Low glucose and high pyruvate reduce the production of 2-oxoaldehydes, improving mitochondrial efficiency, redox regulation, and stallion sperm function

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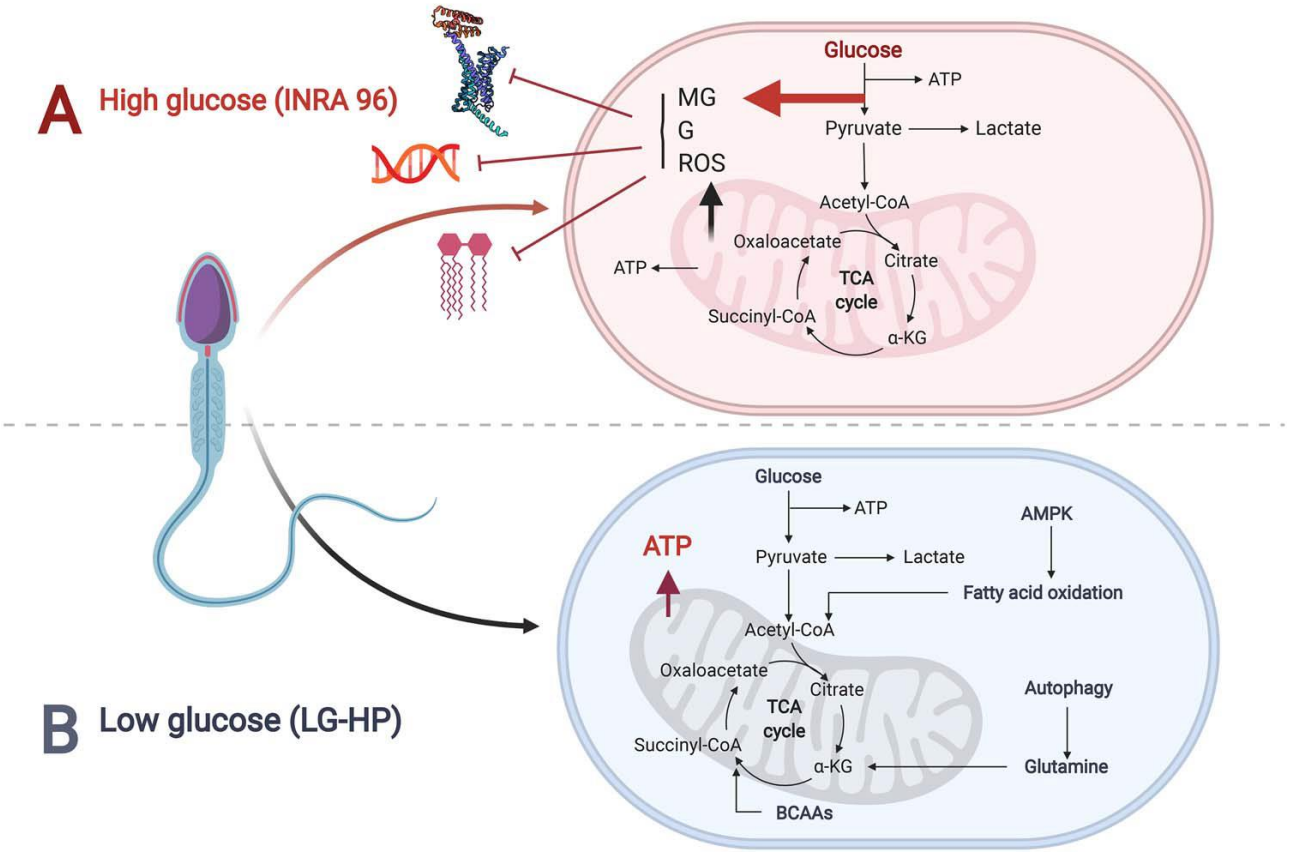
Abstract

Energy metabolism in spermatozoa is complex and involves the metabolism of carbohydrate fatty acids and amino acids. The ATP produced in the electron transport chain in the mitochondria appears to be crucial for both sperm motility and maintaining viability, whereas glycolytic enzymes in the flagella may contribute to ATP production to sustain motility and velocity. Stallion spermatozoa seemingly use diverse metabolic strategies, and in this regard, a study of the metabolic proteome showed that Gene Ontology terms and Reactome pathways related to pyruvate metabolism and the Krebs cycle were predominant. Following this, the hypothesis that low glucose concentrations can provide sufficient support for motility and velocity, and thus glucose concentration can be significantly reduced in the medium, was tested. Aliquots of stallion semen in four different media were stored for 48 h at 18◦C; a commercial extender containing 67 mM glucose was used as a control. Stallion spermatozoa stored in media with low glucose (1 mM) and high pyruvate (10 mM) (LG-HP) sustained better motility and velocities than those stored in the commercial extender formulated with very high glucose (61.7 ± 1.2% in INRA 96 vs 76.2 ± 1.0% in LG-HP media after 48 h of incubation at 18◦C; $P < 0.0001$). Moreover, mitochondrial activity was superior in LG-HP extenders (24.1 ± 1.8% in INRA 96 vs 51.1 ± 0.7% in LG-HP of spermatozoa with active mitochondria after 48 h of storage at 18◦C; $P < 0.0001$). Low glucose concentrations may permit more efficient sperm metabolism and redox regulation when substrates for an efficient tricarboxylic acid cycle are provided. The improvement seen using low glucose extenders is due to reductions in the levels of glyoxal and methylglyoxal, 2-oxoaldehydes formed during glycolysis; these compounds are potent electrophiles able to react with proteins, lipids, and DNA, causing sperm damage.

Summary sentence

High glucose in the extender damages spermatozoa through formation of 2-oxoaldehydes.

Graphical Abstract



Key words: stallion, semen, flow cytometry, metabolism, ROS, GSH, glyoxal, methylglyoxal, glucose, pyruvate

Introduction

For a long time, glucose metabolized through a purely glycolytic pathway, has been considered as the main source of energy for the stallion spermatozoa and thus most commercial extenders have been formulated containing supraphysiological concentrations of glucose [1] with use recommended under anaerobic conditions. However, sperm metabolism is complex as are the interactions between glycolysis, the Krebs Cycle and oxidative phosphorylation [2-5]. The application of proteomics and metabolomics to the study of the spermatozoon, is revealing that this highly specialized cell has complex metabolic pathways and a much higher level of metabolic plasticity than was previously thought [2, 6, 7]. Moreover, the link between metabolism and redox regulation arises as a key factor underlining sperm biology as occurs in somatic cells [8, 9].

This recent research disputes the paradigm of the predominantly glycolytic metabolism of the spermatozoa and indicates that lipid, together with carbohydrate and amino-acid metabolism

contribute to the production of substrates for the Krebs Cycle in male gametes [2, 10, 11]. While glycolysis occurs in the cytosol, the final product of glycolysis, pyruvate, enters the mitochondria and is decarboxylated to Acetyl-CoA by the pyruvate dehydrogenase complex, feeding the Krebs cycle in the mitochondrial matrix. The Krebs cycle then produces NADH and succinate that are oxidized in the inner mitochondrial membrane during oxidative phosphorylation (OXPHOS). The importance of OXPHOS for the stallion spermatozoa is now well described, composing the principal source of ATP for sperm motility and the maintenance of membranes [12].

Mitochondria are, thus, key organelles in spermatozoa metabolism and they are also the source of important signaling molecules for proper sperm functionality, particularly regarding physiological levels of reactive oxygen species (ROS), that if deregulated may trigger the release of death signals [13-17]. New studies in somatic cells indicate that other molecules with origin in the mitochondria play an important regulatory role, particularly through the release of mtDNA and TCA cycle metabolites into the cytosol [18]. Glycolysis is not a perfect process, the Emden-Meyerhof-Parmas pathway includes a series of steps for elimination of phosphates from the trioses phosphates glyceraldehyde 3-phosphate and dihydroxyacetone phosphate [19] and during this process glyoxal (G) and methylglyoxal (MG) are produced continuously, their production being proportional to the concentration of glucose present [20-22]. These products are also generated during lipid metabolism, chemically both are 2-oxo aldehydes, and due to their adjacent carbonyl groups, 2-oxoaldehydes are strong electrophiles that react rapidly and spontaneously with nucleophiles from proteins, lipids and DNA forming advanced glycation end products (AGEs). These compounds are potentially cytotoxic and mutagenic.

Our hypothesis is that since current research indicates that the stallion spermatozoa has limited glycolytic capacity glucose levels in storage media for stallion spermatozoa can be significantly reduced. Moreover, we hypothesize that supraphysiological concentrations of glucose present in commercial extenders are in fact detrimental to the spermatozoa due to increased production of 2 -oxoaldehydes.

Material and methods

Reagents and media

Monochlorobimane, methyl α-ketoglutarate 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 was purchased from Beckman Coulter (Indianapolis, In USA). For mass spectrometry analysis, O-benzylhydroxylamine hydrochloride (O-BHA) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), Glyoxal and Methylglyoxal were supplied by Sigma-Aldrich (St. Louis, MO, USA). LC-MS grade formic acid and acetonitrile were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA. Ultra-pure deionized water (> 18.2MΩ·cm) was produced from Millipore Milli-Q Gradient system (Millipore, Bedford, MA, USA).

