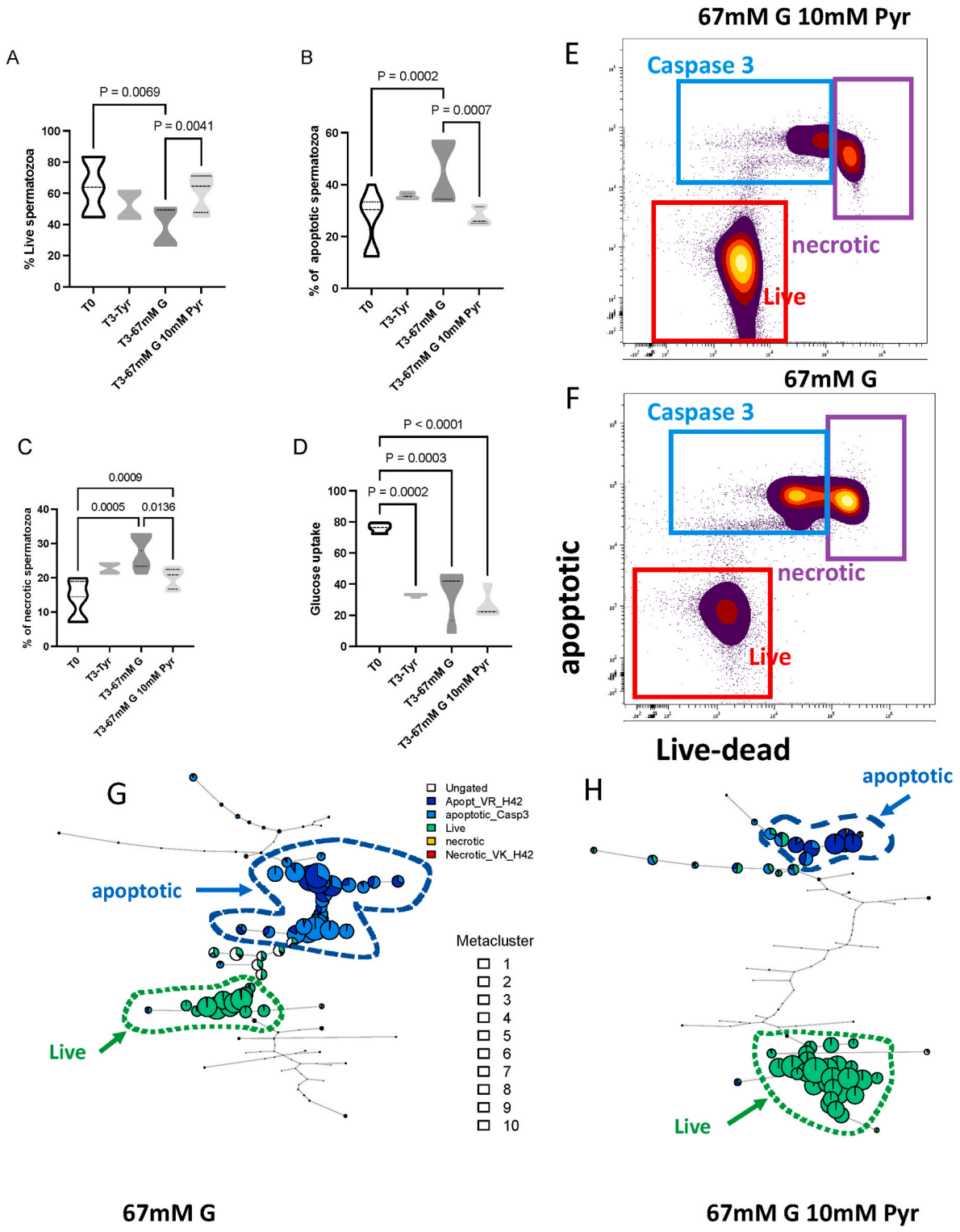

Fig. 2. Effect of high pyruvate on the viability and glucose uptake of stallion spermatozoa incubated at 37 °C for 3 h. Stallion ejaculates were processed as indicated in material and methods, and at times 0 and 3 h of incubation samples were analyzed by FC for viability and apoptotic changes (caspase 3 and Annexin-V). Moreover, glucose uptake was determined using the fluorescent glucose analogue 2-NBDG. A) Changes in the percentage of viable spermatozoa. B) Apoptotic (Caspase 3+) spermatozoa. C) Percentage of necrotic spermatozoa. D) Incorporation of glucose, 2-NBDG fluorescence intensity (RFU). E and F) Representative cytograms (2D density plots) E) Stallion spermatozoa incubated in the 67 mM Glucose, 10 mM pyruvate media. F) Stallion spermatozoa incubated in the 67 mM Glucose media. G and H) Representative FlowSOM clustering analysis performed in Cytobank, G) Stallion spermatozoa incubated in the 67 mM Glucose media H) Stallion spermatozoa incubated in the 67 mM Glucose, 10 mM pyruvate media. Data are expressed as means ± SEM and are derived from 12 independent experiments (4 stallions 3 replicates each n = 12.

