The stallion spermatozoa: A valuable model to understand the interplay between metabolism and redox (de)regulation in sperm cells

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ABSTRACT.

Significance Proper functionality of the spermatozoa depends on the tight regulation of their redox status, at the same time these cells are very energy demanding, and in the energetic metabolism, principally in the electron transport chain (ETC) in the mitochondria, reactive oxygen species (ROS) are continuously produced, but also in the Krebs Cycle and during the beta-oxidation of fatty acids. **Recent advances** Additionally, in the glycolysis, elimination of phosphate groups from glyceraldehyde 3-phosphate and dihydroxyacetone phosphate originates as byproducts glyoxal (G) and methylglyoxal (MG); these products are 2-oxoaldehydes. The presence of adjacent carbonyl groups make them strong electrophiles that react with nucleophiles of proteins, lipids and DNA, forming advanced glycation end products (AGEs). **Critical Issues.** This mechanism is behind subfertility in diabetic patients; in the animal breeding industry, commercial extenders for stallion semen contain a supraphysiological concentration of glucose that promotes methylglyoxal production, constituting a potential model of interest. **Future directions.** Increasing our knowledge on sperm metabolism and its interactions with redox regulation, may improve current sperm technologies in use, and shall provide new clues to for the understanding of infertility in males. Moreover, stallion spermatozoa due to its accessibility, intense metabolism, and suitability for proteomics/metabolomic studies may constitute a suitable model for studies of the regulation of metabolism and the interaction between metabolism and redox homeostasis.

Key words: spermatozoa, extenders, glucose, ROS, metabolism, methylglyoxal,

INTRODUCTION

Spermatozoa are characterized by highly active energetic metabolism, and detailed studies using proteomic approaches show that significantly present pathways in the male gamete, are proteins with metabolic functions (6,7,50,63,100,159), sustaining the importance of the metabolism in these cells. The energetic metabolism is a process in which ATP is generated from the oxidation of nutrients. Consists of reactions in which biological molecules are oxidized to simpler molecules; energy released in these processes is harnessed to phosphorylate ADP to ATP (144,167).

Redox reactions must be tightly regulated and are key components of the metabolism; transfer of electrons from reduced organic molecules to acceptors, NAD^+, $NADP^+$ or oxygen are the base of redox reactions. Reactive oxygen species (ROS) like $O_2^{\bullet -}$ and H_2O_2 are byproducts of redox reactions occurring in the metabolism.

Spermatozoa are provided of sophisticated antioxidant systems in the seminal plasma (52), and in the spermatozoa itself (45,46,83,111,112,114,119,129) to maintain these reactions under control. In the functionality of the spermatozoa, redox reactions play a major role; reversible oxidation of thiols in cysteine residues of key proteins constitute an "on-off" switch for the regulation of key spermatic functions. In case these redox reactions lose proper regulation, these residues may experience irreversible oxidation leading to the alteration of the function and ultimately death of the spermatozoa (135) (Fig 1).

While is widely accepted that the human spermatozoa are cells that produce energy mainly through glycolysis (24,156), recent research shows an important metabolic plasticity (6,18,24); is possible the influence of oviductal factors regulating the switch among metabolic pathways in the spermatozoa (145). However, the mechanisms regulating the switch from predominance of one type of metabolism to another remain largely unknow, although recent research is providing important information in this topic as will be discussed in pertinent sections of this review (66,180-182).

On the contrary, growing scientific evidence shows that oxidative phosphorylation in the horse is the main route producing ATP to be used for motility and to support the integrity and functionality of the plasmalemma (34,36,37,57,59,137,140,160) in the spermatozoa. The stallion spermatozoa have a limited glycolytic activity although glycolysis may support sperm velocity through glycolytic enzymes in the flagellum (37), but, despite this scientific evidence, commercial extenders contain supraphysiological amounts of glucose.

Moreover, recent evidence show that the spermatozoa have an important metabolic plasticity, these cells can use amino-acids, sugars, and fatty acids and source of energy (100). The identification of the receptor for insulin in the spermatozoa, highlights the sophisticated metabolism of the male germ cells (2,11,139). The supraphysiological concentrations of glucose seen in diabetic conditions in human beings cause male infertility. The molecular pathways leading to sperm malfunction in diabetic patients share many of the aspects seen in stallion spermatozoa stored in extenders containing high glucose concentrations (8,9,73,77,81,89,96,97,138,152).

The commerce of equine semen for artificial insemination is an important aspect of horse breeding (133). The generalized introduction of artificial insemination and other techniques of assisted reproduction in the second half of the past century caused a big expansion of this commerce of genetic material. The majority of the semen extenders were formulated by that time, are based on high concentrations of glucose, well beyond of physiological concentrations of this hexose, and are still in use. Initially extenders were designed with this concentration of sugar to provide physiological osmolality and a source of energy (16,171). However, the formulation of classic extenders enters in conflict with current scientific information on the sperm metabolism.

The aim of this review is to offer an updated overview of the current knowledge regarding the cross-link between metabolism and redox regulation as a major factor determining sperm viability, using the stallion spermatozoa as a model of the sperm malfunction caused by supraphysiological concentrations of glucose. For this, the terms "oxidative stress", "redox regulation", "spermatozoa" "interaction between metabolism and redox regulation/oxidative stress in the spermatozoa", were compiled after exhaustive literature search in the Web of Science and PubMed. We only included articles published in English and available until the end of September 2021.

Applicable selected publications referring to these concepts in other cellular models were included to provide comparative insights.

Overview of sperm physiology

These cells are formed in the germinal epithelium in the testis; this is formed by germ cells in different stages of development, intermingled with Sertoli cells that proportionate structural support and nursing. The formation of the spermatozoa is highly regulated and complex phenomena, involving serial steps of stem cell proliferation and renewal, genetic remodeling, and reduction of chromosomes accompanied by major morphological transformations (32) .

The first step of the spermatogenesis is the differentiation of spermatogonia from a stem cell pool. The next step, spermatocytogenesis is characterized by numerous mitotic divisions increasing the number of spermatogonia. Then a meiotic phase includes duplication and exchange of genetic information and two meiotic divisions to form round haploid spermatids. During the spermiogenesis phase round spermatids experience compaction, silencing of DNA, elongation of the nucleus, and most of the histones are replaced by transition proteins and then by protamines. Development of the tail of the spermatozoa from the centriole, the acrosome (from the Golgi´s apparatus), the mitochondria relocate to the midpiece, and other organelles and most of the cytoplasm are lots in this phase. Finally, morphologically mature spermatozoa are released in the lumen of the seminiferous tubules during spermiation (19,55,150,153). Proteomic studies disclose critical metabolic changes during sperm maturation, affecting proteins involved in redox regulation and lipid and carbohydrate metabolism (146).