Semen collection and processing

Semen was collected from 5 stallions of different breeds individually housed at the Veterinary Teaching Hospital of the University of Extremadura, Cáceres Spain, and 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 protocols used at our center. After collection the semen was immediately evaluated and processed in the adjacent laboratory. The ejaculate was extended 1:2 in INRA-96 extender (IMV L'Aigle, France), centrifuged at 600 x g for 10 min to remove the bulk of the seminal plasma and then re-suspended in basic 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_2PO4, 20 mM HEPES, 5 mM glucose, 21.7 mM NaL-Lactate, 1 mM Na Pyruvate, 15 mM NaH_2CO_3, 0.3% v/v BSA) pH 7.4 [23] and various experimental media as outlined below. The original medium was named G and the medium in which glucose was substituted by 2-Deoxy-d-glucose was named 2-DG. All samples were adjusted to a concentration of 25 x 10^6 total spermatozoa/ml.

Experimental design

The sperm suspension was split into sub-samples for control and experimental treatments, in one glucose was substituted by 5mM 2-DG, and two further experimental groups with 5mM 2-DG plus 20 or 100 µM of the permeable analogue of oxoglutarate, dimethyl-oxoglutarate (M2OX). Samples were then incubated at 37º C and after 1 and 3 hours of incubation aliquots were removed for measurement of sperm motility and velocities using computer assisted sperm analysis (CASA) and viability, mitochondrial membrane potential and GSH content using flow cytometry.

In a second set of experiments aliquots of the same ejaculates were split into control and experimental samples. The commercial extender INRA 96 and Tyrode's media were used as controls, and aliquots of stallion spermatozoa were incubated in two modified versions of Tyrode's media: low glucose high pyruvate (LG-HP) and low glucose high pyruvate supplemented with 100 mM of methyloxoglutarate (LG-HP-OXO). The components of these media are given in supplementary table 1. Split samples of stallion spermatozoa were incubated at a concentration of 25 x 10^6 spermatozoa at 18ºC for 48 hours. Motility, velocities, viability, mitochondrial membrane potential, GSH content, reactive oxygen species, static oxidation reduction potential and total antioxidant capacity were measured after 24 and 48h of storage at 18ºC, this temperature was chosen to avoid cold shock to the membranes [24]. Additional independent ejaculates from the same stallions were collected and processed for the proteomic study. Finally, the levels of the 2-oxoaldehydes glyoxal (G) and methylglyoxal (MG) were measured using mass spectrometry (HPLC/MS) in ejaculates conserved in the media described in all the control and experimental samples. For all the experiments at least three independent ejaculates from each of the 5 stallions were collected and processed (n= 15 replicates).

Flow cytometry

Two different flow cytometers were used in this study. A Cytoflex LX® flow cytometer (Beckman Coulter, Indianapolis, In USA) equipped with ultraviolet, violet, blue, yellow, red and infrared lasers and a Cytoflex® flow cytometer equipped with violet, blue, yellow and red lasers were used. The instruments were calibrated daily using specific calibration beads provided by the manufacturer. Compensation for spectral overlap was performed before each experiment. Files were exported as FCS files and analyzed using Flowjo V 10.7 Software (Ashland, OR, USA). Unstained, single-stained,

and Fluorescence Minus One (FMO) controls, were used to determine compensations and positive and negative events, as well as to set regions of interest as described in previous publications from our laboratory [25-27].

Measurement of GSH, viability and mitochondrial membrane potential

Intracellular GSH was measured using monochlorobimane 10μM adapted to previously published protocols [28, 29], optimized for GSH detection in flow cytometry [30] which were also adapted to equine spermatozoa by our laboratory [31, 32]. Mitochondrial membrane potential (JC-1) and sperm viability (DRAQ7) were also simultaneously assessed. In brief, sperm aliquots (2.5 x 10^6 sperm/mL) were stained with JC-1 for measurement of mitochondrial membrane potential 1μM (20 minutes in the dark at r.t.), monochlorobimane (MCB) 10 μM for measurement of GSH and DRAQ7 3μM for measurement of live spermatozoa (10 minutes in the dark at r.t). Briefly, after assessment of flow quality, doublets and debris were gated out, monochlorobimane 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 at 690 nm. Unstained and single stained spermatozoa were used to set compensations and regions of interest as previously described in our laboratory[33].

Measurement of GSH, viability, mitochondrial membrane potential and reactive oxygen species (ROS)

In this protocol stallion spermatozoa washed in PBS (2.5 x 10^6 sperm/mL), were stained with JC-1 for measurement of mitochondrial membrane potential 1μM, monochlorobimane (MCB) for measurement of GSH 10 μM, ViaKrome 808 for measurement of live and dead spermatozoa (2.5 μL of the reconstituted solution as indicated by the manufacturer) and CellRox deep Red 5 μM for measurement of reactive oxygen species , and were incubated at 37ºC for 30 minutes (JC-1, CellRox Deep Red and ViaKrome 808) and 10 minutes at r.t with monochlorobimane (MCB). Briefly, after assessing flow quality, doublets and debris were gated out, monochlorobimane 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 488nm excitation and 530 nm emission (monomers), CellRox Deep Red at 644 nm excitation and 655 emission and Viakrome 808 at 854nm excitation and 878 emission. After each incubation period, the samples were washed and

resuspended in PBS before they were run through the flow cytometer. Unstained, single stained, and stimulated (positive controls) spermatozoa were used to set compensations and regions of interest as previously described in our laboratory [34, 35]; the use of a different laser for excitation of every probe (and two for excitation of JC-1) allowed a 5-color experiment with minimal spectral overlap. For all the probes used the percentages of positive spermatozoa were measured, except for monochlorobimane (GSH measurement) for which the relative fluorescence units were measured. The gating strategy is depicted and fully described in figure 6.2 and the legend for figure 6.2. In brief after measurement of flow quality and elimination of debris and doublets, live spermatozoa were identified (Fig 6.2 B) and for mitochondrial membrane potential (Fig 6.2 E-H) and GSH content (Fig 6.2 I-L) measurements were only performed on the live population and analysis of ROS production (Fig. 6.2 M-P) was performed on the whole population (live and dead spermatozoa). FCS files were exported to FlowjoV 10.7 Software (Ashland, OR, USA). All of the events from every single replicate for each experimental group were concatenated together in a single FCS, and analyzed, dot plots were presented as density plots comprising $1x10^6$ events (spermatozoa) each.

Computational flow cytometry

The data from each five-color protocol described in the material and methods were exported as FCS files from the flow cytometer and loaded and analyzed using FlowjoV 10.7 Software (Ashland, OR, USA). For computational analysis, data from all the replicates for each treatment were concatenated and single cell events analyzed, providing single files including all the replicates for every treatment. The files obtained after concatenation were exported as FCS files. Flow cytometry files were then analyzed using non-linear dimensional reduction techniques (t-SNE) on a gate for live cells as previously described [36] .

Analysis of the proteins with metabolic function

Independent ejaculates were used to study the proteome of the stallion spermatozoa, paying special attention to proteins involved in the regulation of sperm metabolism. Proteomic analysis of stallion spermatozoa was performed as described in previous publications [10, 37]. In brief, proteins were extracted from stallion spermatozoa and analyzed using UHPLC/MS/MS (Agilent 1290 Infinity II Series UHPLC, Agilent Technologies, Santa Clara, CA, USA) equipped with an automated multisampler module and a High Speed Binary Pump coupled to an Agilent 6550 Q-TOF Mass Spectrometer. Data

processing and analysis was performed using a Spectrum Mill MS Proteomics Workbench (Rev B.04.01, Agilent Technologies, Santa Clara, CA, USA).

Identification of proteins with metabolic function in stallion spermatozoa

Due to the increased detail available for the human proteome in terms of annotation, the equine annotations obtained in the previous step were transformed to their human orthologs using g:Profiler (https://biit.cs.ut.ee/gprofiler/orth). Pathway enrichment analysis and visualization was also performed using g:Profiler (https://biit.cs.ut.ee/gprofiler/gost) and Cytoscape analysis using Reactome (https://reactome.org).

Network analysis

Cytoscape (https://cytoscape.org) plug-in ClueGo was used to identify functionally grouped gene ontology terms in equine seminal plasma as previously described [38, 39]. A kappa score of 0.52 was used and pathways were set at $P<0.01$.

Measurement of oxidation-reduction potential

Oxidation-reduction potential (ORP) was measured using the MioxSYS® diagnostic system (Englewood CO, USA) [40-42]. This technology measures the static oxidation reduction potential (sORP) over 4 min, measuring the potential of an electrochemical cell under static conditions, followed by measurement of the capacity for oxidation reduction potential (cORP), the total amount of readily oxidizable molecules. In brief, 30μL of the sperm suspension was loaded onto the sample port of the pre-inserted disposable sensor, at which point measurement begins. After 4 min, the static oxidative-reduction potential (sORP) is provided in millivolts (mV). According to the manufacturer sORP is measured while applying a low oxidizing current (1nA) to the sample. After allowing 1 min and 50 s for equilibration the reader takes two measurements per second over a 10 s period of the difference in potential between the working and reference electrode in mV. Subsequently cORP are measured applying a linearly increasing oxidizing current until the charge rapidly changes between the working and reference electrode, indicating that all readily oxidizable molecules have been oxidized, providing a measurement of antioxidant capacity reserve (cORP) [42].

The time until the charge changes is used to calculate the number of electrons needed to cause charge changes and is reported in μCoulomb (μC).