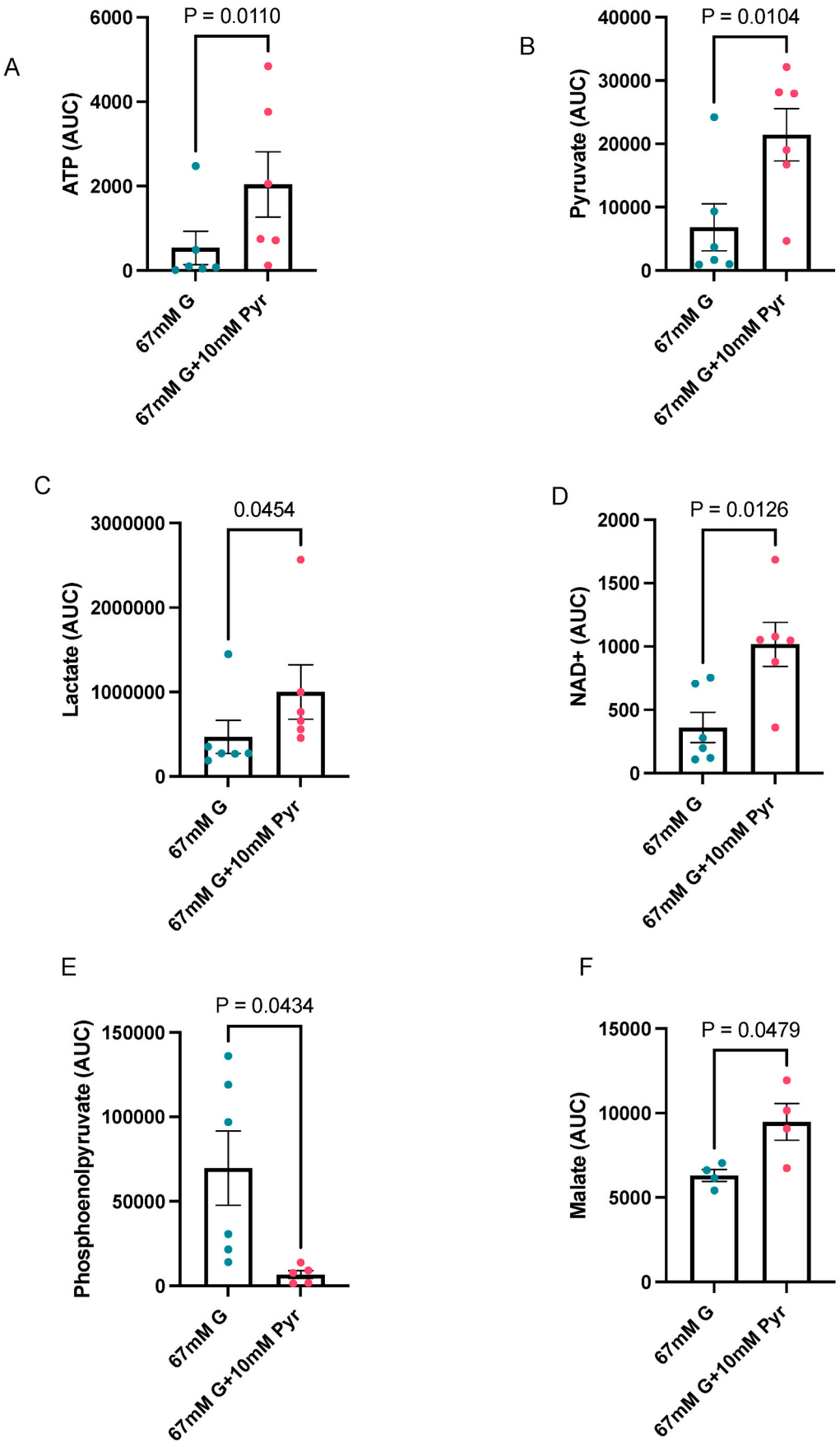

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Fig. 3. Metabolic study of stallion spermatozoa incubated in different media High glucose 67 mM (HG) and and High Glucose (67 mM) High Pyruvate (10 mM). A) Changes in the relative amounts of ATP in stallion spermatozoa B) Changes in the relative amounts of Pyruvate, C) Changes in the relative amounts of Lactate D) Changes in the relative of NAD + E) Changes in the relative amounts of phosphoenolpyruvate F) Changes in the relative amounts of Malate. Data are expressed as means ± SEM and are derived from 3 independent experiments (3 stallions 3 replicates each n = 9).