Cellular generation of ROS was identified for the first time in the spermatozoa (166), bull spermatozoa were able to generate H_2O_2 as consequence of cellular respiration. The H_2O_2 produced inhibited cellular respiration; to prevent this effect was concluded that bovine spermatozoa had mechanisms for the elimination of H_2O_2 at a low rate, and thus maintaining H_2O_2 at physiological concentrations. The concept of ROS as toxic byproduct of sperm metabolism without any role was largely considered in the past; but nowadays, it is known that crucial functions of the spermatozoa are redox regulated, and redox regulation is nowadays a major area of research in the study of sperm biology (38,83,92,110,116-118,120). Countless cellular processes are redox regulated.

In spermatozoa, redox regulation has been extensively studied in relation to capacitation (5,43,83,93,110,154,155,173). The maturational process that sensitizes spermatozoa to recognize and fertilize the oocyte is termed capacitation. This process includes changes in the spermatozoa such as loss of cholesterol from the plasma membrane, removal of coating materials from plasmalemma, a rise in intracellular Ca^{2+}, increase in intracellular cAMP, and an increase in the phosphorylation in tyrosine of numerous key proteins.

During capacitation bicarbonate stimulates the oxidation of cholesterol forming oxysterols that are removed from the membrane by albumin(1,17,179) Different aspects are worth mentioning in the context of the present review; the first relates to the fact that cryopreservation impairs this oxidative mechanism, that can explain why cryopreserved spermatozoa have reduced fertility (21). The stallion spermatozoa do not capacitate efficiently in vitro, this is one of the reasons explaining the poor results with conventional IVF in the horse.

This particular aspect has been recently review(84), and the reader is referred to it for further details; however, the possibility that this fact may relate to the specific redox regulation in the stallion spermatozoa is an intriguing possibility that warrants to be further research. Intracellular glutathione (GSH) is much higher in horses than in other domestic species, perhaps this relates to known difficulties to capacitate in vitro.

Other membrane changes are linked to plasma hyperpolarization of the plasmalemma (15,28,43), and spermatozoa alkalinization (38). Not all the spermatozoa in the ejaculate are able to capacitate, only a subpopulation can experience capacitation (43,93). Redox chemistry regulates Tyrosine phosphorylation (38,68,85,113,115,120,148,155,174). Motility may be regulated by tyrosine phosphatases (PTPs) (38,47), which are intracellular targets for ROS (61). The activity of PTPs depends on a conserved cysteine (Cys) residue, which oxidation inhibits the enzyme (39,69), ROS also are able to activate kinases. In addition to hydrogen peroxide, other species such as hydrogen sulfide and lipid peroxides (LPO) can inactivate PTPs (48), its activity is regulated through the reversible oxidation of specific cysteine residues in target proteins (69).

Reduction of oxidized cysteine (Cyss) residues is necessary in order for them to function in a reversible manner, thus depending on the adequate availability of reducing molecules, including the peroxiredoxin (PRDX) family of antioxidant enzymes (69). PRDXs are present in the spermatozoa (83,92,110,128) being critical for the proper sperm functionality of these cells. To reverse the Cyss residue peroxiredoxins use thioredoxin or GSH. Reduction of sulphinic acid (SO_2H the higher oxidation state) precise sulfiredoxin or sestrins (69,76). This reversible and sequential oxidation of PRDXs permits tight regulation of peroxiredoxin functionality, a model of regulation defined as a “floodgate” (177,178).

The spermatozoa are cells rich in thiols (86) associated with proteins, indicating that regulated redox reactions are a major regulation mechanism. Spermatozoa are transcriptionally silent cells being thus dependent on post transcriptional modification of proteins for their regulation.

Mitophagy has been recently described in spermatozoa (10), this process depends of redox reactions on of Cys residues on specific proteins; this is the case of Cys-dependent proteases (69). In this sense, the cysteine protease HsAtg4 is target of H_2O_2, that oxidizes a residue close to the protein's catalytic site (149).

Other functions depending of regulated oxidation -reduction reactions are the control of motility (47), and the formation of the sperm reservoir through binding of the spermatozoa to the oviductal epithelium (64,65,162). The pattern of motility changes in the female genital tract, from a pattern, termed activated motility characterized by linear progression to a pattern characterized by the high amplitude of the flagellar movement and high lateral amplitude of the head displacement; this latter kind of movement, hyperactivated motility, is necessary to penetrate the oocyte.

The transition of one to another kind of movement occurs in response to changes in the environmental conditions in different sections of the female genitalia through the activation of a pH-dependent calcium sensor (30). Recently, has been found an EF-hand-calcium-binding domain-containing protein-9 (EFCAB9) that forms a complex with the CatSper subunit CATSPER ζ and is necessary for pH-dependent and Ca^{2+} sensitive activation of the CatSper channel; EFCAB9 interacts with CATSPER ζ in a Ca^{2+} sensitive manner and dissociates at elevated pH, this protein is an intracellular pH-dependent Ca^{2+} sensor that triggers changes in sperm motility (71,88).

All these changes imply that the spermatozoa demand a high amount of energy that is also dependent on their physiological status, nowadays, in spite that either more glycolytic or more oxidative phosphorylation dependent species exist, growing scientific consensus indicates that both forms of the metabolism cooperate to provide energy to the spermatozoa, however, the mechanisms controlling the switch from the predominance of one or another strategy for energy production still are poorly understood. Next, we will provide an overview of sperm metabolism, recent findings on its regulation on spermatozoa, and its implications on redox regulation.