UHPLC/MS measurement of glyoxal and methylglyoxal

The sperm pellet containing $100x10^6$ spermatozoa, was re-dissolved in 300 μL of milliQ water and sonicated for 5 sec. Immediately afterwards, it was centrifuged at 6272 x g at 4° C for 3 min and the supernatant was subjected to a derivatization procedure. The derivatization reagent was O-BHA coupled to EDC, which catalyzed the derivatization reaction. This reagent is suitable for derivatizing acids, ketones and aldehyde groups. The derivatization was carried out following the procedure for analysis of short chain fatty acids [43] with modifications. Specifically, 100 μL of supernatant was incubated with 20 μL of 0.1M BHA in MeOH and 20 μL of 0.25M EDC in MeOH at 35 °C for 1 h. After incubation, 50 μL was diluted 20 fold in 50% methanol, and 500 μL of dichloromethane was added to liquid-liquid extraction. Finally, a volume of dichloromethane (containing the derivatized analytes) was evaporated until dry. The residue was reconstituted in 100μL of 50% aqueous MeOH, vortexed briefly and 5 μL was injected on UHPLC-MS/MS. The effectiveness of the process was evaluated. Calibration was carried out by standard addition technique. Therefore, the standards for glyoxal and methylglyoxal were prepared in supernatant samples spiked with increasing amounts of each, following the same derivatization procedure. The derivatized glyoxal and methylglyoxal (BHA-Gly, and BHA-MGly) were analyzed by UHPLC-MS/MS. An Agilent 1290 Infinity II UHPLC coupled with 6470 triple quadrupole (QqQ) (Agilent Technologies, Waldbronn, Germany) was used. The UHPLC was equipped with a built-in auto-degasser, binary pump and column thermostat. Zorbax C18 column, 100 mm x 2.1 mm, 1.8 um (Agilent, CA) was used for the separation step working at 25 ºC. The LC-MS interface was ESI with jet stream. Nitrogen was used as nebulizing gas, drying gas, sheath gas and collision gas. The mobile phase was composed of two solutions: A, aqueous with 0.5 % formic acid and B, acetonitrile with 0.5 % formic acid. A binary gradient was applied with a flow rate of 0.4 mL/min.: 0-5 min 20% B, 5-10 min linear increase from 20 to 100% B and maintained until minute 12, followed by re-equilibration of the column up to minute 15. The BHA-Gly and BHA-MGly was eluted at 6.4 and 6.8 minutes, respectively. The ionization source parameters, operating in positive polarity, were optimized injecting 3 mg/L of BHA-Gly and BHA-MGly. The best sensitivity was obtained with the following ionization source parameters: drying gas temperature at 200ºC, nebulizer at 25 psi, drying gas flow at 12 L/min., sheath gas temperature and flow rate at 350 ºC and 10 L/min.,

respectively, capillary voltage at 3500 V and fragmentor to 110 V. The MRM conditions were optimized by injecting the same solution at different collision energies (CE). The transitions were from 269 to 91 (at 20 eV CE) and to 144.1 (at 10 eV CE) for BHA-Gly and 283 to 91 (at 25 V CE) and to 181.1 (at 10eV CE) were the selected MRM from sensitivity and selectivity point of view. The quantification transitions were 269 to 91 and 283 to 91 for BHA-Gly and BHA-MGly, respectively.

Statistical analysis

Sperm samples were obtained from ejaculates from 5 different stallions. All experiments were repeated at least three times with independent samples (three separate ejaculates from each of the donor stallions). The normality of the data was assessed using the Kolmogorov-Smirnoff test. A one-way ANOVA followed by Dunnett's multiple comparisons test were performed using GraphPad Prism version 7.00 for Mac, La Jolla California USA, www.graphpad.com, in this way every treatment was compared with each samples own controls and all other treatments. Differences were considered significant when $P < 0.05$ and results are displayed as means ± SEM.

Results

Characterization of the metabolic proteome of the stallion spermatozoa

Analysis of gene ontology of biological processes (GO-BP) and reactome pathways enriched in proteins with metabolic function of the stallion spermatozoa, using human orthologs, revealed the key role of pyruvate and the Krebs Cycle (Figure 1 AB). The reactome pathways most significantly enriched were the Cytric Acid Cycle REAC :R-HAS-71403, Pyruvate metabolism and the Citric Acid (TCA) cycle REAC:R-HAS-71406 and the citric acid (TCA) cycle and respiratory electron transport REAC:R-HSA-1428517 (Fig 1A). Network analysis using Cytoscape and the ClueGo app also revealed the major role of pyruvate metabolism (Fig 1B).

Inhibition of glycolysis improves the percentage of live spermatozoa

Split samples from the same stallion and ejaculate were incubated for up to three hours in the presence of glucose (G) 5 mM and the non-metabolizable glucose analogue 2-deoxyglucose (2-DG) 5

mM, and the percentage of live spermatozoa was evaluated flow cytometrically. When G was substituted by 2DG a significant increase in the percentage of live spermatozoa was observed after one and three hours of incubation at 37ºC, from 53.7± 5.9% in samples incubated in the presence of glucose to 74.5± 0.63 in samples incubated in the presence of 2DG (*P*<0.05) (Fig 2 A).

Inhibition of glycolysis modifies intracellular GSH and improves mitochondrial function

Since recycling of oxidized glutathione (GSSG) depends on the availability of reducing power in the form of NADPH, that can be produced in the pentose phosphate pathway (PPP), we investigated whether inhibition of glycolysis affected intracellular GSH. It was observed that substitution of G by 2-DG did not modify intracellular GSH in stallion spermatozoa after 1 hour of incubation (Fig 2 C), but reduced intracellular GSH after three hours of incubation, from 1.8 ± 0.17 in G samples to 1.5 ±0.12 in 2DG samples (*P*<0.05; Fig 2 F). Interestingly this difference was due to an increase in the relative amount of GSH in glucose samples, since the amount of GSH remained constant in 2DG treated aliquots (Fig 2 C). The percentage of spermatozoa with high mitochondrial membrane potential ($\Delta\Psi$m) increased in samples containing 2 DG both after one and three hours of incubation. After 1 hour the percentage of spermatozoa with high $\Delta\Psi$m was seen to be increased from 67.1 ± 6.5 in samples containing glucose to 82.6 ± 2.2% in samples containing 2DG (*P*<0.0001; Fig 2 B). After three hours of incubation the percentage of high $\Delta\Psi$m increased from 54.4± 7.2 to 75.5 ± 3.6 % (*P*<0.001) (Fig 2 E).

Oxoglutarate maintains GSH in the absence of glucose

Since reducing equivalents can be obtained via different metabolic pathways, we investigated whether oxoglutarate can rescue the drop in GSH in the absence of glucose. Stallion spermatozoa were incubated for three hours at 37ºC in the presence of glucose, 2-DG and in the presence of 2-DG with 20 or 100 μM of methyl oxoglutarate, a cell permeable form of oxoglutarate. GSH content (as mean florescence intensity units) was 1.8 ± 0.17 in samples supplemented with glucose and this dropped to 1.4 ±0.12 in media containing 2-DG (*P*<0.05). Methyl oxoglutarate prevented the drop in GSH when glucose was substituted by 2-DG where GSH content was 2.0 ± 0.9 relative fluorescence units at 100 μM (Fig 2 I)

Inhibition of glycolysis reduces sperm motility and velocity

When G was substituted by 2-DG after 1 hour of incubation at 37ºC there was a drop in the percentage of motile spermatozoa from 84.5 ± 2.0 to 65.8 ± 3.2 (*P*<0.001; Fig 3 A-B). The velocities of motile spermatozoa were also reduced. In samples incubated in media containing glucose after 1 hour of incubation at 37ºC VCL was 200.7 ± 9.9 μm/s, while in samples in which glucose was substituted by 2-DG VCL dropped to 102.2 ± 2.7 μm/s (P<0.001; Fig. 3 C-E).

Low glucose concentration maintains motility for longer periods of time

The previous experiments showed that stallion spermatozoa can survive without glucose, although motility and especially velocities were reduced. In order to test if a reduced amount of glucose is sufficient to maintain motility, we developed a low glucose media, supplemented with pyruvate and oxoglutarate that readily feed the TCA cycle. The commercial extender INRA 96 was used as a control, and aliquots of stallion spermatozoa were incubated in Tyrode's media and two modified versions of Tyrode's media. Low glucose high pyruvate (LG-HP) and low glucose high pyruvate supplemented with 100 μM of methyl oxoglutarate (LG-HP-OXO). The components of these media are given in supplementary table 1. Split samples of stallion spermatozoa were incubated at a concentration of 25 x 10^6 spermatozoa at 18ºC for 48 hours. Motility and velocities were measured after 24 and 48 hours. Motility was better in all variants of the Tyrode's media than in INRA 96 after 24 and 48 hours. After 24 hours of storage at 18º C total motility was 68.4 ± 1.11% in INRA 96, 77.9 ± 1.1% in Tyrode's (*P*<0.0001) and 79.2 ± 1.0% (*P*<0.0001) and 76.7 ± 1.2 % (*P*<0.01) respectively in LG-HP and LG-HP-OXO (Fig 4A). The percentage of linear motile spermatozoa followed the same tendency, the most significant differences with respect to the INRA 96 control were observed in the LG-HP and LG-HP-OX groups (Fig 4 D F). With aliquots extended in INRA 96 and Tyrode's showing percentages of linear motility of 53.0 ± 1.6 % and 58.0 ± 1% respectively (*P*<0.01) and samples extended in LG-HP and LG-HP-OX showing significantly higher linear motility; 60.3 ± 0.86 and 60.0 ± 1.1% respectively (*P*<0.01 and *P*<0.0001) (Fig 4B). After 48 hours of storage, better motilities were observed in spermatozoa stored in the LG-HP groups. Total motility was 61.7 ± 1.2% in the INRA 96 group and 76.2 ± 1.0% in samples stored in the LG-HP extender (*P*<0.0001; Fig 4 C). Interestingly the motility values were more homogeneous in the LG-HP extender (Fig 4C red circle). The percentage of linear motile spermatozoa after 48h of storage followed the same tendency (Fig 4 D).