3.2. High pyruvate in the media maintains the viability of stallion spermatozoa

Spermatozoa incubated in Tyrode's with 67 mM glucose and 10 mM pyruvate for 3 h at 37 °C maintained viability along the incubation period (64.03 ± 5.5 vs 61.3 ± 3.6), while spermatozoa incubated in 67 mM Glucose-Tyrode's showed a decrease in viability after 3 h of incubation (64.03 ± 5.5 vs 38.01 ± 5.0, $P = 0.0069$; Fig. 2A). Accordingly, viability after the incubation period was significantly higher in the HGHP group than in the HG group (Fig. 2A; $P = 0.0041$).

3.3. High glucose in the media increases apoptosis which is prevented in the presence of high pyruvate

Spermatozoa incubated in the 67 mM Glucose Tyrode's media showed an increased percentage of apoptotic (Live-Caspase 3 positive) spermatozoa after 3 h of incubation in comparison with aliquots from the same ejaculate incubated in the 67 mM G-10 mM pyruvate media (26.9 ± 2.9 vs 13.9 ± 2.2 $P = 0.007$; Fig. 2 B).

3.4. High glucose in the media increases the percentage of necrotic spermatozoa that is reduced in the presence of high pyruvate

Incubation of stallion spermatozoa in media containing high glucose, 67 mM increased the percentage of necrotic spermatozoa, from 13.7 ± 1.7% at the beginning of the incubation period to 27.8 ± 2.1 after 3 h of incubation at 37 °C (Fig. 2; $P = 0.0005$), however, the presence of 10 mM pyruvate in the 67 mM glucose media, reduced the increase in the percentage of necrotic spermatozoa to 20.1 ± 0.9 % ($P = 0.0136$; Fig. 2C).

3.5. Glucose uptake

The ability of spermatozoa to incorporate exogenous glucose decreased along the incubation period ($P < 0.001$; Fig. 2D). No differences were observed among different media after 3 h of incubation.

3.6. Metabolomics UHPLC/MS/MS

We investigated changes in metabolites associated with glycolysis and the TCA cycle. In the spermatozoa incubated in the high pyruvate media, we observed significant increases in the relative amounts (area under the curve) in ATP (Fig. 3 A; $P = 0.0110$), NAD^+ (Fig. 3D; $P = 0.0126$), and lactate (Fig. 3C; $P = 0.0454$). Pyruvate increased in the high glucose high pyruvate media (Fig. 3 B; $P = 0.0216$) and Phosphoenolpyruvate was significantly reduced in spermatozoa incubated in the high pyruvate media (Fig. 3; $P = 0.0434$). The TCA cycle compound malate were also significantly increased in stallion spermatozoa incubated in the high glucose-high pyruvate media (Fig. 3F; $P = 0.0135$).

3.7. Role of the pentose phosphate pathway (PPP) and lactate dehydrogenase (LDH) on motility and velocities

Improvements seen with the addition of 10 mM pyruvate to the 67 mM glucose media, may involve the activation of the PPP, providing reducing power in form of NADPH and thus preventing oxidative-induced damage. Another possible route may involve the metabolization of pyruvate through LDH, providing NAD^+ to improve glycolysis. To test these hypotheses, we incubated stallion spermatozoa in the high glucose-high pyruvate media in the presence of the inhibitor of the G6PD 6-Aminonicotinamide to prevent the use of glucose through the PPP, and in the presence of the LDH inhibitor oxamate. This is a structural analog of pyruvate that inhibits LDH activity. The percentage of total motile spermatozoa was reduced in the presence of the LDH inhibitor oxamate after 3 h of incubation, from 85.7.5 ± 1.1% at the beginning of the incubation period, to 78.0 ± 1.9 in the presence of oxamate (Fig. 4A; $P = 0.0447$). The VCL increased in the presence of oxamate, concerning the initial values (Fig. 4C; $P = 0.0048$).

3.8. Role of the pentose phosphate pathway (PPP) and lactate dehydrogenase (LDH) on stallion sperm viability

Inhibition of G6PD, caused a reduced viability ($P = 0.0282$; Fig. 5A). Oxamate reduced sperm viability from 76.2 ± 1.4 % in aliquots incubated in the 67 mM G 10 mM Pyruvate to 69.6 ± 1.1 % in the presence of Oxamate ($P < 0.0001$; Fig. 5A). The percentage of necrotic spermatozoa increased in the presence of oxamate, from 23.4 ± 1.3 in the HGHP media to 30.1 ± 1.1 in the presence of oxamate (Fig. 5C; $P < 0.0001$). The percentage of apoptotic spermatozoa increased in presence of oxamate (Fig. 5B; $P = 0.0110$).