A brief overview on sperm energetic metabolism

Glycolysis in the spermatozoa.- The metabolization of glucose to pyruvate is contemplated as the main route producing of ATP in the spermatozoa of humans (176) and boars (98), particularly in aerobiosis (121). Sperm incorporates hexoses through specific transporters GLUTs (23). After incorporation, hexokinase phosphorylates glucose to glucose 6-phosphate. Then can follow different pathways, pentose phosphate pathway, glycogen synthesis, and glycolysis. The enzyme pyruvate dehydrogenase oxidized pyruvate to Acetyl CoA. The NAD accepts the electrons released in this process forming $NADH^+$. Pyruvate was considered the main glycolytic product used by the mitochondria to feed the tricarboxylic acid or Krebs cycle, however, nowadays is known that reduction of pyruvate to lactate under aerobic conditions occurs (Warburg-Like effect) (121).

In the mitochondria lactate is oxidized to pyruvate in the *Mitochondrial Lactate Oxidation Complex*; intra-mitochondrial oxidation of lactate contributes to mitochondrial energetics (20). Evidence of the role of lactate in the metabolism of stallion spermatozoa has been recently reported (36), with the discovery of a lactate dehydrogenase in the mitochondrial matrix that transforms lactate into pyruvate (159), lactate is more effective than pyruvate sustaining the motility of the stallion spermatozoa (36). The importance of lactate for sperm metabolism was evidenced in the late 70s of the past century when Storey and Kayne (157) described the aerobic oxidation of lactate in rabbit sperm mitochondria.

Monocarboxylate transporters (MCTs) are present in the spermatozoa (20), specifically, the MCT1 is located in the sperm head (54). In a similar fashion as occurs in the horse, bovine spermatozoa use lactate for sperm motility as efficiently, or even better, than glucose (74). Sertoli Cells in the testis secrete lactate instead of glucose to fuel sperm motility constituting a cell-to-cell lactate shuttle (20,60). Sertoli cells, as stallion spermatozoa, convert glucose to lactate under aerobic conditions to support mitochondrial respiration; it has been postulated that Sertoli cells have a "Warburg like" metabolism, with a highly active glycolytic machinery and preferential production of lactate, even in presence of high oxygen (121).

As previously mentioned, evidence of oxidation of lactate to pyruvate is present in horse and in boar spermatozoa; in boars inhibition of oxidation of external lactate in presence of the MCT inhibitor α-cyano-4-hydroxicinnamate and by the inhibitor of LDH oxamate occurs, evidencing that lactate is transported into mitochondria to be oxidized to pyruvate (20,60). Different reports indicate that a gluconeogenesis linked glycogen metabolism is present in spermatozoa (4,13,130); lactate is converted to glycogen by the mature spermatozoa, and; in dogs, at least, is considered to play a major role a source of energy for capacitation.

Pentose Phosphate Pathway (PPP).- The PPP is the principal source of NADPH, though NADPH can also originate by degradation of metabolites of the tricarboxylic acid cycle (TCA), and by the oxidation of fatty acids and utilization of ketone bodies (29,42,70,131). The PPP comprises two branches. The oxidative produces NADPH and ribonucleotides; the first reaction is the dehydrogenation of glucose-6-phosphate by glucose-6-phosphate dehydrogenase (G6PD) to yield 6-phosphogluconolactone (6PGL) and the reduction of one molecule of NADP+ to one of NADPH (131); 6PGL is then hydrolyzed spontaneously or by the action of 6-phosphogluconolactonase (PGL) into 6 phosphogluconate. Then, this product generates ribose 5-phosphate and the second molecule of NADPH, by the action of 6-phosphogluconolactonase (91).

The nonoxidative branch comprises reversible reactions using glycolytic intermediates, as fructose 6-phosphate and glyceraldehyde 3 phosphate to be converted in pentose phosphates in a reversible manner (131). In the context of this review the production of NADPH as reducing power to recycle oxidized glutathione (GSSG) to reduced glutathione (GSH), is the principal role of this pathway in spermatozoa (44,105,168,169).

However, the roles of NADPH in the synthesis of fatty acids in the spermatozoa also has to be considered, especially in the context of recent proteomics studies (6), suggesting that this pathway may be active in spermatozoa (100). In ejaculated spermatozoa, the main function of the PPP, is the production of reducing power, and thus the PPP is tailored to accelerate the oxidative branch and redirect the non-oxidative branch to re-synthesize fructose 6 phosphate to be transformed to glucose 6 phosphate and feed the oxidative branch (131).

Tricarboxylic acid Cycle.- The tricarboxylic acid cycle (TCA) is a series of reactions occurring in a closed-loop (103). The cycle starts with the reaction of acetyl CoA (2C) with oxaloacetate (OAA, 4C) giving citrate (6C). The acetyl CoA derives from the oxidation of pyruvate, fatty acids, and the metabolism of different amino acids, especially leucine, isoleucine, and tryptophan. Recent data indicate that lipid and amino acids metabolism are present in the spermatozoa of different species (6,100,159,163).

Pyruvate may derive from citrate by the mitochondrial enzyme acetyl CoA synthetase short-chain family member 1 (ACSS1), present in the stallion spermatozoa (100). The citrate is transformed in its isomer, isocitrate and the cycle follows with two reactions of oxidative decarboxylation; isocitrate is converted to the 5C α-ketoglutarate (α-KG) and shortly afterward to the 4C succinyl CoA, liberating two molecules of CO_2 and two NADH.

The next step is the conversion of succinyl CoA to succinate, a reaction coupled to the generation of GTP, that can be transformed to ATP (103). Succinate is oxidized to fumarate (4C), and two hydrogen atoms are relocated to FAD originating two $FADH_2$, through the action of succinate dehydrogenase (SDH), this enzyme also participates in the electron transport chain (ETC). Then fumarate converts into malate and this into oxaloacetate that combines with another molecule of acetyl CoA to close and continue the cycle (103). The first step in the TCA is the generation of citrate from acetyl CoA and oxalacetate, the cycle can be fed at diverse points, such as the transformation of pyruvate to OAA by pyruvate decarboxylase and the glutaminolysis, that is the conversion of glutamine in glutamate and then to α-ketoglutarate (104).

Moreover, the oxidation of lactate oxidation is an important mechanism sustaining the TCA cycle (102). Both pathways may be also relevant in spermatozoa.

Oxidative phosphorylation. - Mitochondria are semiautonomous organelles crucial for cellular energetics, that through oxidative phosphorylation (OXPHOS) produce most ATP in the cell (170). These organelles have many other important functions, such Ca^{2+} regulation, control of the lifespan of the spermatozoa, and production of ROS with regulatory functions; for example, recently has been described that capacitation in the human spermatozoa is dependent of ROS generation in the spermatozoa, and is independent of the presence of glucose, suggesting that human spermatozoa have a notable metabolic plasticity (26).