Low glucose concentration improves sperm velocities in stallion spermatozoa stored for long periods

Circular velocity was significantly higher in all the Tyrode's based extenders in comparison with INRA 96 at every time point considered (Fig 5 A). Circular motility after 24h of incubation at 18ºC was 112.3 ± 2.2 μm/s in aliquots conserved in INRA 96 while it was 214.5 ± 6.9 (P<0.001), 226.7 ± 7.6 (P<0.0001) and 212.3 ± 6.8 μm/s (P<0.0001) in samples conserved in Tyrode's, LG-HP and LG-HP-OX respectively. A similar pattern was observed in straight line and average velocities after 24 h of conservation at 18ºC (Fig 5 B- C). After 48 hours of storage at 18ºC, the tendency was maintained, with better velocities in all Tyrode's based extenders; VCL was 111.6 ± 4.3 μm/s in the INRA 96 extender, 191.1 ± 3.57 μm/s in Tyrode's (P<0.0001), 203 ± 3.2 μm/s in LG-HP (P<0.0001), that was also significantly different from Tyrode's (P<0.01). In samples extended in LG-HP-OX circular velocity after 48 hours of storage was 168.7 ± 6.4 μm/s (P<0.001). Straight line and average path velocities followed the same trend as VCL (Fig 5C-E), but in this case VSL and VAP were also significantly improved in LG-HP (P<0.001) with respect to samples stored in the Tyrode's basal medium.

Low glucose concentration improves mitochondrial membrane potential in stallion spermatozoa stored for long periods

Media formulated with low glucose, showed an improved capacity to sustain high mitochondrial activity in stallion spermatozoa. After 24 hours of storage at 18ºC, the percentage of spermatozoa with high mitochondrial membrane potential was 25.4 ± 2.0 in samples extended in INRA 96, while this was 58.4 ± 4.0 (P<0.0001), 63.5± 4.9 (P<0.0001), 64.6 ± 5.9 (P<0.0001) and 62.7 ± 529 (P<0.0001) in samples stored in Tyr, LG-HT and LG-HT-OX media respectively. The same effect was maintained after 48 hours of storage (Fig 6), but at this point in time, samples stored in LG-HP media also showed better mitochondrial membrane potential than samples stored in Tyrodes (51.1±0.7 vs 43.6±1.5% P<0.01). After 24 h storage at 18º C samples stored in Tyrodes showed lower viability in comparison with those kept in INRA 96 (P<0.001). After 48 h of storage samples stored in the LG-HP-OX medium showed lower viability (P<0.05).

Low glucose concentrations reduce ROS and increases GSH content in stallion spermatozoa

In low glucose extenders, the percentages of live cells producing significant amounts of ROS were lower than in samples extended in INRA 96 or Tyrode's media. After 24 h of storage at 18◦C, the percentage of cells producing ROS was 61.1 ± 1.4 and 64.3 ± 1.3% in aliquots stored in INRA 96 and Tyrode's medium, respectively. In samples stored in LG-HP and LG-HP-OX, the percentage of ROS positive cells dropped to 45.4 ± 4.7% ($P < 0.01$) and to 40.1 ± 5.9% ($P < 0.01$; Figure 6D). At the same time, GSH content increased in LG-HP extenders, from 10 162 ± 731.7 r.f.u in INRA 96 to 15 553 ± 912 and 15 242 ± 1327 r.f.u in samples stored in LG-HP and LG-HP-OX, respectively ($P < 0.001$, $P < 0.01$). ROS production and GSH content followed the same trend after 48 h of storage (Figure 6G and H). GSH content was higher in Tyrode's and LG-HPOX extended samples after 48 h of incubation (Figure 6G).

Total antioxidant capacity reserve is higher in LG-containing media

The sORP and the antioxidant capacity reserve (cORP) were measured after 48 h of storage at 18◦C. Samples stored in LG-HP-OX showed a slight, although significant, increase in the sORP with respect to those stored in INRA 96 (12.3 ± 0.1 vs 11.7 ± 0.0 mV/106 spermatozoa; $P < 0.001$). On the other hand, samples stored in Tyrode's and LG-HP media showed an increased antioxidant capacity reserve ($P < 0.05$; Supplementary Figure S1B).

Production of glyoxal and methylglyoxal is reduced in low glucose high pyruvate media

The production of glyoxal and methylglyoxal 2-oxoaldehydes was measured after 24 and 48 h of conservation at 18◦C in stallion spermatozoa extended in different media with varying glucose concentrations. After 24 h of incubation, the concentration of G was 1.7 ± 0.1 ng/100 × 106 spermatozoa in aliquots extended in INRA 96 and 1.1 ± 0.1 ng/100 × 106 spermatozoa in samples extended in LG-HP medium ($P < 0.01$; Figure 7A). The same effect was observed after 48 h of storage at 18◦C ($P < 0.01$; Figure 7B). The production of methylglyoxal was also reduced in media with low glucose; after 24 h of incubation, the concentration of MG was 2.3 ± 0.2 ng/100 × 106 spermatozoa in aliquots stored in INRA 96, whereas it was 1.3 ± 0.2 ng/100 × 106 spermatozoa in aliquots stored in LG-HP and LG-HP-OXO ($P < 0.05$; Figure 7C). After 48 h of incubation, the production of MG was reduced in all media with respect to INRA 96, and the concentration of MG was 2.7 ± 0.3 ng/100 × 106 spermatozoa in samples stored in INRA 96, 1.8 ± 0.3 ng/100 × 106 spermatozoa in aliquots stored

in Tyrode's ($P < 0.05$), 1.6 ± 0.2 ng/100 × 106 spermatozoa when stored in LGHP ($P < 0.05$), and 1.8 ± 0.4 ng/100 × 106 spermatozoa in aliquots extended in LG-HP-OX ($P < 0.05$; Figure 7D).

DISCUSSION

Although current research indicates that in the stallion spermatozoa metabolism glycolysis is not the main pathway producing ATP, commercial extenders for stallion spermatozoa are formulated with very high glucose concentrations [1]. The main role of glycolysis is in providing the precursors for the Krebs cycle under aerobiosis. However, the role of glycolysis in supporting glycolytic enzymes all along the flagella should also be considered [4, 5].

In this study the major pathways in the metabolic proteome of the stallion spermatozoa were examined, showing the predominance of the Krebs cycle and pyruvate metabolism. Moreover in the present study inhibition of glycolysis had no effect on sperm viability or mitochondrial activity, in fact it caused significant improvements in both parameters. Despite the fact that motility and velocity parameters were reduced under this condition, these findings will argue in favor of the importance of the numerous glycolytic enzymes localized in the flagella [5], supporting sperm motility through glycolysis [12]. However, the use of 2-DG may cause ATP depletion due to the action of the sperm hexokinase phosphorylating 2-DG, making it difficult to establish the real cause of low ATP levels.

In a previous study [16] we showed that reduced motility and velocities in the presence of 2-DG were not present in media without glucose, suggesting that reduced motility and velocities caused by 2-DG were most probably due to ATP depletion due to futile 2-DG phosphorylation. Although after 1 hour of incubation there were no changes in GSH, inhibition of glycolysis caused reduced GSH content after 3 hours of incubation at 37ºC. This finding is probably explained by reduced flux to the pentose phosphate pathway (PPP) and thus diminished generation of reduced power in the form of NADPH to recycle GSSG into GSH [44]. However, NADPH can be generated in the Krebs cycle. To test if this occurs in the spermatozoa, aliquots were incubated in presence of the cell permeable analogue of oxoglutarate, methyl-oxoglutarate. This compound was able to restore GSH to levels comparable to those observed when glycolysis was not inhibited.

Overall these results may suggest that an important plasticity exists in the stallion spermatozoa, and underlines the close interplay between oxidant species and metabolism [44]. In view of these previous findings we tested the hypothesis that extenders with a low glucose concentration could be effective for long term preservation of spermatozoa. For this, aliquots of stallion ejaculates were extended in different low glucose media at 18ºC for 48 hours. A commercial extender for equine semen that has been successfully used at this temperature [24] and contains 67 mM glucose in HEPES supplemented Hank's solution [1] was used as a control. Although the composition of this extender differs from that of the Tyrode's base used in the modified extenders, not only in terms of glucose concentration, but the main difference, in addition to much higher glucose concentration, is the presence in the commercial media of purified milk fractions to provide membrane protection to the spermatozoa during storage at 4ºC. However, samples were stored at 18 ºC, and this difference may not be substantial at this temperature.

The composition and concentration of salts and the HEPES buffer are comparable in all extenders used; this, in addition, to the fact that the commercial extender is also used at temperatures ranging from 15ºC to 20ºC [24] in horses considered as "poor coolers", makes this a good control, especially considering that it is widely used in Europe, and the hypothesis being tested was that the high glucose concentrations found in commercial extenders can be substantially reduced.