3.9. Glucose uptake

Glucose uptake was higher at the beginning of the incubation period and was reduced thereafter. Inhibition of G6PD in the high glucose high pyruvate media increased the glucose uptake ($P < 0.0001$, Fig. 5D).

3.10. Inhibition of the PPP and LDH impacted mitochondrial membrane potential and GSH content in stallion spermatozoa

Both, inhibition of the PPP and LDH impacted mitochondrial membrane potential and GSH content in stallion spermatozoa. Mitochondrial membrane potential (measured as R.F.U) was reduced in the presence of 6-Amin and Oxamate (Fig. 6 A; $P = 0.0329$ and $P = 0.0217$ respectively). Also, GSH content in the spermatozoa was reduced in the presence of oxamate (Fig. 6 B; $P < 0.0001$).

4. Discussion

We investigated whether the concentration of pyruvate interacts with a high concentration of glucose. Incubation of stallion spermatozoa for 3 h in Tyrode's media (5 mM Glucose) and 67 mM glucose Tyrode's, reduced sperm motility and kinematics, but the presence of 10 mM pyruvate in the 67 mM glucose Tyrode's, prevented the drop in motility after 3 h of incubation, maintaining the motility values observed at the beginning of the incubation period. Sperm viability also showed a similar trend, with a significant drop after 3 h of incubation at 37 °C in the Tyrode's 67 mM glucose, which was prevented in the presence of 10 mM pyruvate. While the negative effects of supraphysiological concentrations of glucose have been reported [10], the mechanisms behind the protection provided by pyruvate in high glucose media remain to be understood. We investigated mechanisms involved in this improvement; possible hypotheses may include that pyruvate improves and/or regulates the use of glucose, either through the activation of the pentose phosphate pathway providing reducing power or providing NAD^+ through the reduction of pyruvate to lactate. Other mechanisms and non-canonical metabolic pathways cannot be excluded [34,35]. The toxic effects of high glucose in the sperm storage media have been recently described, related to the excessive production of toxic oxoaldehydes, like glyoxal and methylglyoxal during glycolysis [10]. Excess

