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An integrated overview on the regulation of sperm metabolism (glycolysis-Krebs cycle-oxidative phosphorylation)

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ABSTRACT

An overview of the sperm metabolism is presented; using the stallion as a model we review glycolysis, Krebs Cycle and oxidative phosphorylation, paying special attention to the interactions among them. In addition, metabolism implies a series of coordinated oxidation-reduction reactions and in the course of these reactions reactive oxygen species (ROS) and reactive oxoaldehydes are produced ; the electron transport chain (ETC) in the mitochondria is the main source of the anion superoxide and hydrogen peroxide, while glycolysis produces 2-oxoaldehydes such as methylglyoxal as byproducts; due to the adjacent carbonyl groups are strong electrophiles (steal electrons oxidizing other compounds). Sophisticated mechanisms exist to maintain redox homeostasis, because ROS under controlled production also have important regulatory functions in the spermatozoa. The interactions between metabolism and production of reactive oxygen species are essential for proper sperm function, and deregulation of these processes rapidly leads to sperm malfunction and finally death. Lastly, we briefly describe two techniques that will expand our knowledge on sperm metabolism in the coming decades, metabolic flow cytometry and the use of the "omics" technologies, proteomics and metabolomics, specifically the micro and nano proteomics/metabolomics. A better understanding of the metabolism of the spermatozoa will lead to big improvements in sperm technologies and the diagnosis and treatment of male factor infertility.

1. Introduction

Dr. Duane L Garner made great contributions to the field of animal reproduction, and in particular to spermatology. The research of the authors has been clearly influenced by Dr. Garner's legacy, particularly his contribution to the development of flow cytometry (flow spermetry), and its application to the understanding of the biology of this particular cell. Among many other inputs to the field, Dr. Garner made important contributions to the study of the sperm mitochondria with the aid of flow cytometry (Garner et al., 1997; Garner and Thomas, 1999; Gravance et al., 2000, 2001). Mitochondria are the central hub for sperm metabolism, and also control numerous functions in spermatozoa, including the regulation of their lifespan, Ca^{2+} control and signaling functions. Other reviews and previous research covered all these aspects and the reader is referred to these for further detailed information (Cummins, 2001; Lieburg-Apers et al., 2015; Plaza Davila et al., 2015; Darr et al., 2016a; Plaza Davila et al., 2016; Vertika et al., 2020; Boguenet et al.,

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2021).

The last decade has been witness to an unprecedented development of numerous techniques that is accelerating our knowledge about sperm biology. The strong foundation provided by basic research is essential for the improvement of sperm biotechnologies and the improvement of the comprehension and treatment of male factor infertility (Barratt et al., 2017; Cairo Consensus Workshop, 2020), among the techniques recently developed are the advances in flow cytometry, or flow spermetry, with the increased number of available probes, technical improvements in flow cytometers and the development of computational flow cytometry increasing the number of events and parameters measured in each single event. Modern flow cytometers allow 5–6 simultaneous parameters to be easily measured in 10^6 spermatozoa (Ortega-Ferrusola et al., 2017; Peña et al., 2018b; Ortiz-Rodriguez et al., 2021). In addition "omics" sciences (Fig. 1.), i.e. proteomics, genomics and metabolomics (Amaral et al., 2013, 2014a; Swegen et al., 2015; Asghari et al., 2017; An et al., 2018; Fu et al., 2019; Griffin et al., 2020; Long, 2020; Martin-Cano et al., 2020; Memili et al., 2020; Xu et al., 2020; Gaitskell-Phillips et al., 2021c) have led to a much greater understanding of sperm biology, and will surely further expand our knowledge on sperm biology in the coming years.

Mammalian spermatozoa have intense metabolic activity; proteomic studies and their corresponding bioinformatic analysis identify that, among the most abundant proteins and pathways in the male gamete, are those involved in metabolism (Amaral et al., 2013, 2014a; Swegen et al., 2015; Griffin et al., 2020; Martin-Cano et al., 2020; Gaitskell-Phillips et al., 2021a). Energetic metabolism consists of a series of reactions in which biological molecules are oxidized to simpler ones, and the energy released is used to phosphorylate adenosine diphosphate (ADP) to adenosine triphosphate (ATP) (Quijano et al., 2016; Trostchansky et al., 2016). Redox reactions and the tight regulation are key components of metabolism; these reactions are the transfer of electrons from reduced organic molecules to acceptors, nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide phosphate ($NADP^+$) or oxygen. Reactive oxygen species (ROS) like the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) are byproducts of these reactions; to maintain these reactions under control, spermatozoa are provided with sophisticated antioxidant systems in both seminal plasma (Gaitskell-Phillips et al., 2020) and the intracellular space (Ozkosem et al., 2016; Lee et al., 2017; O'Flaherty and Matsushita-Fournier, 2017; Fernandez and O'Flaherty, 2018; O'Flaherty, 2018; Fernandez et al., 2019; O'Flaherty et al., 2019; O'Flaherty, 2020).