All media used in the experiment had the same pH and osmolality. Two low glucose extenders (1mM), in which pyruvate was present at 10mM were formulated. The reactome pathways identified in our study confirmed previous studies and proteomic research indicating that this metabolite is used very efficiently by the stallion spermatozoa [10, 14, 45]. Methyl-oxoglutarate was also tested in one of the defined media. Computer assisted sperm analysis showed significant improvements in all the parameters studied in the low glucose media. The percentage of total motile spermatozoa was significantly higher in all defined media, with significant improvements in the LG-HP and LG-HP-OX media with respect to both total and progressive motility after 48 hours of storage (Fig 4 C-D). These improvements were manifested even more in sperm velocities. These findings can be easily explained; low glucose concentrations are sufficient to feed the glycolytic enzymes of the flagella [46], while reducing glucose toxicity. The localization of the high affinity glucose transporter GLUT 1 all along the flagellum in stallion spermatozoa supports this hypothesis [47].

Another important point to consider when explaining our results is that during glycolysis, production of toxic 2-oxoaldehydes is unavoidable [48, 49]. These compounds, and particularly methylglyoxal are produced during elimination of the phosphate groups from glyceraldehyde 3-phosphate and dihydroxyacetone phosphate [19]. These 2-oxoaldehydes are potent electrophiles that may oxidize proteins, lipids and nucleic acids and thus are potentially cytotoxic and mutagenic. Also, high glucose may have a direct effect, inducing cell toxicity through mitochondrial toxicity and increased production of reactive oxygen species [50-54]. In order to measure if reduced production of 2 oxoaldehydes occurs in low glucose media, levels of glyoxal and methylglyoxal were measured at 24 and 48 h of storage at 18ºC. The amount of glyoxal was significantly reduced in LG-HP media in comparison with INRA 96 both after 24 and 48 h storage; methylglyoxal was reduced in both LG-HP and LG-HP-OXO after 24 h of storage with respect to INRA 96 and was reduced in all media with respect to INRA 96 after 48h of storage. These findings indicate that 2 oxoaldehydes are produced in higher amounts during storage of stallion spermatozoa in media containing high levels of glucose, and cause sperm damage. This is the first time that sperm toxicity induced by high glucose concentration in the extender has been described. This finding challenges current procedures in use for storage of stallion spermatozoa in refrigeration and provides new clues which can be used to improve this technology.

Reduced percentages of ROS positive spermatozoa extended in LG-HP extenders were found, and this favors this hypothesis. Additionally, the role of glycolysis in stallion spermatozoa seems to be a mechanism which may support motility, feeding the glycolytic enzymes in the flagella [5, 12, 45], although the main source of ATP in the stallion spermatozoa is OXPHOS [7, 14, 17, 45]; thus low concentrations may suffice to accomplish this function, while reducing glucose toxicity as demonstrated measuring the levels of MG and G in our study. The improved velocities seen in all LG media also work in favor of this hypothesis. Low glucose media also improved other sperm functions. In particular, mitochondrial activity was enhanced in all Tyrode's based media throughout the storage time periods. The percentage of viable spermatozoa (those with intact membranes) was more constant in low glucose media and at the same time low glucose extenders showed lower percentages of cells showing significant ROS production linked to higher GSH content, since detoxification of 2 oxoaldehydes, especially methylglyoxal is linked to GSH [22, 55, 56], lower production of MG explains that GSH levels can remain high in low glucose media.

Flow cytometry indicated that efficient mitochondrial activity was linked to production of $O_2^{\bullet-}$ and increased GSH content. Recent reports link increased production of $O_2^{\bullet-}$ in spermatozoa to mitochondrial activity and fertility [14, 17, 57, 58]. Additionally, the concomitant increase in GSH in the same cells, indicates that redox homeostasis was maintained in these spermatozoa, and thus oxidative stress did not occur [8]. Nowadays oxidative stress is defined as a disruption of redox signaling and control rather than as increased production of ROS [59].

Overall, our findings support previous research indicating the predominance of pyruvate metabolism, and provide the first published evidence of glucose toxicity, due to increased levels of the 2 oxoaldehydes, glyoxal and mainly methylglyoxal found in stallion spermatozoa stored in commercial extenders with high glucose concentrations. Low glucose concentration may permit a more efficient mitochondrial activity boosting sperm metabolism when substrates for an efficient TCA cycle are provided. For the first time we demonstrated that excess glucose in the media increases the amount of unmetabolized glucose and causes increases in the levels of 2 oxoaldehydes formed during glycolysis. Unmetabolized glucose may also stimulate direct ROS production in the mitochondria [60], causing mitochondrial malfunction, further increasing ROS production with entry into a self-exacerbating loop of ROS production. The findings reported here are readily applicable to the development of new extenders for semen conservation and stress the importance of further research into sperm metabolism and the relationship between energetic metabolism and redox regulation.

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Figures

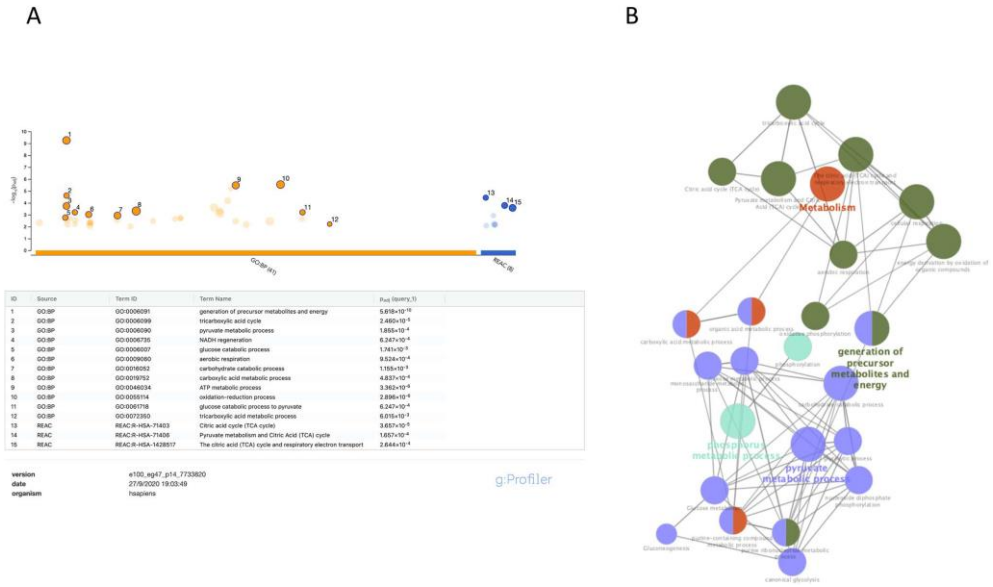

Figure 1.- A) g:GOST multiquery Manhattan plot showing comparative enrichment analysis of sperm proteins involved in metabolism. Gene Ontology (GO) for biological processes (BP) in orange and Reactome pathways in dark blue (all using human orthologs) are given. The P values are depicted on the y axis and in more detail in the results table below the image. B) CLueGO network analysis of proteins in stallion spermatozoa involved in metabolism. To reduce redundancy of GO terms, the fusion option was selected. Reactome pathways of functionally grouped networks with terms are indicated as nodes (Benjamini–Hochberg p value < 0.01), linked by their kappa score level (≥ 0.52) where only the label for the most significant term per group is shown.

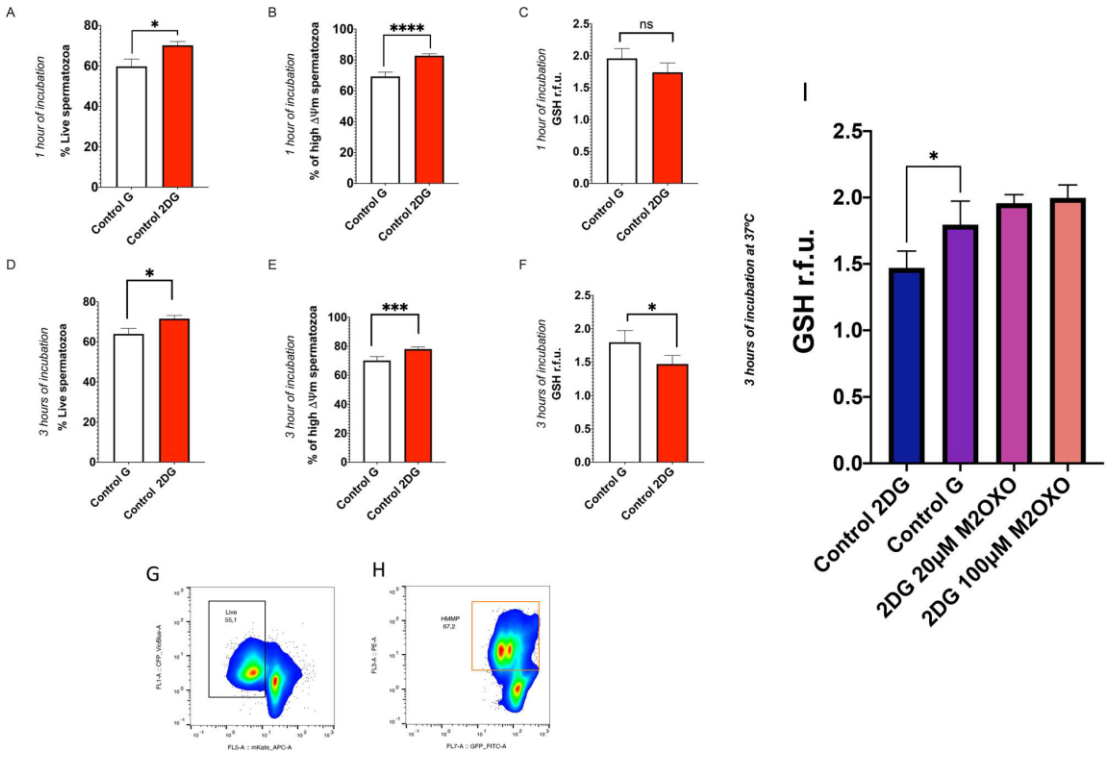

Figure 2. Effect of the inhibition of glycolysis on the viability, mitochondrial membrane potential and relative GSH content in stallion spermatozoa. Stallion spermatozoa were processed as described in material and methods and split aliquots incubated in presence of glucose 5mM or the glycolysis inhibitor 2- deoxy-glucose 5mM. After 1 hour of incubation at 37ºC inhibition of glycolysis resulted in improved viability (A) (P<0.05), better mitochondrial membrane potential (B) (P<0.0001) and no changes in relative GSH content (C). 2) After 3 hours of incubation, inhibition of glycolysis resulted in more viable spermatozoa (D) P<0.05, improved mitochondrial membrane potential (E) P<0.001, but reduced GSH content (F) (P<0.05). G and H are representative cytograms of the assays. I) Effect of oxoglutarate on GSH content, addition of 20 and 100 µM methyl-oxoglutarate reverted the reduction in GSH when glycolysis was inhibited.