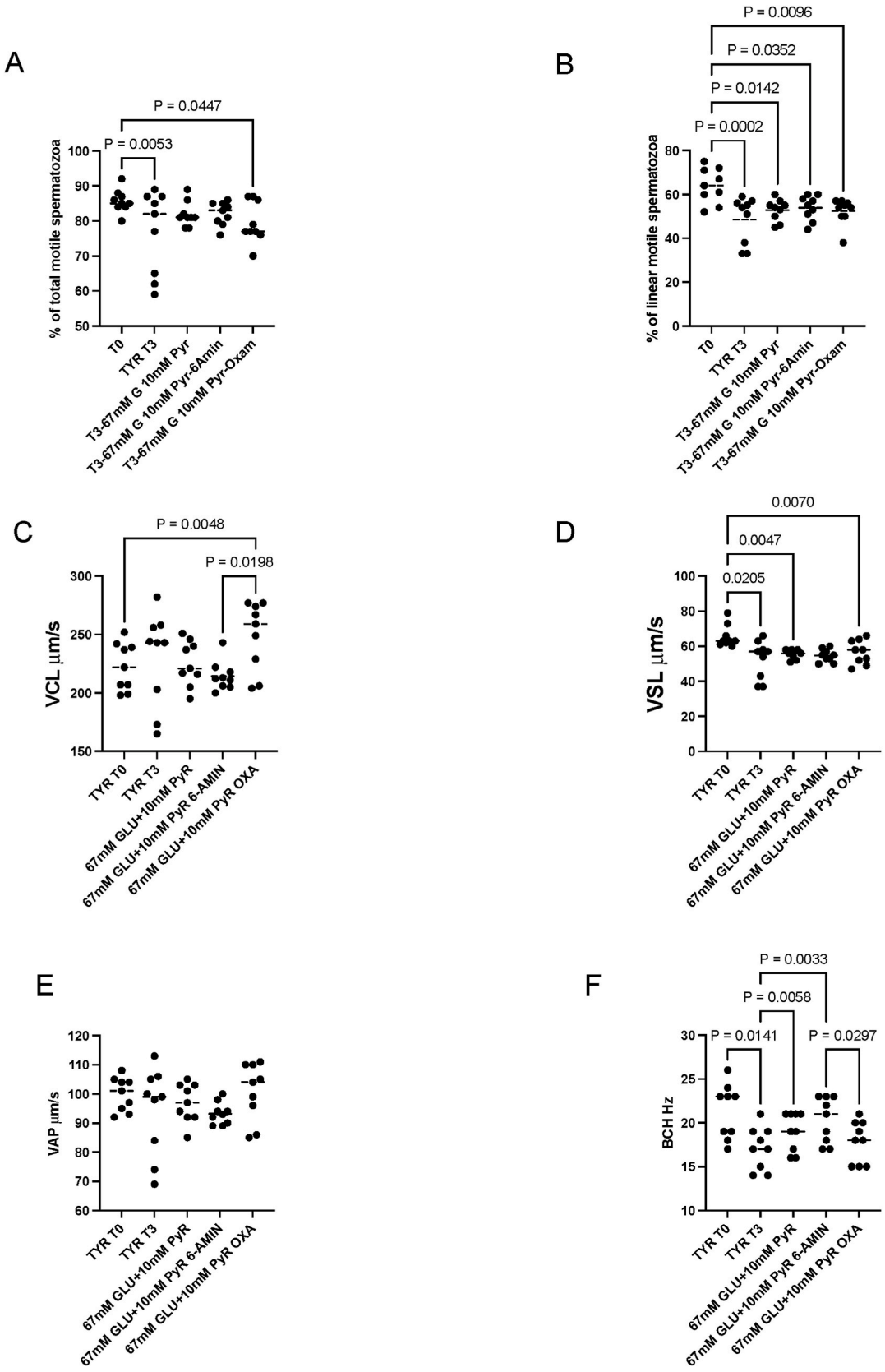

Fig. 4. Effect of inhibition of 6 phosphate dehydrogenase (6GDP) and lactate dehydrogenase (LDH) on stallion sperm motility and velocities. A) Percentage of total motile spermatozoa (CASA). B) Percentage of linear motile spermatozoa. C) Circular velocity (VCL; µm/s), D Straight line velocity (VSL; µm/s), E) Average path velocity (VAP; µm/s) F) Beat cross frequency (BCF; Hz). Data are expressed as means ± SEM and are derived from 3 independent experiments (3 stallions 3 replicates each n = 9).