While, traditionally, human spermatozoa have been considered as purely glycolytic cells (Storey, 2008; Calvert et al., 2019), it has been recognized recently that there is an important metabolic plasticity (Amaral et al., 2013; Calvert et al., 2019; Boguenet et al., 2021) and it is likely that factors in the milieu of the oviduct could induce deviation from a predominant metabolic pathway to another (Reynolds et al., 2017). In horse spermatozoa, growing scientific evidence indicates that oxidative phosphorylation is the principal mechanism producing energy both for motility and maintenance of membrane integrity (Gibb et al., 2014, 2015; Peña et al., 2015; Plaza Davila et al., 2015; Darr et al., 2016a, b; Plaza Davila et al., 2016; Swegen et al., 2016). Furthermore, spermatozoa can metabolize amino-acids, sugars, and fatty acids (Amaral et al., 2013; Martin-Cano et al., 2020). The recent discovery of the insulin receptor in spermatozoa underpin the complex metabolism of these cells (Aquila et al., 2005; Pitia et al., 2017; Aitken et al., 2021). In humans, supraphysiological concentrations of glucose occurring in diabetic conditions cause male infertility, and the mechanisms causing sperm malfunction share many of the aspects observed in horse spermatozoa stored in high glucose extenders (Amaral et al., 2006; Mallidis et al., 2007, 2009; Karimi et al., 2011; La Vignera et al., 2012; Liu et al., 2015; Pergialiotis et al., 2016; An et al., 2018; Imani et al., 2021; Simas et al., 2021). In this context, it is noteworthy that the trade of male gametes for artificial insemination is an important aspect in the horse breeding industry (Peña et al., 2011). This industry experienced a big expansion with the generalized introduction of artificial insemination and other assisted reproduction techniques in the second half of the past century. Most semen

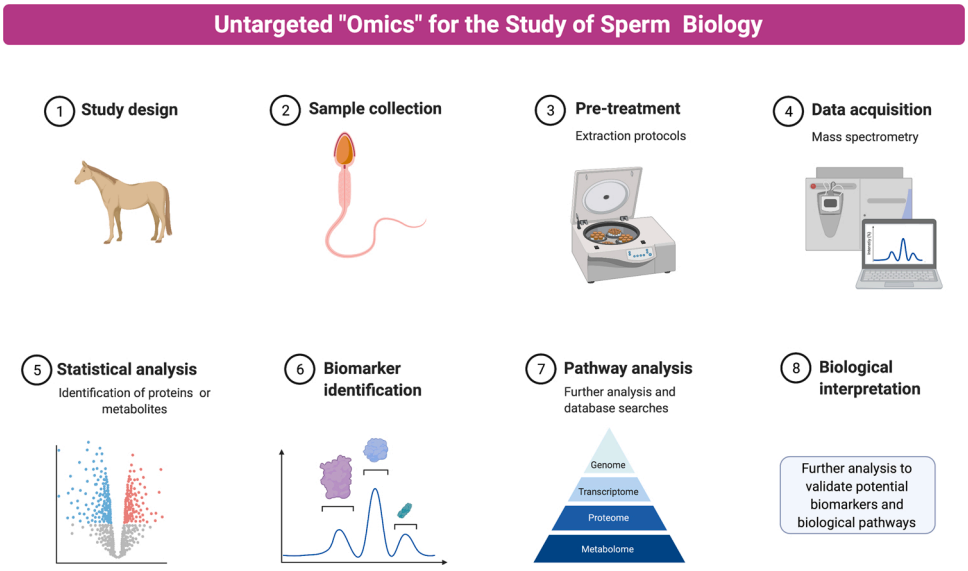

Fig. 1. Overview of the workflow in the "omics" technologies used in the study of sperm biology.

extenders were formulated around that time with large concentrations of glucose, well beyond the physiological concentrations of this hexose. According to initial research, extenders incorporated this sugar to provide physiological osmolality and a source of energy (Blanchard et al., 1987; Varner et al., 1988). However, current knowledge on horse sperm metabolism enters in conflict with the formulation of classic extenders. Therefore, in this review we will provide an updated overview of the current knowledge on sperm metabolism, using the horse as a model, and its cross talk with redox regulation as a major factor controlling sperm survival. Furthermore, techniques for the study of sperm metabolism will be briefly described.