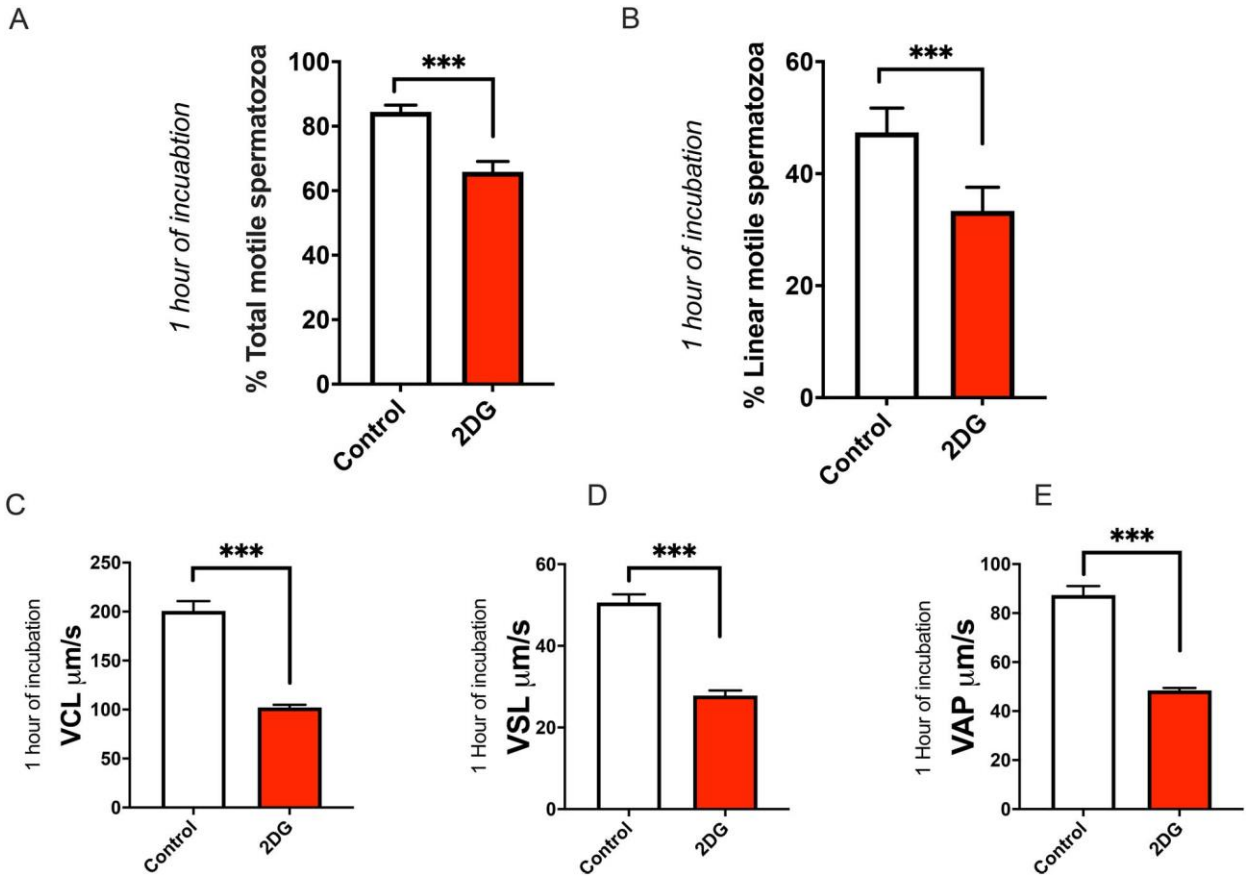

Figure 3. Effect on inhibition of glycolysis on stallion sperm motility and velocities. Inhibition of glycolysis by 2-DG resulted in reduced percentages of total motile ($P<0.001$) and linearly motile ($P<0.001$) spermatozoa. Sperm velocities were also reduced; circular velocity (VCL), straight line velocity (VSL) and average path velocity (VAP) were reduced ($P<0.001$).

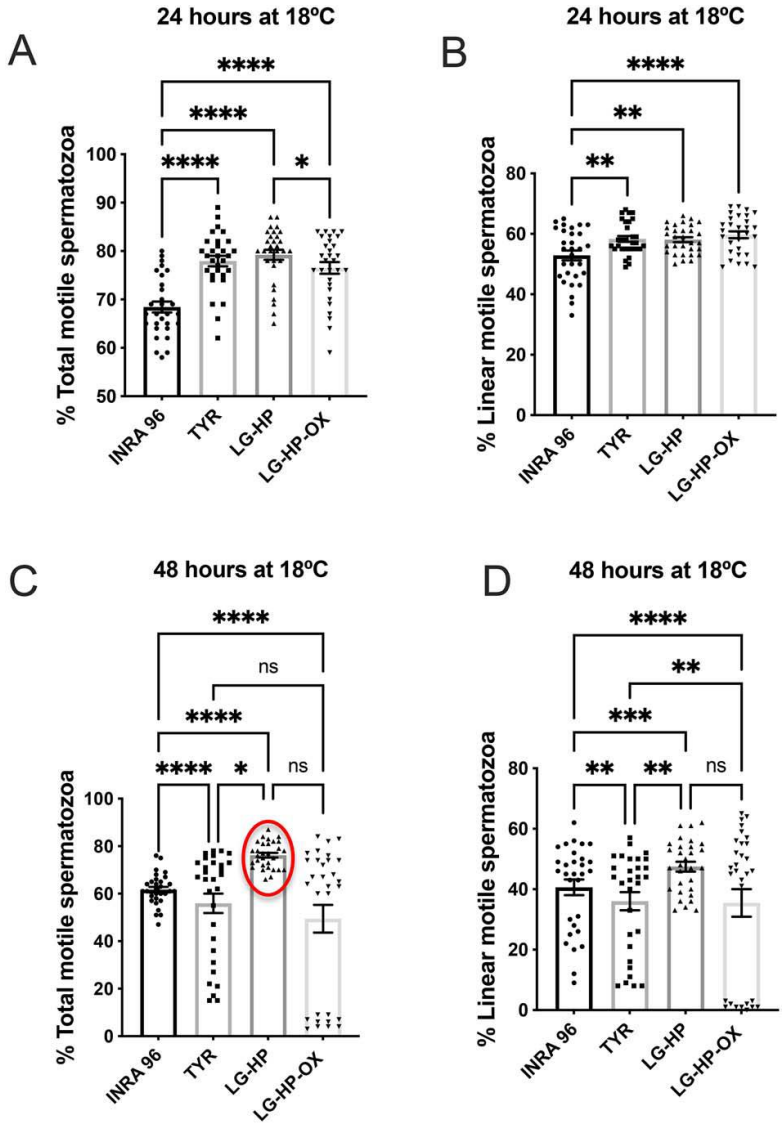

Fig 4.- Storage of stallion spermatozoa in low glucose high pyruvate (LG-HP) both supplemented (LG-HP-OX) and unsupplemented with oxoglutarate sustains motility. Aliquots from the same ejaculate were stored at 18ºC for 48 hours in a commercial extender, INRA 96™, in Tyrode's media (Tyr) and two modified Tyrode's media with low glucose (1 mM) and high pyruvate (10mM) LG-HP, and a modified LG-HP supplemented with 100 µM methyl-oxoglutarate LG-HP-OX. A-C: percentages of total motile spermatozoa after 24 and 48 hours of storage at 18ºC. B-D: percentages of linear motile spermatozoa after 24 and 48 hours of storage at 18ºC. ns: not significant, * $P<0.05$, ** $P<0.01$*** $P<0.001$ **** $P<0.0001$