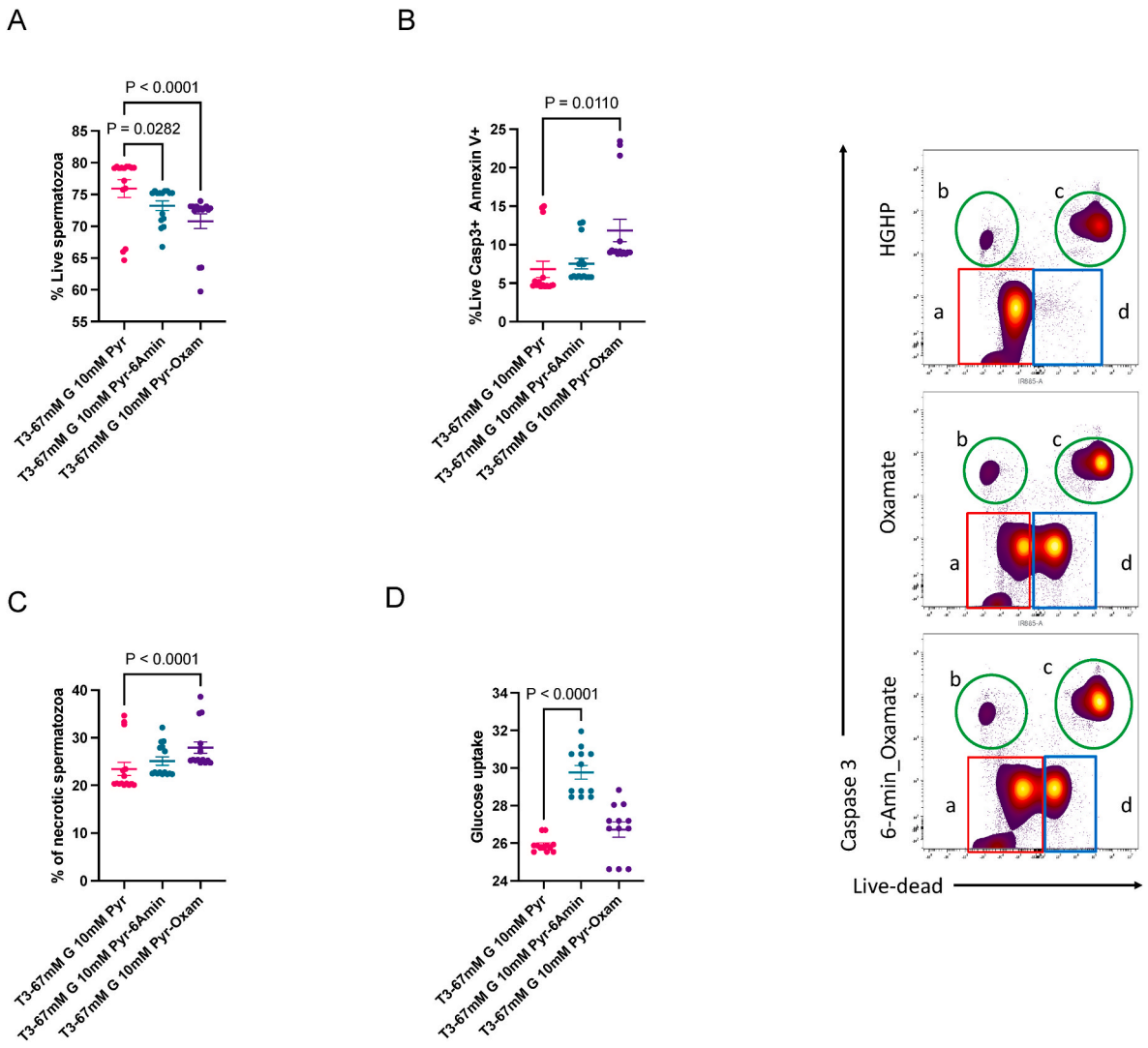

Fig. 5. Effect of inhibition of 6 phosphate dehydrogenase (6GDP) and lactate dehydrogenase (LDH) on the viability and glucose uptake of stallion spermatozoa. Stallion spermatozoa were processed as described in the material and methods and incubated in the presence of Oxamate (inhibitor of LDH) or 6- aminonicotinamide (inhibiting the glucose 6 phosphate dehydrogenase (6GDP)). A) Effect on the percentage of viable spermatozoa. B) Effect on the percentage of Caspase 3/Annexin V positive spermatozoa C) Effect on the percentage of necrotic spermatozoa. D) Effect on glucose uptake. Representative cytograms of the assay. Data are expressed as means ± SEM and are derived from 3 independent experiments (4 stallions 3 replicates each n = 9).

glucose depletes GSH and predisposes spermatozoa to experience ferroptosis [36]. To determine if in the presence of high glucose and high pyruvate, the mechanism is related to an increased activity of the pentose phosphate pathway (PPP) we inhibited the enzyme G6PD; while no major effects were seen in motility the percentage of viable spermatozoa was significantly reduced as were the percentage of spermatozoa showing a high mitochondrial membrane potential. Interestingly the incorporation of glucose was significantly increased in the presence of the PPP inhibitor. These results suggest that reducing power (NADPH) produced in the PPP has a role in maintaining sperm viability and in mitochondrial activity; the increased incorporation of glucose when the G6PD is inhibited is interpreted as a compensatory mechanism, in an attempt of the spermatozoa to maintain an adequate flux of glucose through the PPP.

Our findings also argue for the existence of additional mechanisms, probably through the regulation of NADH/NAD^+, explaining the effect of pyruvate improving sperm function in the presence of high concentrations of glucose. This mechanism may involve the reduction of pyruvate into lactate through lactate dehydrogenase (LDH). Cytosolic NAD^+ is used during the conversion of glyceraldehyde 3-phosphate to 1, 3 bisphosphoglycerate, by the glyceraldehyde 3-P dehydrogenase (GADPH) generating NADH and sustaining a continuous flux through glycolysis [37], then NAD^+ is regenerated in the ETC in the mitochondria. However, if NAD^+ requirements (i.e. under high glucose concentration) are high and surpass NAD^+ regeneration capability in the mitochondria, alternative mechanisms to oxidize NADH must be present [35]. Lactate dehydrogenase (LDH) reduces pyruvate to lactate in the cytosol, a reaction coupled with the oxidation of NADH to NAD^+. This reaction occurs in the presence of oxygen and is termed aerobic glycolysis or Warburg effect [38]. Furthermore, lactate can be incorporated in the mitochondrial matrix [39] through monocarboxylate transporters (MCT) and further oxidized to pyruvate reducing NAD^+ to NADH, feeding the TCA cycle and providing reducing equivalents to the ETC in the mitochondria [35]. Recent research is stressing the importance of pyruvate for sperm function [10,14,19,40–43] Interestingly the role of pyruvate was already proposed in the past century [43]. To test this hypothesis, we incubated aliquots of stallion spermatozoa in media with high glucose and high pyruvate and inhibited LDH with oxamate. Inhibition of LDH in the high glucose, high pyruvate media, caused a significant reduction of sperm motility, viability, and the percentage of spermatozoa with active mitochondria, findings that argue in favor of our hypothesis. Although we used a concentration of oxamate validated