2. Glycolysis

Glycolysis is the conversion of glucose into pyruvate, and is considered the principal metabolic pathway for the obtention of ATP in human (Williams and Ford, 2001) and pig (Marin et al., 2003) spermatozoa. Spermatozoa incorporate exogenous hexoses through specific transporters, GLUTs (Bucci et al., 2011). Once in the sperm cytoplasm, glucose is phosphorylated to glucose 6-phosphate, and then the use of different pathways is possible, including the pentose phosphate pathway (PPP), glycogen synthesis and glycolysis in which pyruvate is produced. The pyruvate molecule is further oxidized by the enzyme pyruvate dehydrogenase to Acetyl CoA. The electrons released in this process are accepted by NAD forming $NADH^{+}$. Although, for a long time, pyruvate was considered the main glycolytic product entering the mitochondria to feed the tricarboxylic acid or Krebs cycle, at present the reduction of pyruvate to lactate is thought to occur when there are aerobic conditions. Posterior intramitochondrial oxidation of lactate to pyruvate in the so called Mitochondrial Lactate Oxidation Complex makes pyruvate obtained after intra-mitochondrial oxidation of lactate a key substrate for mitochondrial energetics (Brooks, 2018). Evidence of the importance of lactate in the energetic metabolism of horse spermatozoa has recently been reported, lactate seems more efficient than pyruvate at sustaining horse sperm motility (Darr et al., 2016b); a lactate dehydrogenase (LDH) detected in the mitochondrial matrix converts lactate to pyruvate (Swegen et al., 2015). The importance of lactate was initially reported about 50 years ago; Storey and Kayne (Storey and Kayne, 1977) described the aerobic oxidation of lactate in rabbit sperm mitochondria. Monocarboxilate transporters (MCTs) have been detected in spermatozoa (Brooks, 2018), particularly MCT1 has been identified in the sperm head (Garcia et al., 1995). Also, in bull spermatozoa, lactate maintains sperm motility as well, or even better, than glucose (Inskeep and Hammerstedt, 1985). Interestingly, in the testis, Sertoli cells secrete lactate rather than glucose to fuel sperm motility, thus, the relation Sertoli cells with spermatozoa constitute a cell-to-cell lactate shuttle (Gladden, 2004; Brooks, 2018). As previously described in this manuscript, evidence of oxidation of lactate to pyruvate has been detected in horse spermatozoa, and also in pig spermatozoa, in which oxidation of external lactate is inhibited in the presence of the MCT inhibitor α-cyano-4-hydroxicinnamate and by the LDH oxamate inhibitor, indicating that lactate is transported into sperm mitochondria where it is oxidized to pyruvate (Gladden, 2004; Brooks, 2018). Results from different studies indicate gluconeogenesis-linked glycogen metabolism is present in spermatozoa (Ballester et al., 2000; Palomo et al., 2003; Albarracin et al., 2004). In this model lactate is incorporated by mature spermatozoa, and converted into glycogen; in dogs, at least, this is considered to have a major function in providing energy for capacitation.

3. The dark side of glycolysis

Formation of ATP from glucose is not a perfect process; this pathway includes a series of steps for the elimination of phosphates. Phosphate is eliminated from the triose phosphates glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) (Deponte, 2013). During this process, glyoxal (G) and methylglyoxal (MG), which are both 2-oxoaldehydes, are continuously produced. These products are also generated during lipid metabolism. It has recently been reported that these compounds are produced during storage of horse spermatozoa in commercial extenders that are formulated with very large glucose concentrations (Ortiz-Rodriguez et al., 2021). It has also been reported that with using extenders formulated with 1 mM glucose and 10 mM pyruvate, sperm functionality can be maintained and production of 2-oxoaldehydes significantly reduced. Due to the adjacent carbonyl groups (Fig. 2), 2-oxoaldehydes are strong electrophiles that rapidly and spontaneously react with nucleophiles from proteins, lipids and DNA originating advanced glycation end products (AGEs) (Nevin et al., 2018).

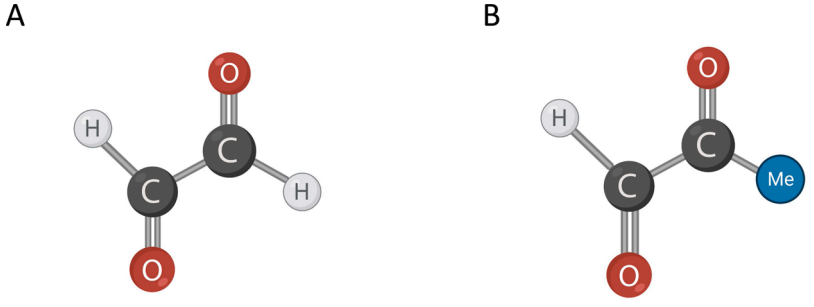

Fig. 2. Chemically glyoxal (A, left) and methyl glyoxal (B, right) are 2-oxoaldehydes. Due to their adjacent carbonyl groups 2-oxoaldehydes are strong electrophiles that rapidly and spontaneously react with nucleophiles from proteins, lipids and DNA causing significant sperm damage. These compounds are byproducts of glycolysis, and their production is proportional to the amount of glucose present in the media.

Furthermore, MG can form adducts with superoxide dismutase 1 (SOD-1) impairing the antioxidant action of this enzyme and promoting oxidative stress (Polykretis et al., 2020). Consistent with this finding, results from recent research from our laboratory indicated SOD-1 was one of the most important antioxidant systems in mammalian spermatozoa (Gaitskell-Phillips et al., 2021a,b). This is a similar process as occurs with hydrogen peroxide, which functions as a regulatory molecule but may induce marked cellular damage if redox homeostasis is lost. Control mechanisms involving conjugation of 2-oxoaldehydes with glutathione (GSH) exist (Quijano et al., 2016). In this sense, the importance of GSH for sperm functionality has recently been stressed with the finding of the Slc7a11 x-CT glutamate/cystine antiporter in horse spermatozoa (Ortiz-Rodriguez et al., 2019, 2020). This antiporter exchanges intracellular glutamate for extracellular cystine and is constitutively expressed in horse spermatozoa; very few cells constitutively contain this protein including the thymus, spleen and brain (Conrad and Sato, 2012). The mRNA transcript has been detected in the testis and *Slc7a11* knockout mice are sub-fertile (Hamashima et al., 2017). This protein is upregulated in many cancers cell lines (Banjac et al., 2008; Koppula et al., 2018). Once incorporated, cystine is reduced intracellularly to cysteine and used for GSH synthesis (Ortega-Ferrusola et al., 2019; Ortiz-Rodriguez et al., 2019). The evidence indicating GSH synthesis in spermatozoa also include the presence of the necessary enzymes, with the identification of glutathione synthetase (GSS) and gamma-glutamylcysteine ligase (GCLC); functional studies using the specific GCLC inhibitor, L-Buthionine sulfoximide (BSO); and direct measurement of GSH using mass spectrometry (Ortega-Ferrusola et al., 2019).