In the OXPHOS pathway enzymes are coordinated by a cascade of oxidation-reduction reactions organized in protein complexes (I-V); these are located in the inner mitochondrial membrane (170), there are also two soluble factors, cytochrome c (situated in the mitochondrial intermembrane space) and coenzyme Q. This set of proteins is known as the electron transport chain (ETC). The ETC transfers electrons to reduce molecular oxygen to water, in this process is generated energy that is used to produce ATP.

The ETC is coupled to the TCA cycle through the electron transport carriers NADH and $FADH_2$, which donate electrons to the ETC. The complexes I and II in the ECT mediate the transfer of two electrons from NADH and $FADH_2$ respectively, to coenzyme Q; this latter can also receive electrons from the metabolism of fatty acids, amino acids, and choline. The reduced coenzyme Q donates two electrons to complex III and transfers these two electrons to cytochrome C, to reduce O_2 to water. This series of redox reactions cause changes in the conformation of the ETC that responds pumping out H^+ to the intermembrane space origination an electrochemical gradient known as mitochondrial membrane potential ($\Delta\Psi m$). This can be measured using probes like JC-1(132,136).

The H^+ driven force generated by complexes I, III and IV is used by the ATP synthase (complex V) to produce ATP phosphorylating ADP. The dependency of the ATP generated in the ETC in the stallion spermatozoa has been revealed by numerous studies (34-37,57,59,62,100,122,135,140,159,164,172).

The well-known production of the radical superoxide $O_2^{\bullet-}$ in the mitochondria, is linked to the activity of the ETC, which is reported to be a major source. The $O_2^{\bullet-}$ derives from the addition of a single electron to O_2, up to 2% of total oxygen is converted to $O_2^{\bullet-}$ under physiological conditions in the complexes, I and III (37,140) . Other sources of mitochondrial ROS are different flavoenzymes in the mitochondria. A high rate of mitochondrial ATP production is linked to increased production of mitochondrial ROS being this effect specially relevant in the equine spermatozoa (57).

Once the mechanism for control the redox homeostasis are overpassed, excessive ROS impairs both the stability of the ETC proteins and also the transcription of mitochondrial proteins (180) leading to sperm malfunction and finally dead; different forms of ROS triggered sperm death have been described, these forms resemble apoptosis and ferroptosis, but with specific sperm particularities(3,123,126). The link between excessive production of ROS and sperm malfunction have been recognized for decades, both in human reproductive medicine and animal breeding, and antioxidants are frequently used in the treatment of male factor infertility (41,99,101,134,151), however aspects related to the interaction between sperm metabolism and redox regulation have received much less attention both in human and animal spermatology.

Generation of Reactive Oxygen and Nitrogen Species in the TCA cycle and in the ETC

The generation of $O_2^{\bullet-}$ in the mitochondria is well-known and has been previously discussed. Specific enzymes of the TCA cycle can be major sources of ROS, specifically the α-Ketoglutarate dehydrogenase and glycerophosphate dehydrogenase (161). Unregulated production of ROS is behind sperm malfunction; being ETC in the mitochondria the main source of ROS in the spermatozoa (37,140). The ability of $O_2^{\bullet-}$ to diffuse across membranes, is limited by its anionic character with most of the reactions occurring in the mitochondria. Principal reactions of $O_2^{\bullet-}$ are the spontaneous or catalyzed dismutation to H_2O_2, reaction with FeS centers, and the reaction with nitric oxide $^{\bullet}NO$ leading to the generation of peroxynitrite ($ONOO^-$)(144,167).

Hydrogen peroxide, which is a non-radical oxidant, can diffuse across cellular membranes. The H_2O_2 is a weak oxidant that through the reversible oxidation of thiols in cysteine residues has important regulatory functions, however, its reaction with metal centers can produce the highly toxic hydroxyl radical ($^{\bullet}OH$). Although peroxynitrite is a stable molecule, is a potent oxidant that reacts with CO_2 and electrophilic transition metal centers yielding different potent oxidants, such as nitrogen dioxide (NO_2), the carbonate radical ($CO_3^{\bullet-}$), and oxo-metal complexes. The peroxynitrous acid (ONOOH) can experience a proton catalyzed dissociation to NO_2 and $^{\bullet}OH$. All these radical species derived from peroxynitrite may oxidize, peroxidize and nitrate many mitochondrial components. Nitric oxide ($^{\bullet}NO$) and nitric oxide derived species, are also regulatory molecules in the sperm mitochondria (109,183).

Lipid metabolism.

Fatty acid metabolism provides energy to the spermatozoa, recent proteomic studies stress the importance of fatty acids supplying energy to these cells (6,12,53,100,106,158,159), through mitochondrial beta-oxidation of short, (less than 8 carbons), medium (between 8 and 12 carbons) and long-chain fatty acids to acetyl CoA, that enters the Krebbs Cycle and then the energy released is used in the ETC to generate ATP through OXPHOS. Evidence supporting the presence of this metabolic pathway, at least in human (6,7) and stallion spermatozoa (159), include proteomic studies and functional experiments in which the use of etomoxir (an inhibitor of carnitine palmitoyl-transferase I) to inhibit beta-oxidation reduces sperm motility. The human spermatozoa, using peroxisomal enzymes, are able to oxidize long-chain fatty acids (VLCFA) (6,7).

A recent study using bovine spermatozoa as a model, provides evidence of the use of saturated fatty acids to produce ATP via mitochondrial ß-oxidation(75) to sustain linear motility. Bovine spermatozoa incorporate saturated fatty acids through the CD36 and GOT2 channels located in the mid-piece. Then they are metabolized through mitochondrial ß-oxidation (75). Some reports also indicate the intrinsic saturated fatty acids may also be used as a source of energy by the spermatozoa (6,53,94). Recent proteomic studies from our laboratory suggest that lipid metabolism may be predominant in stallion spermatozoa (100). Interestingly, stallions showing a high activity of this metabolic pathway are more resistant to the stress of cryopreservation, showing better sperm quality after freezing and thawing (50). As a metabolic process, mitochondrial ß-oxidation of fatty acids causes the formation of $O_2^{\bullet -}$ and H_2O_2 (161).