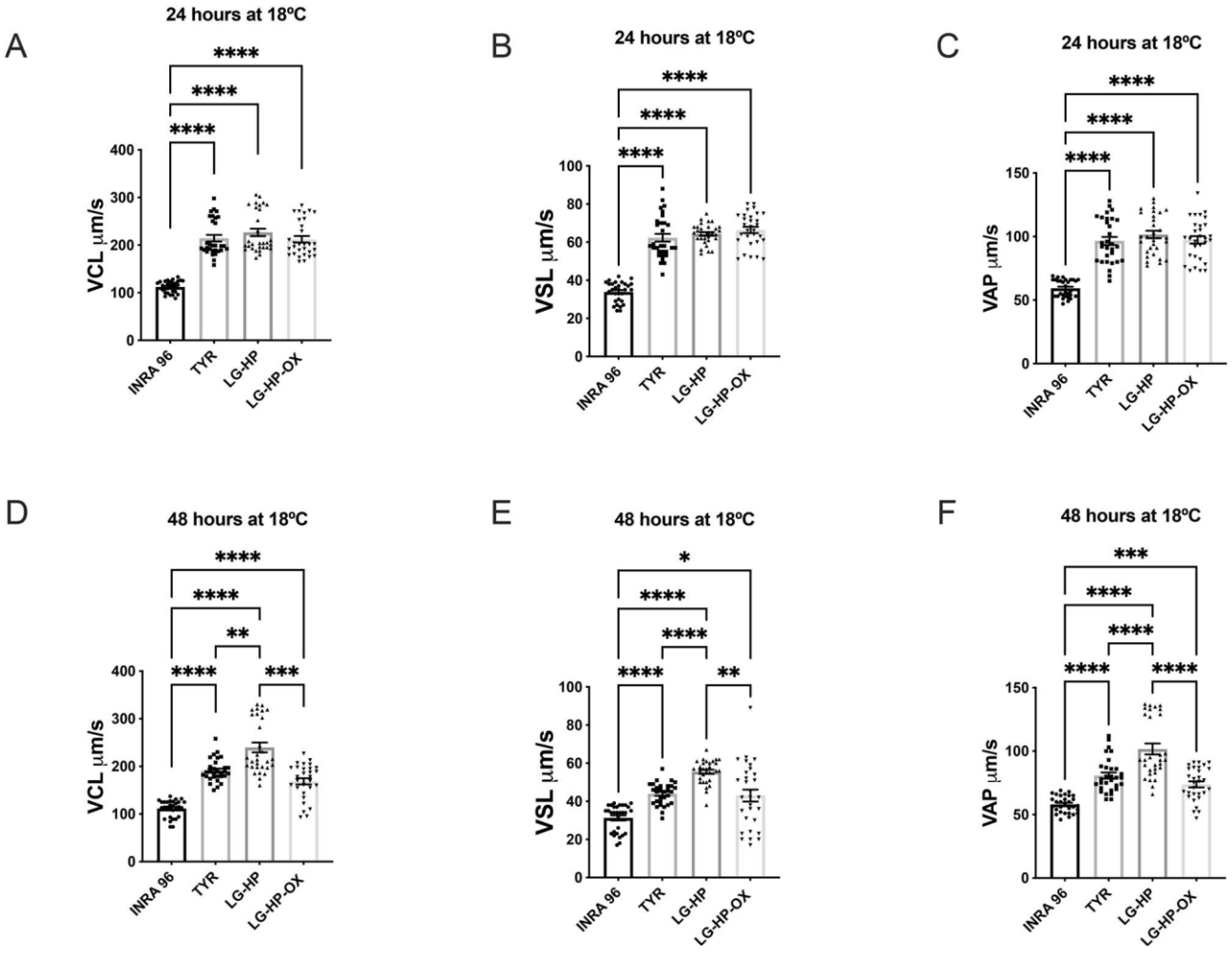

Fig 5.- Storage of stallion spermatozoa in low glucose high pyruvate (LG-HP) both supplemented (LG-HP-OX) and unsupplemented with oxoglutarate sustains sperm velocities. Aliquots from the same ejaculate were stored at 18ºC for 48 hours in a commercial extender INRA 96™, in Tyrode's media (Tyr) and two modified Tyrode's media with low glucose (1 mM) and high pyruvate (10mM) LG-HP, and a modified LG-HP supplemented with 100 µM methyl-oxoglutarate LG-HP-OX. A-D: circular velocity µm/s (VCL) after 24 and 48 hours of storage at 18ºC. B-E: Straight line velocity µm/s (VSL) after 24 and 48h hours of storage at 18ºC. C-F: Average path velocity µm/s (VAP) after 24 and 48h hours of storage at 18ºC, * P<0.05, ** P<0.01***P<0.001 ****P<0.0001

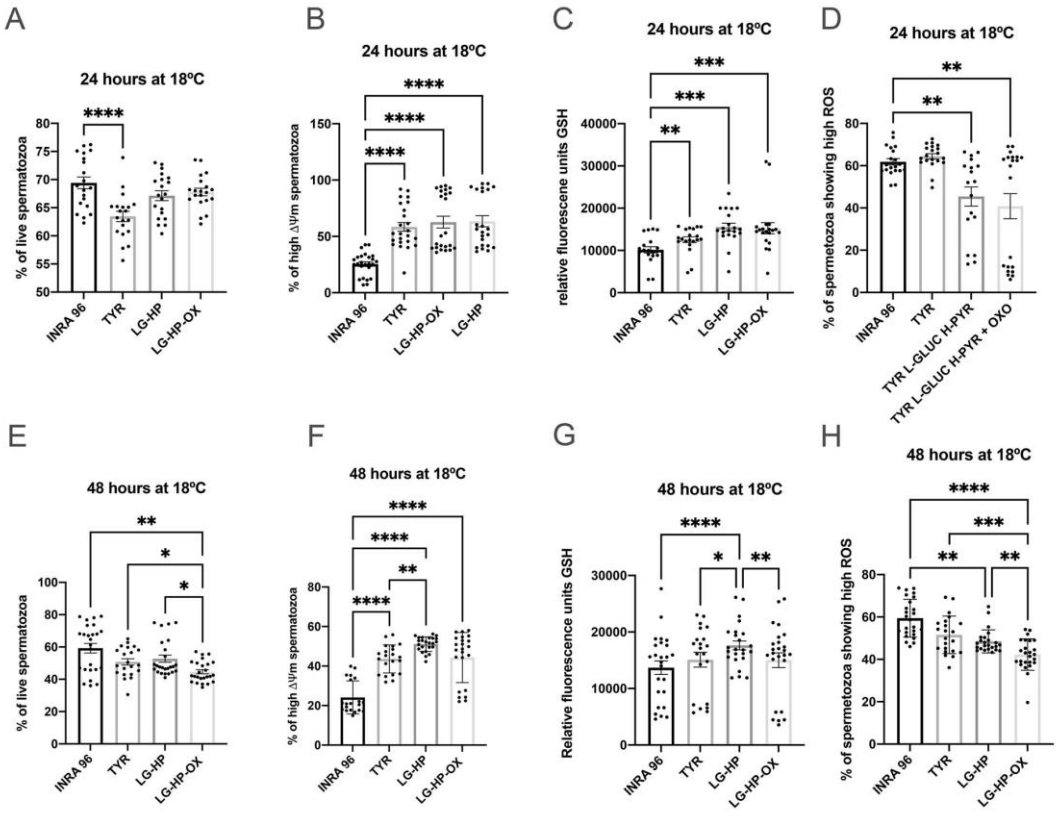

Fig 6.- Storage of stallion spermatozoa in low glucose high pyruvate (LG-HP), both supplemented (LG-HP-OX) and unsupplementedwith oxoglutarate, sustains viability and mitochondrial activity. Aliquots from the same ejaculate were stored at 18◦C for 48 h in a commercial extender, INRA 96, in Tyrode's medium (Tyr), and two modified Tyrode's media with low glucose (1 mM) and high pyruvate (10 mM) LG-HP and a modified LG-HP supplemented with 100 µM methyloxoglutarate, LG-HP-OX. (A–E) Percentage of viable spermatozoa after 24 (A) and 48 (E) h of storage at 18◦C. (B–F) Percentage of spermatozoa showing high mitochondrial activity after 24 (B) and 48 h (F) of storage at 18◦C. (C–G) Relative GSH content of spermatozoa after 24 (C) and 48 h (G) of storage at 18◦C. (D–H) Percentage of viable cells showing significant production of reactive oxygen species (ROS) after 24 (D) and 48 h (H) of storage at 18◦C. ∗P <0.05; ∗∗P <0.01; ∗∗∗P <0.001; ∗∗∗∗P <0.0001. (6.2) Gating strategy and density dot plots showing the flow cytometry analysis performed. Samples were stained and processed as described in the Materials and Methods. Monochlorobimane was used to measure GSH at a peak excitation of 405 nm and emission of 450/45 nm BP. Mitochondrial membrane potential was measured using JC-1, and this probe was detected at a peak excitation of 511 nm and emission of 596 nm (aggregates representing mitochondria with high membrane potential) and 488 nm excitation and 530 nm emission (monomers, representing spermatozoa with low mitochondrial membrane potential). The amount of reactive oxygen species was measured using CellRox Deep Red at 644 nm excitation and 655 nm emission. Finally, the percentages of live and dead spermatozoa

were measured using Viakrome 808 at 854 nm excitation and 878 nm emission. The use of five different lasers, one for each probe and two (blue and yellow) to excite the JC-1 probe, allowed the development of a five-color panel with minimal spectral overlap. In the first step and after assessing the flow quality, doublets and clumps were identified and gated out comparing SSC-H versus SSC-A (6.2A). Then, cells in this gate were analyzed for the measurement of live and dead spermatozoa (6.2B), dead cells were excluded, and live cells were used to determine the percentage of spermatozoa showing high mitochondrial membrane potential (MMP; 6.2E–H) and the GSH content expressed as relative fluorescence units (r.f.u; 6.2I–L). Finally, the percentage of stallion spermatozoa showing high production of reactive oxygen species was measured in the whole sperm population (live and dead) (6.2M–P).