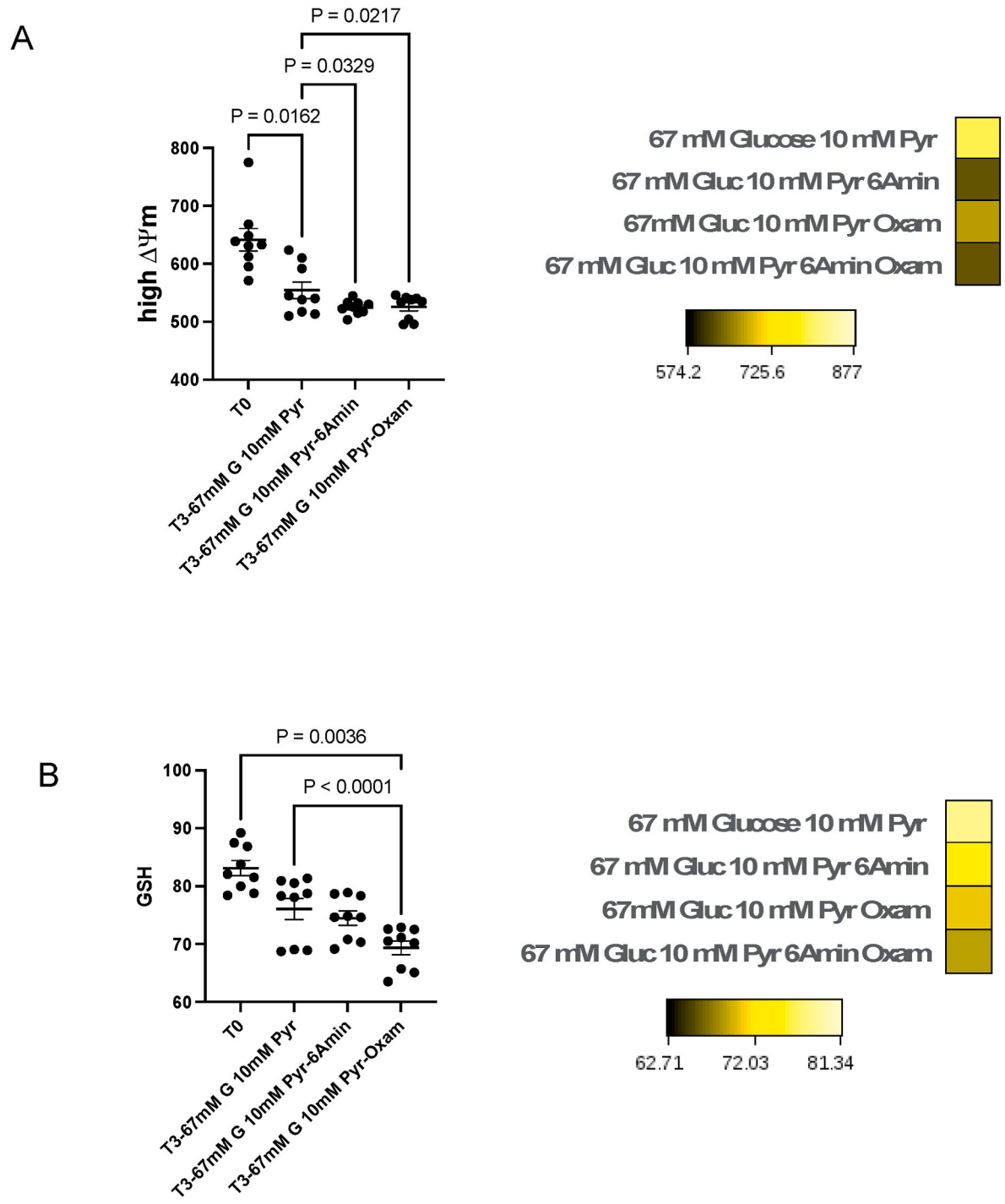

Fig. 6. Effect of inhibition of 6 phosphate dehydrogenase (6GDP) and lactate dehydrogenase (LDH) on the mitochondrial membrane potential (A) and the GSH content in the spermatozoa (B). Data are expressed as means ± SEM and are derived from 3 independent experiments (3 stallions 3 replicates each n = 9).

for mammalian spermatozoa [24], a time and concentration experiment is warranted to investigate possible stallion-specific responses to this inhibitor.

From being considered merely a waste product of metabolism, recent discoveries show that lactate is a major circulating carbohydrate fuel, and also enables the uncoupling of carbohydrate-driven mitochondrial energy metabolism from glycolysis, furthermore, lactate and pyruvate together serve as a redox buffer that equilibrates the NADH/NAD^+ ratio [34]. Pyruvate may operate through distinct mechanisms, mitochondrial oxidation of lactate to pyruvate may produce NADH and donate electrons to the electron transport chain, on the other hand, reduction of pyruvate to lactate with the concomitant production of NAD^+ from NADH may boost glycolysis in the cytosol. Pyruvate is rapidly reduced by LDH, and providing exogenous pyruvate increases the NAD^+/NADH ratio in cells [44,45]. The metabolic study conducted in stallion spermatozoa shows increased amounts of lactate and NAD^+ in spermatozoa incubated in the high glucose high pyruvate media, in addition, overall ATP content was also higher in spermatozoa incubated in high glucose high pyruvate media, showing a more efficient energetic metabolism in these cells.