Metabolic disfunction, production of ROS in the spermatozoa

As previously discussed, energy metabolism consists of reactions of oxidation of biological molecules to simpler compounds, the energy liberated in this process, which is thermodynamically favorable, is used to phosphorylate ADP, producing ATP (144,167). In the redox electrons are transferred from reduced molecules to molecules such as NAD^+, $NADP^+$ or oxygen that are the final acceptors of electrons and are crucial elements of the energetic metabolism. ROS are products of these reactions that participate in cellular physiology, but if deregulated can cause severe cellular damage.

Hydrogen peroxide (H_2O_2) and the superoxide ion ($O_2^{\bullet-}$), are not very reactive and can be tightly regulated by antioxidant enzymes. However, upon the reaction of $O_2^{\bullet-}$, with nitric oxide $^{\bullet}NO$, peroxynitrite ($ONOO^-$) is produced. On the other hand, in the reaction of H_2O_2 with Fe^{2+} of Fe^{3+} the highly reactive hydroxyl radical ($^{\bullet}OH$) is formed. Lipids, proteins, and DNA are targets of the attack of these highly reactive radicals if the redox homeostasis is lost. Also, enzymes regulating metabolism are targets of this attack; this is one of the factors explaining why mitochondrial oxidative attack leads to further production of ROS (37,143), activation of glycolysis impaired OXPHOS.

This suggests that ROS may deregulate glycolysis and deregulated glycolysis may deregulate redox homeostasis (87). Overall, the intimate relation between energetic metabolism and reactive oxygen species shall be considered as a critical hub explaining both sperm physiology and sperm malfunction (57,58,83,92,135).

Formation of electrophilic 2-oxoaldehydes.

During glycolysis, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (40), lose phosphate groups, during this step glyoxal (G) and methylglyoxal (MG) are continuously produced. Methylglyoxal and glyoxal are 2-oxo aldehydes. These 2-oxoaldhydes due to their adjacent carbonyls (Fig 2), are strong and highly reactive electrophiles that attack nucleophiles in proteins, lipids, and DNA, causing the formation of advanced glycation end products (AGEs) (Fig 3) (107). In spite of their cytotoxicity and their capacity to damage cellular DNA, these compounds, under proper regulation, may also have regulatory functions.

Furthermore, MG can form adducts with Superoxide dismutase 1 (SOD1) and reduce the ability of the cells to maintain redox homeostasis (141). This is particularly important in the horse; SOD1 is the most important constituent of the antioxidant system in the spermatozoa (50,51).

When stallion spermatozoa is extended and stored in commercial media containing very high glucose concentrations the production of these 2-oxoaldehydes is unavoidable (125). Recent proteomics studies link higher amounts of the enzyme fructose bisphosphate aldolase and poor motility and velocities; this is the enzyme involved in splits of 1, 6 fructose bisphosphate in dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G3P), are precursors of G and MG (49). Production of G, and especially MG was concomitant with reduced motility and sperm velocities, a drop in mitochondrial membrane potential, and an increment of the production of ROS (49,125).

To the contrary, in extenders formulated with 1mM glucose the production of M and MG is significantly reduced, linked to better sperm functionality(125). The GSH is a major mechanism of control of 2 oxoaldehydes(144). In this sense, the understanding of the role of GSH in the regulation of sperm redox homeostasis has been recently expanded with the finding of the SLC7A11 x-CT glutamate/cystine antiporter in the stallion spermatozoa (126,127) (Fig 4). Moreover, incorporation of cystine may be critical, since the transsulfuration pathway seems incomplete in stallion spermatozoa (127). This antiporter is constitutively expressed in the spermatozoa and exchanges intracellular glutamate for extracellular cystine.

It is interesting that spermatozoa is one of the few cells constitutively expressing the SLC7A11 along with thymus, spleen and brain(33); mRNA is present in testis and the SLC7A11 knock out mouse display subfertility (67). The SLC7A11 is upregulated in many cancer cell lines (14,80), interestingly, and as seems to occur as well in the spermatozoa, high expression of the SLC7A11 in cells, is linked to high activity of the PPP (90), with cells expressing high amounts of this protein expressing also high amounts of enzymes involved in the PPP, particularly G6PD. Incorporation of cystine through this antiporter, requires intracellular reduction of cystine to cysteine, this process consumes high amounts of NADPH, generated in the PPP, then cysteine is used for GSH synthesis (124,127).

The evidence showing GSH synthesis in the spermatozoa include the presence in the spermatozoa of the glutathione synthetase (GSS) and gamma-glutamylcysteine ligase

(GCLC), studies demonstrating the activity of the enzymes that synthesize GSH using the specific inhibitor of the GCLC, L-Buthionine sulfoximide (BSO), and the use of mass spectrometry for measurement of GSH (124). Recent findings in spermatogonia support the role of GSH and cystine coupled with the PPP pathway in the redox regulation of these cells (142), moreover and in accordance with our findings in mature stallion spermatozoa the transsulfuration pathway is not a source of cysteine (127).

The concentration of glucose in current extenders must be reconsidered

Glucose and fructose in high concentrations, well above the physiological, have been incorporated to most extenders for semen conservation in animal breeding. However, a growing body of evidence from scientific research, suggests that this approach may be incorrect. While frequently used concentration of glucose in currently used extenders for stallion spermatozoa range from 80 to 300 mM concentrations of glucose in the mare's oviduct are in the micromolar range. Excessive consumption of highly processed sugars is now a major issue in public health, evidences show that excessive consumption increases the risk of diabetes, many types of cancer, and cardiovascular and neurodegenerative diseases (31,78,95).

In a similar fashion, very high concentrations of glucose in the extenders may be causing significant damage to the spermatozoa. For a long time the main source of energy in the spermatozoa was the subject of intense debate, nowadays growing consensus establish that while species-specific differences may be present, spermatozoa can use different pathways to obtain energy. Interestingly, first reports on the metabolic plasticity of the spermatozoa were done in the first decades of the past century by researchers at the Universities of Wisconsin and Pennsylvania (156).

In regard to stallion spermatozoa, and as previously discussed, according to current biochemical research that in spite that glycolysis may be necessary to support sperm velocities (37,79,140), spermatozoa produce most of their energy through OXPHOS; due to its intense mitochondrial activity radical superoxide ($O_2^{\bullet-}$) is also generated as a byproduct.