Glucose and fructose have been the base of most extenders for semen conservation in animal breeding. However, a growing body of evidence obtained from scientific research indicates that this approach needs extensive revision. Extenders for horse spermatozoa currently in use have glucose concentrations ranging from 80 to 300 mM, while oviductal concentrations of glucose are in the micromolar range. Sugars are becoming the new foe to defeat by public health systems and it is now evident that excessive consumption of highly processed sugars contribute to diseases like obesity, diabetes, cardiovascular diseases, many types of cancer and neurodegenerative diseases (Malik et al., 2019; Clinton et al., 2020; Kashino et al., 2021). In a similar fashion, excessive sugar content in extenders may be harmful to spermatozoa. There has been an intense debate among spermatologists regarding the main source of energy in spermatozoa; however, while species-specific differences may occur, spermatozoa are able to use different pathways. It is noteworthy that this was reported as early as the first decades of the past century by researchers at the Universities of Wisconsin and Pennsylvania (Storey, 2008). Regarding horse spermatozoa, recent biochemical research has provided relevant information that can be summarized indicating that these are cells in which oxidative phosphorylation is the main source of energy for maintenance of sperm function and, therefore, there is an abundance of mitochondrial activity and production of the $O_2^{\bullet -}$. However, glycolysis may be necessary to feed glycolytic enzymes in the flagella (Kim et al., 2007; Plaza Davila et al., 2015; Plaza Davila et al., 2016). In addition, results from previous studies suggest that horse spermatozoa have a noteworthy metabolic plasticity, and that amino-acids and fatty acids are relevant sources of energy (Amaral et al., 2013). In particular, proteomic studies indicate that horse spermatozoa have a relevant capacity to metabolize fatty acids (Gibb et al., 2015; Swegen et al., 2015; Martin-Cano et al., 2020). These findings have been conducive to the development of new extenders with the ability to significantly extend the lifespan of spermatozoa while stored in liquid state (Gibb et al., 2015, 2018). These improvements are due to two main factors: reduction of glucose toxicity and a more efficient sperm metabolism. Excess glucose, due to supraphysiological concentrations of inefficient glucose utilization, causes cellular damage (Brownlee, 2001).

Diabetic conditions are extremely prevalent in humans, and thus extensive research has been conducted on the molecular mechanisms behind glucose toxicity. When spermatozoa are extended in currently available commercial media, these germ cells are exposed to supraphysiological glucose concentrations. Physiological glucose in serum in horses is 5 mM (Wright et al., 2019) while oviductal glucose concentrations are much lower, with maximal concentrations reported as being 300 μM (Campbell et al., 1979). Obviously, extenders in use expose horse spermatozoa to supra-physiological glucose concentrations potentially leading to glucose toxicity (Liemburg-Apers et al., 2015) involving different mechanisms, such as production of 2-oxoaldehydes as described in the previous section, direct induction of ROS by glucose; activation of MAP kinase and Ca^{2+}-mediated mitochondrial fission (Nishikawa et al., 2000; Terrell et al., 2012); activation of the polyol pathway consuming NADPH and producing GSH depletion (Brownlee, 2001). Also, hyperglycemia activates a particular metabolic route that involves diacylglycerol (DAG) protein kinase C (PKC) and NADPH-oxidase leading to greater than optimal production of ROS and mitochondrial damage further activating mitochondrial production of $O_2^{\bullet -}$. Excess $O_2^{\bullet -}$ may inhibit glyceraldehyde 3-phosphate dehydrogenase (GAPDH) diverting metabolites upstream of the glycolysis pathway, resulting in increased flux of dihydroxyacetone phosphate (DHAP) to diacylglycerol, that activates protein kinase C (PKC) (Nishikawa et al., 2000; Brownlee, 2001). Furthermore, DHAP is precursor of the oxoaldehyde MG (Ihnat et al., 2007; Ceriello and Testa, 2009). In addition, large glucose concentrations predispose cells to apoptosis, ferroptosis, necroptosis and other types of cell death (LaRocca et al., 2016). Therefore, the situation regarding current protocols used in sperm conservation in the horse breeding industry constitute a natural model of sperm damage induced by hyperglycemia.