The dependence of spermatozoa on the production of reducing equivalents in the form of NADPH in the PPP has been reported in

numerous species [23,46,47], however, investigation of the PPP in stallion spermatozoa is lacking to date. Notwithstanding intense investigation on stallion sperm metabolism has been conducted recently revealing the importance of oxidative phosphorylation, and consequently, the intense production of reactive oxygen species (ROS) in the mitochondria [13,14,41,48–50]. Due to this intense mitochondrial activity, stallion spermatozoa have developed sophisticated mechanisms to control ROS [9,36,51]. Increasing the concentration of pyruvate to 10 mM induced major changes in the functionality of stallion spermatozoa in addition to the improvement in motility. However, inhibition of the PPP did not affect motility but reduced viability, with an important increase in the presence of spermatozoa showing an apoptotic phenotype. Furthermore, inhibition of the PPP reduced mitochondrial membrane potential and GSH content in spermatozoa. Alternative sources of NADPH may be present since G6PD inhibition reduced but not abolished NADPH. Isocitrate dehydrogenase 1 (IDH1), and isocitrate dehydrogenase II (mitochondrial), the latter has been detected in stallion spermatozoa [40] and can be an alternative pathway to generate NADPH in stallion spermatozoa when the PPP is inhibited as has been observed in other cellular models [52]. Thus, a possible explanation for our findings is that pyruvate after conversion to lactate, when the PPP is inhibited, is used to synthesize isocitrate donating hydride to NADP + to generate NADPH in a reaction catalyzed by IDH [53].

A worth mentioning aspect of this study is that shows how the stallion spermatozoa adapts to the conditions of the media they are exposed, glucose incorporation was high at the beginning of the incubation period and decreased thereafter, moreover, inhibition of the G6PD in the 67 mM 10 mM pyruvate increased the incorporation of glucose, but not in other media. Besides, the metabolic phenotype changed and was also reflected in the metabolic study conducted showing the metabolic flexibility of the spermatozoa.

The UHPLC/MS/MS analysis showed that in the presence of 10 mM pyruvate, the relative amounts of pyruvate, lactate, ATP, and NAD^+ increased and phosphoenolpyruvate decreased. This latter finding indicates improved glycolysis in the media. The last and critical step in glycolysis is the conversion of phosphoenolpyruvate into pyruvate, and our findings indicate that this step is much more efficient in the spermatozoa incubated in the high pyruvate media. Moreover, metabolomic data support the initial hypothesis that to a great extent, the improvement seen in the presence of high pyruvate, is linked to the production of NAD^+, mostly through the reduction of pyruvate to lactate. Also, relative amounts of fumarate and malate were increased in spermatozoa incubated in the high glucose-high pyruvate media, suggesting a more efficient TCA cycle [54], and interestingly, in the conversion of fumarate to malate $FADH_2$ is generated, these two electrons are transferred to ubiquinol during complex II of the mitochondrial ETC, this may explain improved mitochondrial functionality seen in our study. In spite that our media contained 21.7 mM Na lactate, the increase in NAD^+ and lactate suggests that the reaction catalyzed by LDH prioritizes the reduction of pyruvate to lactate. In fact, the sperm-specific sperm isoform of LDH (LDH-C) has an affinity for pyruvate >60-fold higher than for lactate [54–56].

In summary, our study shows that increasing the concentration of pyruvate in the media, prevents the damage induced by high glucose concentration, through mechanisms that may involve increasing the efficiency of glycolysis providing NAD^+. Moreover, a better understanding of the energetic metabolism of the stallion spermatozoa is the basis for the improvement of assisted reproductive technologies.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

CRediT authorship contribution statement

Francisco E Martín Cano: Experiments, data analysis, and interpretation. **Gemma Gaitskell-Phillips:** Conducted experiments, writing, and editing (native English speaker). **Laura Becerro Rey:** Conducted experiments. **Eva da Silva:** Conducted experiments. **Javier Masot:** Supervision **Eloy Redondo:** Supervision. **Antonio Silva Rodríguez:** Conducted experiments. **Cristina Ortega Ferrusola:** Supervision, data interpretation. **Maria Cruz Gil:** Supervision, data interpretation. **Fernando J Peña:** Experiment design, Funding acquisition, data analysis and interpretation, writing, and editing.

Declaration of competing interest

All authors declare no potential conflict of interest.

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