The stallion spermatozoa may use amino-acids and fatty acids as relevant sources of energy (6), recent studies using proteomic approaches indicate that stallion spermatozoa oxidize fatty acids (59,100,159). Altogether this new knowledge has been conducted to design new extenders that significantly expand the lifespan of the spermatozoa (56,59). These extenders reduce glucose toxicity and sustain a more efficient sperm metabolism. Supraphysiological concentrations of glucose or inefficient utilization cause cellular damage (22), this situation is characteristic of Diabetic conditions, extremely prevalent in humans, and intensive research on the molecular mechanisms behind glucose toxicity has been done, however, studies on glucose toxicity on the spermatozoa are scarce.

In horses the physiological concentration of glucose is 5 mM, concentrations in the oviduct reported are 300 μM (25), then extenders in use expose stallion spermatozoa to supra-physiological glucose concentrations potentially leading to glucose toxicity (87); this may be due to involving including the excessive generation of the 2- oxoaldehydes MG ang G as described in the previous section. Other mechanisms include direct induction of ROS by glucose, activation of MAP kinase and Ca^{2+} mediated mitochondrial fission (108,165), increased polyol pathway activity, that consumes NADPH impairing the reduction GSSG, depleting GSH (22).

Hyperglycemia activates a pathway that involves diacylglycerol (DAG) protein kinase C (PKC) and NADPH-oxidase; activation of this pathway causes overproduction of ROS and mitochondrial damage. Mitochondrial damages increase mitochondrial production of $O_2^{\bullet-}$ that inhibits 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) (22,108), DHAP is precursor MG (27,72). In addition, high glucose concentration predisposes apoptosis, ferroptosis, necroptosis and other types of cell death (82). The situation of current protocols for conservation seminal dose on the equine breeding industry, is a natural occuring model of hyperglycemia induced sperm damage.

Itaconate regulates the switch between glycolysis and the pentose phosphate pathway

Sperm cells depend on GSH to regulate their redox state, to recycle GSSG NADPH is needed. Recently, a mechanism that the spermatozoa use to adjust the energy metabolism and the redox homeostasis has been disclosed (180,182). In boar spermatozoa, itaconate regulates the switch from glycolysis to the pentose phosphate pathway (Fig 5). Boar spermatozoa incubated in a modified commercial media containing moderate amounts of glucose (30mM) showed increased mitochondrial activity and increased itaconate production. This increased production of itaconate activates the PPP in detriment of glycolysis maintaining redox homeostasis, that in addition results in improved sperm motility (182).

These findings have great importance and provide an explanation on how the spermatozoa adjust their metabolism to changes in the different environments, including different concentrations of glucose (1.4 mM in the seminal plasma to 300μM in the oviduct) (147), that these cells are exposed to, in their travel to the oviduct to fertilize the egg. Regulation of the cellular state through signals released from the mitochondria are common in many cell lines, mitochondrial signals regulating cellular state include, the mitochondrial metabolites fumarate, succinate and itaconate, mitochondrial reactive oxygen species (mt ROS) and mitochondrial DNA (mt DNA) (175).

Recent reports from our laboratory in stallion spermatozoa, showed increased GSH content and improved motility, concomitant with increased succinate, fumarate and malate when GDH-1 was inhibited (126), furthermore recent proteomic studies show that the mitochondrial aconitase hydratase is more abundant in the spermatozoa of stallions with better motility, this enzyme catalyzes the transformation of citrate to isocitrate via cis aconitate (49); these findings suggest that regulation of sperm metabolism and redox status mediated by TCA cycle metabolites is also present in horses.

CONCLUSIONS AND FUTURE DIRECTIONS

Spermatozoa are cells with intense demand for energy, that vary along their life cycle. Specifically, their journey through the female reproductive tract to reach and fertilize the egg, the preparatory processes of capacitation and the acrosome reaction depend on adequate sources of energy. The energetic metabolism involves oxidation-reduction reactions and the production of ROS is unavoidable. The maintenance of redox homeostasis is necessary for sperm functionality, thus, the study of the interactions between metabolism and redox reactions is a critical field to improve the understanding of infertility in humans and sperm biotechnologies in humans and animals (Fig 6).

Numerous reasons make the stallion a good model for redox-metabolic studies in the spermatozoa; the intense mitochondrial activity and the intense production of ROS as subproducts, the recent discovery of sophisticated redox regulatory mechanisms, and finally the glucose-induced toxicity caused by some commercial extenders. Particularly relevant is becoming the presence of very high concentration of glucose in commercial extenders for stallion spermatozoa, the molecular mechanisms behind this glucose-induced damage may constitute a model for infertility seen in diabetic patients, being a model for the study of infertility in this disease.

AUTHORS CONTRIBUTIONS

All authors contributed towards researching, writing, discussing, and editing the manuscript.