4. Pentose phosphate pathway (PPP)

The PPP is the main source of NADPH, although this compound can also result from degradation of products of the tricarboxylic acid cycle (TCA), and from the oxidation of fatty acids and utilization of ketone bodies (Horecker, 2002; Patra and Hay, 2014; Dick and Ralser, 2015; Cherkas et al., 2020). The PPP comprises two branches, the oxidative branch that leads to the generation of NADPH and ribonucleotides, and the nonoxidative branch that consists of reversible reactions that recruit glycolytic intermediates to be converted into pentose phosphates in a reversible manner (Patra and Hay, 2014). In the oxidative branch, the first reaction is the dehydrogenation of glucose-6-phosphate by the action of the glucose-6-phosphate dehydrogenase (G6PD) enzyme to produce NADPH and 6-phosphogluconolactone, that is then hydrolyzed by phosphoglucolactonase into 6-phosphogluconate. The oxidative decarboxylation

of 6-phosphogluconate is then catalyzed by 6-phosphogluconate dehydrogenase to yield NADPH again and ribulose-5-phosphate that is converted into ribose-5-phosphate (Patra and Hay, 2014). In the context of this review, it is noteworthy that the production of NADPH for the reduction of oxidized glutathione (GSSG) into reduced glutathione (GSH) is the main function of this pathway in spermatozoa (Urner and Sakkas, 1999, 2005; Miraglia et al., 2010; Evdokimov et al., 2015).

5. Tricarboxylic acid cycle

The citric acid cycle, Krebs cycle or tricarboxylic acid cycle (TCA) is a series of reactions occurring in a closed loop (Martinez-Reyes and Chandel, 2020). This cycle is initiated with a reaction combining acetyl-CoA (2C) with oxaloacetate (OAA, 4C) to form citrate (6C). Acetyl-CoA can be generated from the oxidation of pyruvate, from fatty acids and from the metabolism of amino acids, particularly leucine, isoleucine, and tryptophan. Results from recent studies indicate that lipid and amino acid metabolism has largely been neglected in spermatozoa (Terrell et al., 2011a; Amaral et al., 2013; Swegen et al., 2015; Martin-Cano et al., 2020). Also, pyruvate can be produced from the conversion of citrate by the mitochondrial enzyme acetyl-CoA synthetase short chain family member 1 (ACSS1), and this enzyme has been detected in recent proteomic studies in horse spermatozoa (Martin-Cano et al., 2020). Citrate is converted into its isomer, isocitrate, and the cycle continues with two oxidative decarboxylation reactions; isocitrate is converted into α-ketoglutarate (α-KG, 5C) and shortly afterwards to succinyl-CoA (4A), releasing two molecules of CO_2 and generating two NADH. The next step involves the conversion of succinyl-CoA into succinate; this reaction is coupled to the generation of guanosine-5′-triphosphate (GTP), which may be converted into ATP (Martinez-Reyes and Chandel, 2020). Succinate is then oxidized to fumarate (4C), and two hydrogen atoms are transferred to flavin adenine dinucleotide (FAD) forming two $FADH_2$ molecules, through the action of the succinate dehydrogenase (SDH) enzyme. Subsequently, fumarate is converted into malate, which is subsequently converted into oxaloacetate that combines with another molecule of acetyl CoA to close the circle and continue the cycle (Martinez-Reyes and Chandel, 2020). Although the initial step in the cycle is the formation of citrate from acetyl-CoA and oxalacetate (OAA), the cycle can be fed at different points, including the conversion of pyruvate to OAA by pyruvate decarboxylase and glutaminolysis, which is the conversion of glutamine into glutamate and then to α-ketoglutarate (Martinez-Reyes et al., 2016). In addition, lactate oxidation is now recognized as an important molecule feeding the TCA cycle (Martinez-Reyes and Chandel, 2017).

6. Oxidative phosphorylation (OXPHOS)

Mitochondria are semiautonomous organelles essential for cellular energetics producing most ATP through oxidative phosphorylation (OXPHOS) (Vakifahmetoglu-Norberg et al., 2017). This is a metabolic pathway in which enzyme functions are coordinated by a cascade of oxidation-reduction reactions organized in protein complexes (I–V) and two soluble factors, cytochrome c and coenzyme Q. The enzymes are situated in the inner mitochondrial membrane (Vakifahmetoglu-Norberg et al., 2017). This set of proteins is known as the electron transport chain (ETC). The ETC transfers electrons to oxygen, that is reduced to water, and the energy generated in this process is used to produce ATP. The ETC is coupled to the TCA cycle, the latter produces the electron transport carriers NADH and $FADH_2$, that donate electrons to the ETC. In the ETC, complexes I and II mediate the transfer of two electrons from NADH and $FADH_2$, respectively, to coenzyme Q, that can also receive electrons from the oxidation of fatty acids, and the metabolism of amino acids and choline. Complex III receives two electrons from reduced coenzyme Q and transfers these two electrons to cytochrome C to reduce O_2 into water. These series of redox reactions induce conformational changes in the ETC that responds by pumping H^+ into the intermembrane space creating an electrochemical gradient denominated mitochondrial membrane potential, which can be measured using probes like JC-1 (Peña et al., 2018a, b). Also, the H^+ driven force generated by complexes I, III and IV is used by complex V or ATP synthase to phosphorylate ADP and produce ATP. Results from numerous studies reveal that the horse spermatozoon is a cell highly dependent on the ATP generated in the ETC (Terrell et al., 2011b; Gibb et al., 2014, 2015; Plaza Davila et al., 2015; Swegen et al., 2015; Varner et al., 2015; Darr et al., 2016a, b; Plaza Davila et al., 2016; Darr et al., 2017; Ortega Ferrusola et al., 2017; Griffin et al., 2019; Peña et al., 2019; Martin-Cano et al., 2020).