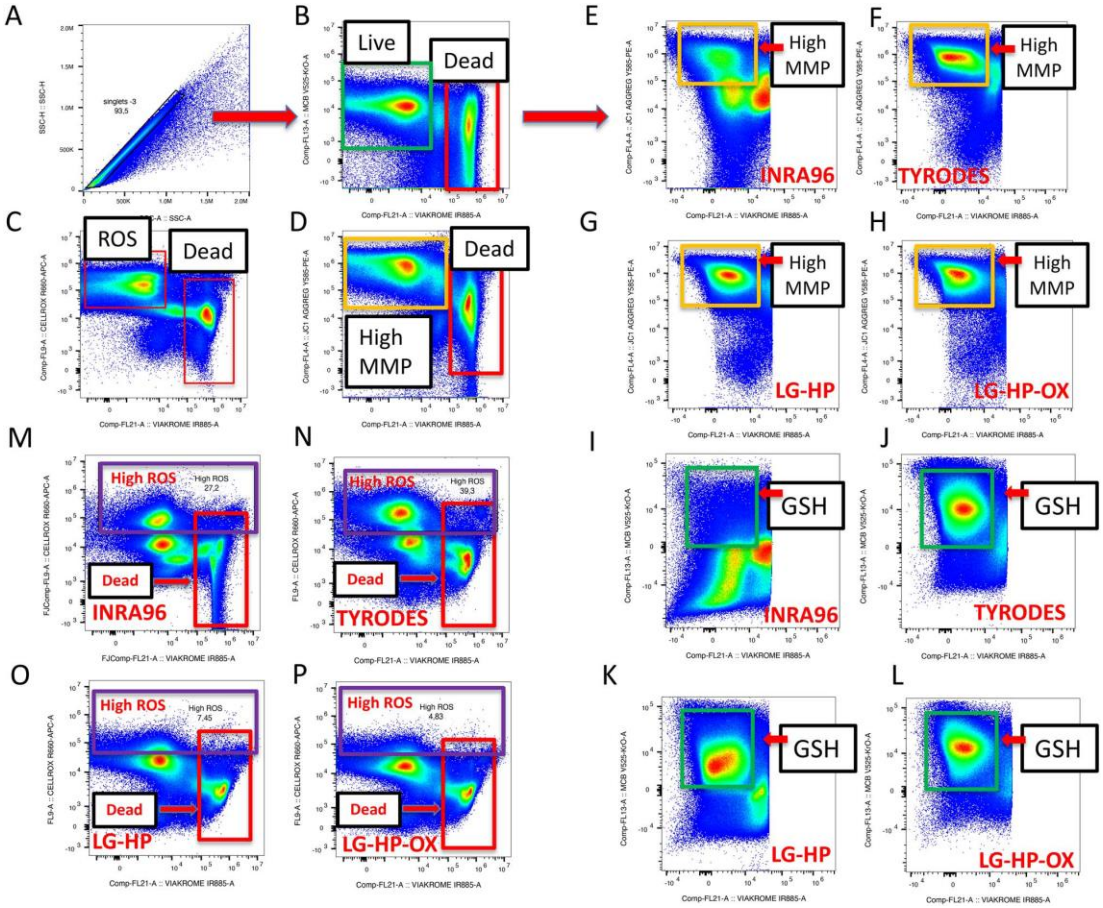

Fig 6.- Continued.

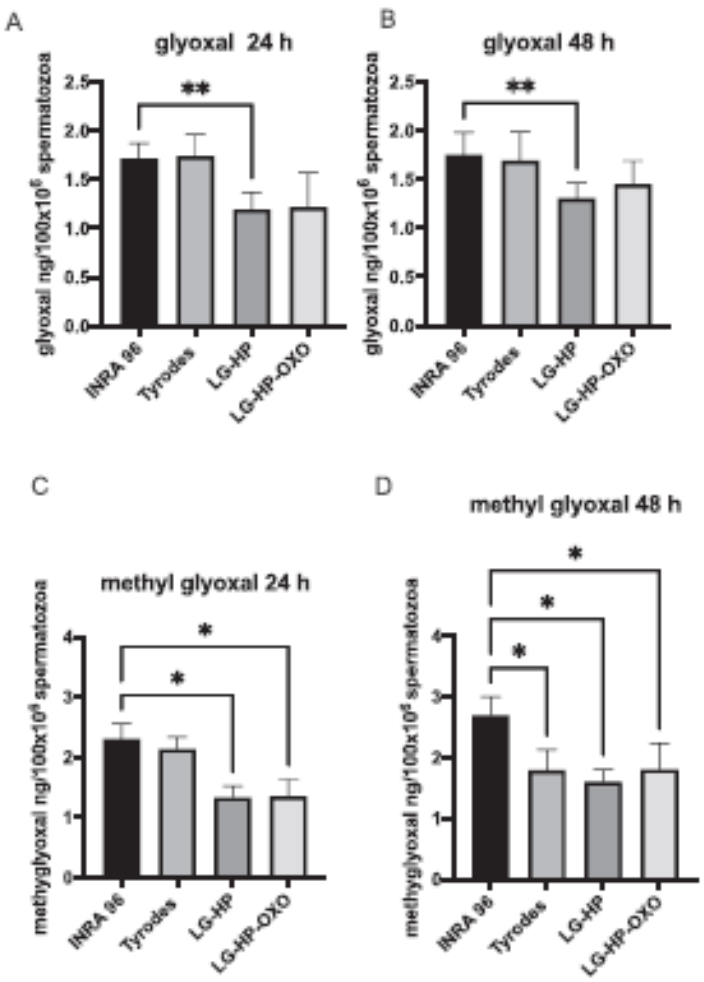

Figure 7.- Production of the 2-oxoaldehydes, glyoxal and methylglyoxal was measured using UHPLC/MS as described in material and methods after 24 and 48h of conservation at 18ºC in stallion spermatozoa extended in different media with different glucose concentration. Changes in the amount of glyoxal in ng/100 x 10^6 spermatozoa after 24 h (A) and 48h (B) of storage at 18ºC and changes in the amount of methylglyoxal in ng/100 x 10^6 spermatozoa after 24 h (C) and 48h (D) of storage at 18ºC * P<0.05, **P<0.01

Supplementary data

	Tyrodes	Low G – High P	Low G – High P + OXO
NaCl	96 mM	96 mM	96 mM
Na Pyruvate	1 mM	10 mM	10 mM
D-Glucose	5 mM	1 mM	1 mM
$NaHCO_3$	15 mM	15 mM	15 mM
Na L-Lactate	21.7 mM	21.7 mM	21.7 mM
$CaCl_2$ * $2H_2O$	2 mM	2 mM	2 mM
KCl	3.1 mM	3.1 mM	3.1 mM
$MgSO_4$ * $7H_2O$	0.4 mM	0.4 mM	0.4 mM
KH_2PO_4	0.3 mM	0.3 mM	0.3 mM
HEPES	20 mM	20 mM	20 mM
Dimethyl-2-oxoglutarate	-	-	0.1 mM
Peniciline G	0.058 g/L	0.058 g/L	0.058 g/L
Dihidrostreptomicyn	0.05 g/L	0.05 g/L	0.05 g/L
BSA	0.03%	0.03%	0.03%

Table S1. Components of the media

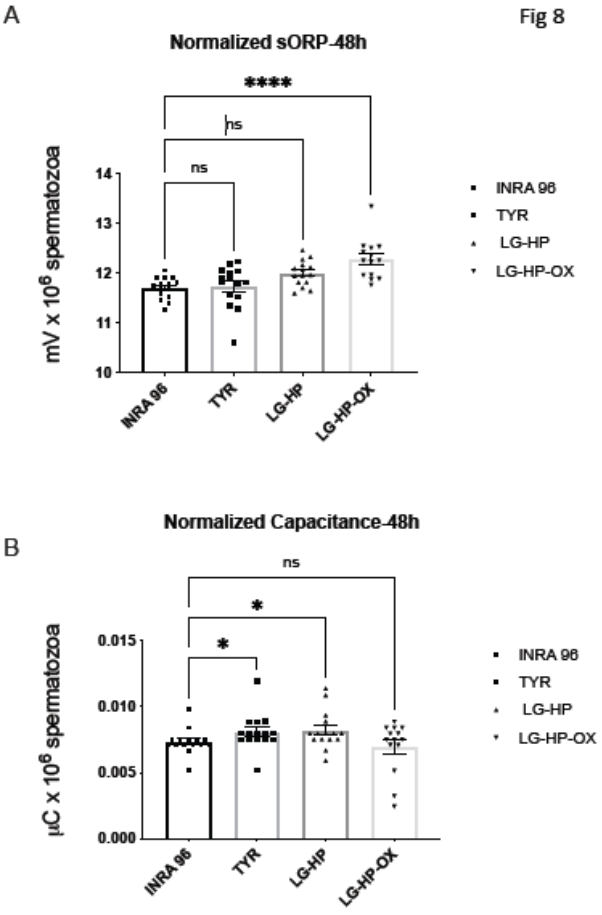

Figure S1B. Static oxidation reduction potential (sORP) and total antioxidant capacity (cORP) in split samples of stallion spermatozoa stored for 48 h in four different extenders. * P<0.05, ns: non-significant.