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ABBREVIATIONS

ACSS:1 acetyl Co A synthetase short-chain family member 1

AGEs: advanced glycation end products

α-KG: alpha-ketoglutarate

BSO: L-Buthionine sulfoximide

DAG: diacylglycerol

DHAP: dihydroxyacetone phosphate

ETC: electron transport chain

G: glyoxal

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

G6PD: Glucose-6-phosphate 1-dehydrogenase

GLUTs: glucose transporters

GSG: glutathione

GSSG: oxidized glutathione

GSS: glutathione synthethase

GCLC: gamma glutamylcysteine ligase

H_2O_2: hydrogen peroxide

LDH: lactate dehydrogenase

MG: methylglyoxal

MCTs: monocarboxilate transporters

$^{\bullet}NO$: nitric oxide

$O_2{\bullet}^-$:superoxide anion

OAA: oxaloacetate

$^{\bullet}OH$: hydroxyl radical

$ONOO^-$:peroxynitrite

OXPHOS: oxidative phosphorylation

PKC: protein kinase C

PPP: pentose phosphate pathway

RNS: reactive nitrogen species

ROS: reactive oxygen species

SDH: succinate dehydrogenase

SLCT7A11 x-CT: soluble carrier family 7 member 11 -glutamate cystine antiporter

SOD1: superoxide dismutase 1

VLCFA: very long-chain fatty acids

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FIGURES

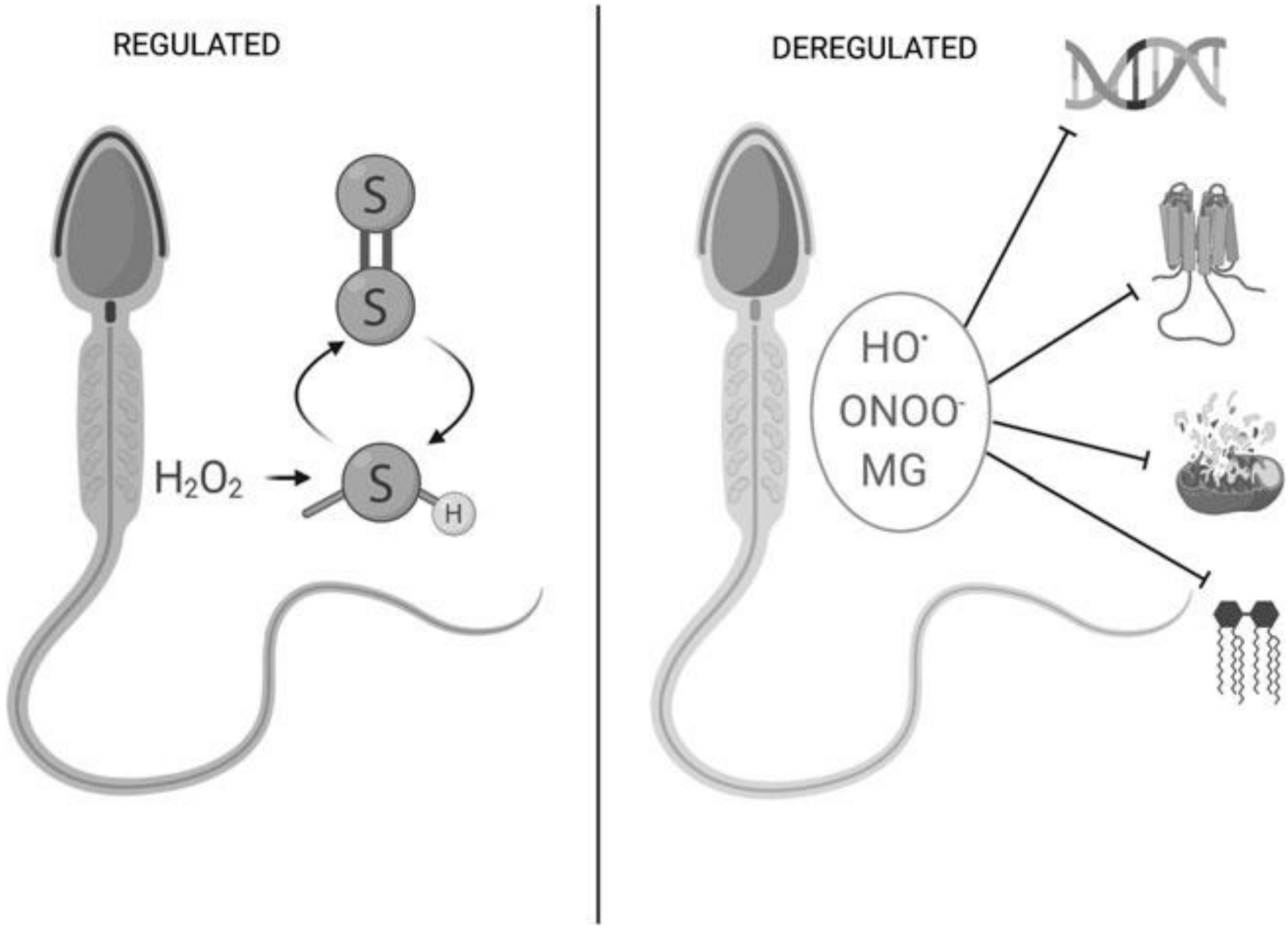

FIG. 1. Overview of the role of ROS in mammalian sperm function. ROS have important regulatory roles in the spermatozoa mainly through the reversible oxidation of thiol groups, principally in cysteine residues. However, if redox homeostasis is lost uncontrolled and unregulated production of ROS causes damage to sperm DNA, lipids, proteins and may trigger the mitochondrial pathway of apoptosis causing sperm demise. ROS, reactive oxygen species.