7. Production of reactive oxygen and nitrogen species in the TCA cycle and ETC

The production of the radical superoxide $O_2^{\bullet -}$ in mitochondria is well recognized, and the ETC is reported to be a major source. The addition of a single electron to O_2 forms $O_2^{\bullet -}$; it is believed that under physiological conditions up to 2% of total oxygen is converted into $O_2^{\bullet -}$. This conversion occurs in complexes I and III. Other sources of mitochondrial ROS exist because these compounds can be produced by different flavoenzymes in mitochondria, and TCA cycle specific enzymes can also be relevant sources of ROS, particularly α-ketoglutarate dehydrogenase and glycerophosphate dehydrogenase (Tahara et al., 2009). While current evidence indicates that this production may be tissue specific, extensive research in spermatozoa has not yet been conducted. Even with this paucity of information, the results reported indicate that unregulated production of ROS is a contributor to sperm malfunctions, and the ETC in mitochondria appears as the main source of ROS in spermatozoa (Plaza Davila et al., 2015; Plaza Davila et al., 2016). The anionic character of $O_2^{\bullet -}$ limits the capacity of this molecule to diffuse across membranes, with most of the reaction occurring in mitochondria. Main reactions are the spontaneous or catalyzed dismutation to H_2O_2, direct reaction with FeS centers and the reaction with nitric oxide $^{\bullet}NO$ leading to the formation of peroxynitrite ($ONOO^-$)(Quijano et al., 2016; Trostchansky et al., 2016). Hydrogen peroxide, a non-radical oxidant, can diffuse across cellular membranes. This is a weak oxidant with important regulatory functions involving the reversible oxidation of thiol in cysteine residues; however, its reaction with metal centers can produce the highly toxic hydroxyl radical ($^{\bullet}OH$). The peroxynitrite anion is a stable molecule and a very potent oxidant able to react with CO_2 and electrophilic transition metal

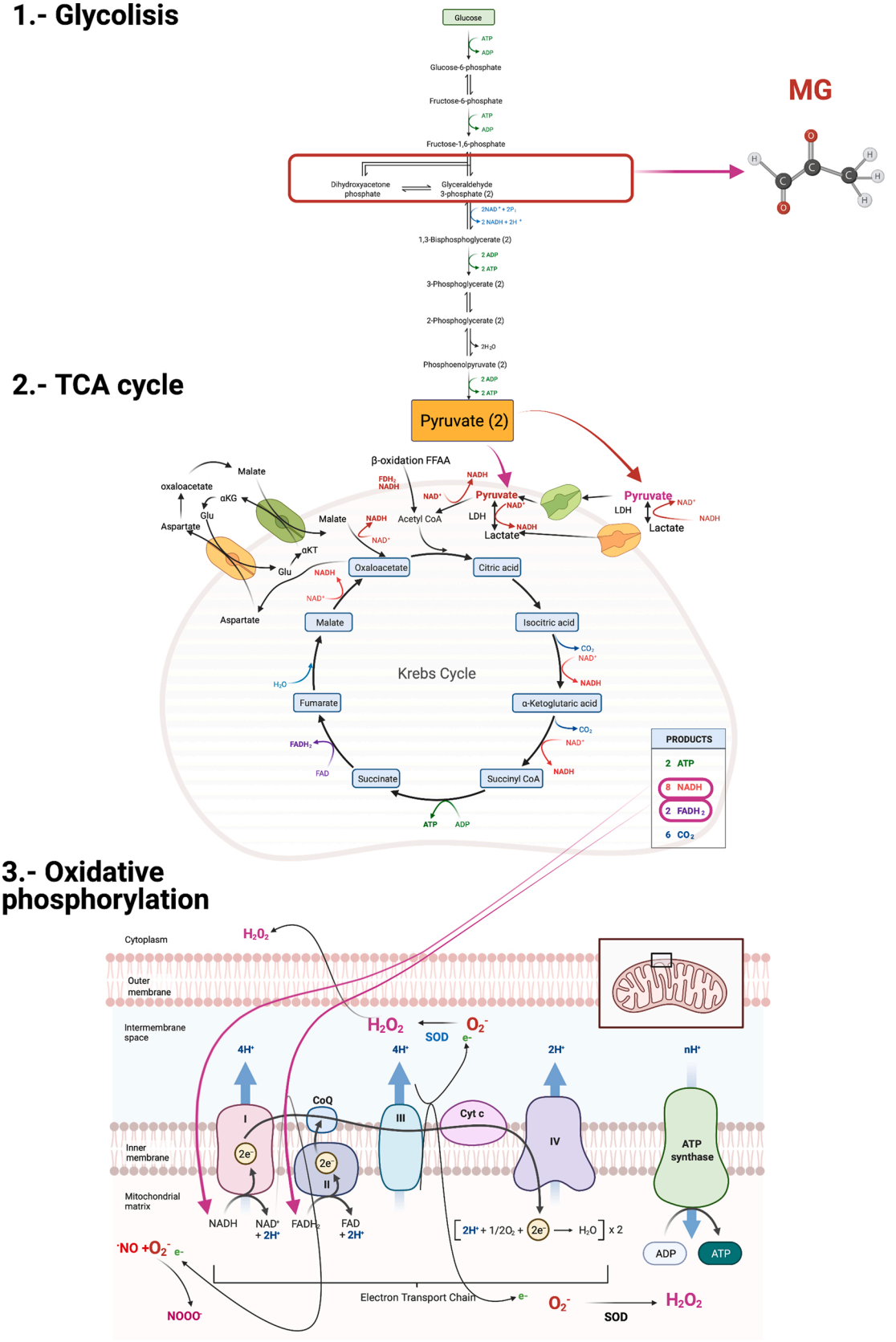

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Fig. 3. Overview of the interactions between glycolysis, TCA cycle and oxidative phosphorylation in spermatozoa. Glycolysis produces pyruvate that feeds the TCA cycle, this produces NADH and $FDAH_2$ that are used as electron transporters in the ETC, that finally produces ATP. As sub-products of glycolysis 2-oxoaldehydes MG and G are produced; however, ROS are byproducts of the ETC. A tight regulation of the interactions between metabolism and redox homeostasis is essential for optimal sperm function.

centers yielding different potent oxidants including nitrogen dioxide (NO_2), carbonate radical (CO_3-$^{\bullet}$) and oxo-metal complexes. Peroxynitrous acid (ONOOH), however, can undergo a proton catalyzed dissociation to NO_2 and $^{\bullet}$OH. All these peroxynitrite-derived radicals can oxidize and induce peroxidation and nitration of many mitochondrial components. Nitric oxide ($^{\bullet}$NO) and nitric oxide derived species, also have important regulatory functions in sperm mitochondria (Zini et al., 1995; O'Bryan et al., 1998). In Fig. 3, an overview of glycolysis, TCA cycle and OXPHOS and the interactions is depicted.

8. The near future

8.1. Use of flow cytometry to evaluate sperm metabolism

Although the most common uses of flow cytometry in veterinary andrology are still measurement of viability, acrosome integrity and DNA fragmentation, flow cytometry is a very versatile technique able to measure multiple parameters in a large number of cells. The research of Dr. Garner was pioneering in the field of the study of sperm metabolism, and the development of flow cytometry assays through JC-1 represent a significant part of the research of Dr. Garner in the field of flow cytometry (Garner et al., 1997; Garner and Thomas, 1999; Gravance et al., 2000, 2001). As previously described in this manuscript, mitochondria constitute the central energetic hub of spermatozoa. Although JC-1 provides significant information, the use of dual excitation, with a yellow laser to excite aggregates (highly active mitochondria, high mitochondrial membrane potential) and a blue laser to excite monomers (inactive mitochondria, relatively lesser mitochondrial membrane potentials), is recommended to obtain more precise data. Additionally, it is important to understand that not all mitochondrial probes are equivalent, whereas probes like JC-1 measure mitochondrial membrane potential, mitotracker deep red measures mitochondrial mass. The reader is referred to recent review and research papers for further details on the topic (Robinson et al., 2015; Peña et al., 2018b; MacDonald et al., 2019). Additionally, fluorescent probes are available to measure low oxygen consuming cells (Hipoxia Greeen Reagent, ThermoFisher Scientific, Waltham, MA, USA), glucose uptake, through fluorescent glucose analogues (Bala et al., 2021), and ATP using fluorescence detectors (Rajendran et al., 2016; Forveille et al., 2019; Fan et al., 2020). However, the real power of flow cytometry for the study of sperm metabolism relies on the ability to apply multiple probes on a single cell base analysis. Recent strategies for single cell metabolic analysis based on flow cytometry have been published. The application of this technique, which is called Met-Flow (Bala et al., 2021), will improve our understanding of sperm biology.

8.2. Mass spectometry (MS)

The progressive introduction of mass spectrometry to the study of spermatozoa has produced unprecedent advances in the comprehension of the biology of these cells; particularly the predominance and plasticity of sperm metabolism (Amaral et al., 2013, 2014b; Codina et al., 2015; Swegen et al., 2015; An et al., 2018; Engel et al., 2019; Boguenet et al., 2020; Chen et al., 2020; Griffin et al., 2020). Due the importance of this technique, understanding its basic principles is necessary for any spermatologist. A mass spectrometer is a device for measuring the mass-to-charge ratio of ionized molecules; the output of a MS is intensity compared with mass to charge ratio (m/z) (Matthiesen and Bunkenborg, 2020). These machines are composed of three main parts: ion source, mass analyzer, and detector. The ion source generates ions by transferring molecules (peptides in the case of proteomics) from the condensed (liquid or solid) phase to gas phase, ionizing them in the process (either positive or negative charge state). The most commonly ionization methods are electrospray ionization (ESI) and matrix assisted laser desorption and ionization (MALDI). The ions produced in the ion source are transferred to the mass analyzer where they are separated according to the mass to charge ratio (m/z). There are different types of analyzers, based on different principles, but all of these instruments ultimately transform the intensities of the signal to a function of m/z values. For example, time of flight instruments accelerates ions and measures the flight time between acceleration and hitting the detector. Measurement of this time enables calculation of accurate m/z values and the results are then visualized by an m/z versus intensity plot also referred as spectrum (Matthiesen and Bunkenborg, 2020). Mass spectrometers are coupled to a liquid chromatography column (for proteomics/metabolomics) or gas chromatography (for metabolomics/lipidomics); this allows acquisition of MS spectra as analytes eluting from the chromatography column, and each spectrum in the tandem mass spectrometer (MS/MS) is further interrogated. For proteomics applications, the peptides are analyzed by the instrument software to select ions that are isolated, fragmented and analyzed by a mass analyzer to generate MS/MS spectrum. Furthermore, the most prominent features of the spectrum are extracted and used to query a protein sequence database, such as uniprot (https://www.uniprot.org), using a specific software that compares the observed fragments to fragments obtained by *in silico* enzyme digestion and fragmentation. An overview of MS technology is depicted in Fig. 4.

A common approach in spermatology is "shot gun" proteomics, where proteins are trypsin digested and a complex mixture of peptides are subjected to high performance liquid chromatography (HPLC) coupled to the mass spectrometer. Another advantage of mass spectrometry is that post-translational modifications of proteins, and the site of the modification can be identified, as well as modifications in amino acids such as phosphorylation, acetylation and methylation, which regulate many sperm functions.

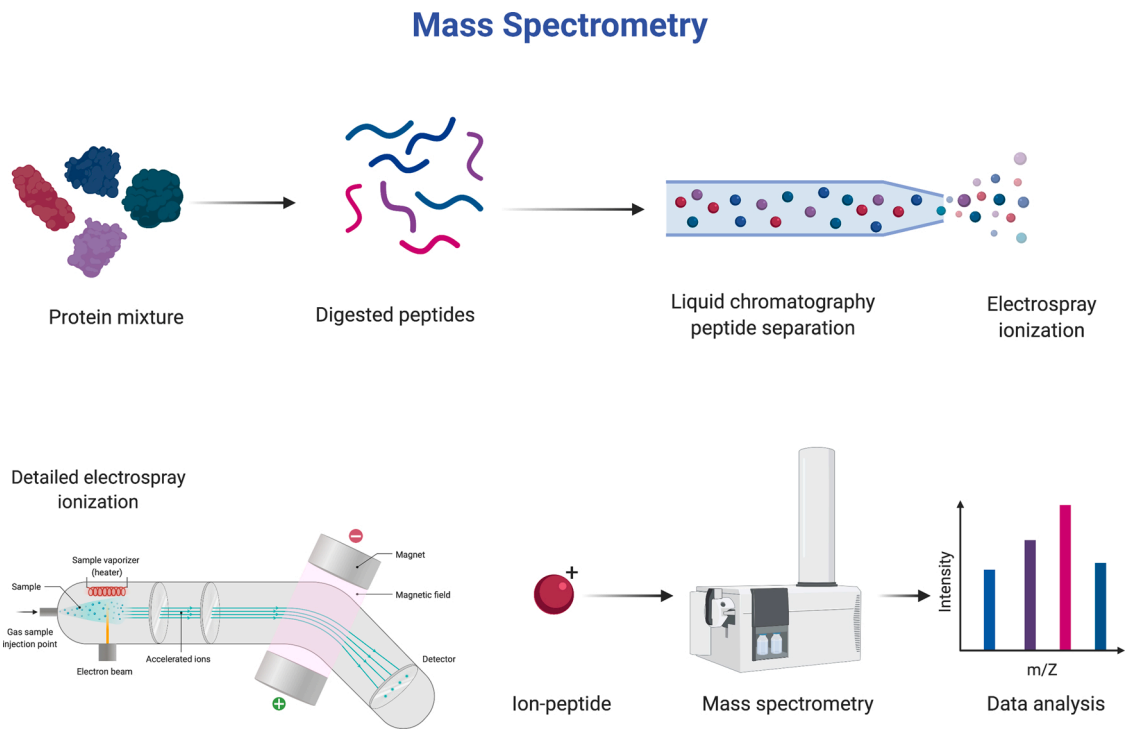

Fig. 4. Overview of the flow of mass spectrometry analysis.

9. Conclusions

Spermatozoa are cells with intense energetic demands that change throughout the lifespan of these gametes. From the time of spermatozoa formation in the germinal epithelia to sperm transport through the female reproductive tract, the processes of capacitation and fertilization depend on adequate sources of energy. Energetic metabolism implies numerous oxidation-reduction reactions and production of reactive oxygen species is unavoidable and proper control of their production is necessary for correct sperm function. Thus, the study of the interactions between metabolism and redox homeostasis is an essential field to improve understanding of male factor infertility in humans and sperm biotechnologies in humans and animals. While most production of reactive oxygen species occurs in the ETC, glycolysis also generates byproducts that are highly reactive electrophiles, mainly methyglyoxal. Results from recent studies reveal sperm damage induced by supraphysiological concentrations of glucose in commercial extenders for horse semen. Interestingly, the molecular mechanisms that result in this glucose-induced damage share numerous aspects with the infertile condition that exists with diabetic patients, thus making for a natural model for the study of infertility that is associated with this disease.

Author contributions

FJP conceived and wrote the manuscript. All authors contributed to literature review, drafting and approved the final version.

Declaration of Competing Interest

The authors report no declarations of interest.

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