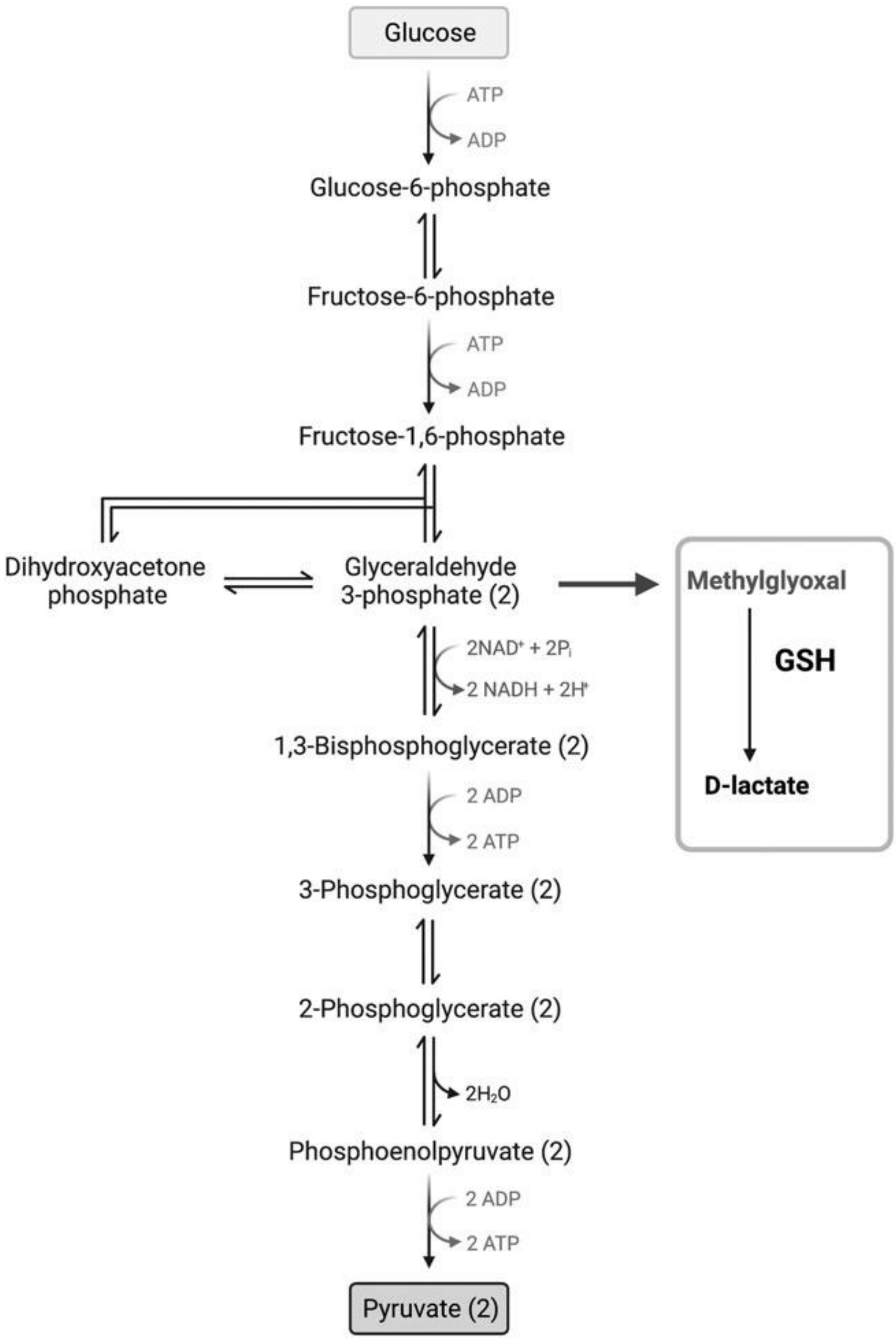

FIG. 2. Overview of glycolysis. This process mainly occurs in spermatozoa under aerobic conditions, the main function being to provide pyruvate to feed the Krebs cycle, although ATP is also generated. Under high-glucose concentrations (diabetic conditions in humans, high glucose containing extenders in animal breeding), the excessive production of MG can induce severe sperm damage. MG, methylglyoxal.

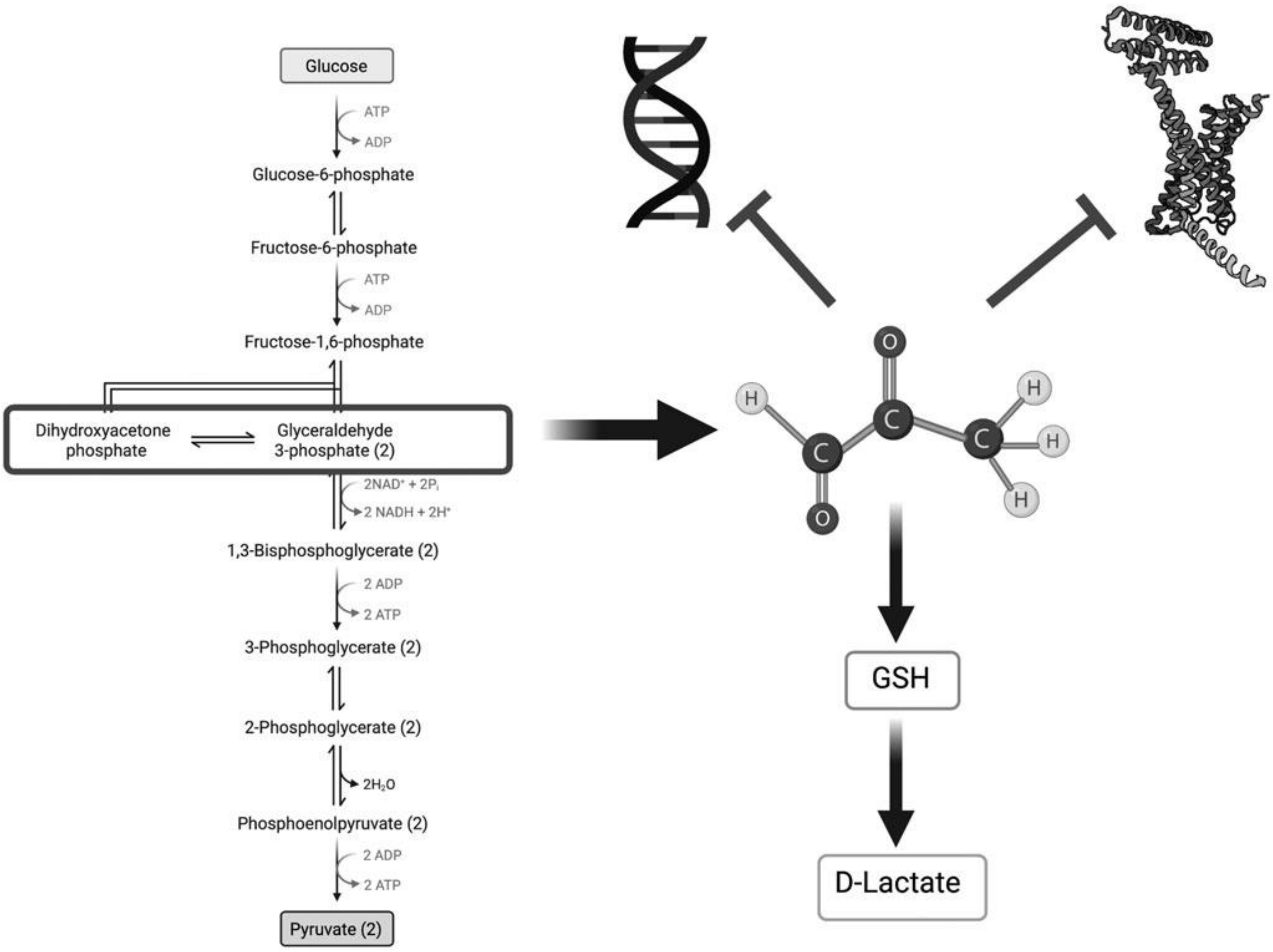

FIG. 3. In the glycolysis, phosphate is eliminated from the trioses phosphates glyceraldehyde 3-phosphate and dihydroxyacetone phosphate; in this process, MG is produced; due to the adjacent carbonyl groups, MG is a strong electrophile that rapidly and spontaneously reacts with nucleophiles from proteins and DNA. MG is detoxified by the glyoxalase system to d-lactate, with the participation of GSH. GSH, glutathione.

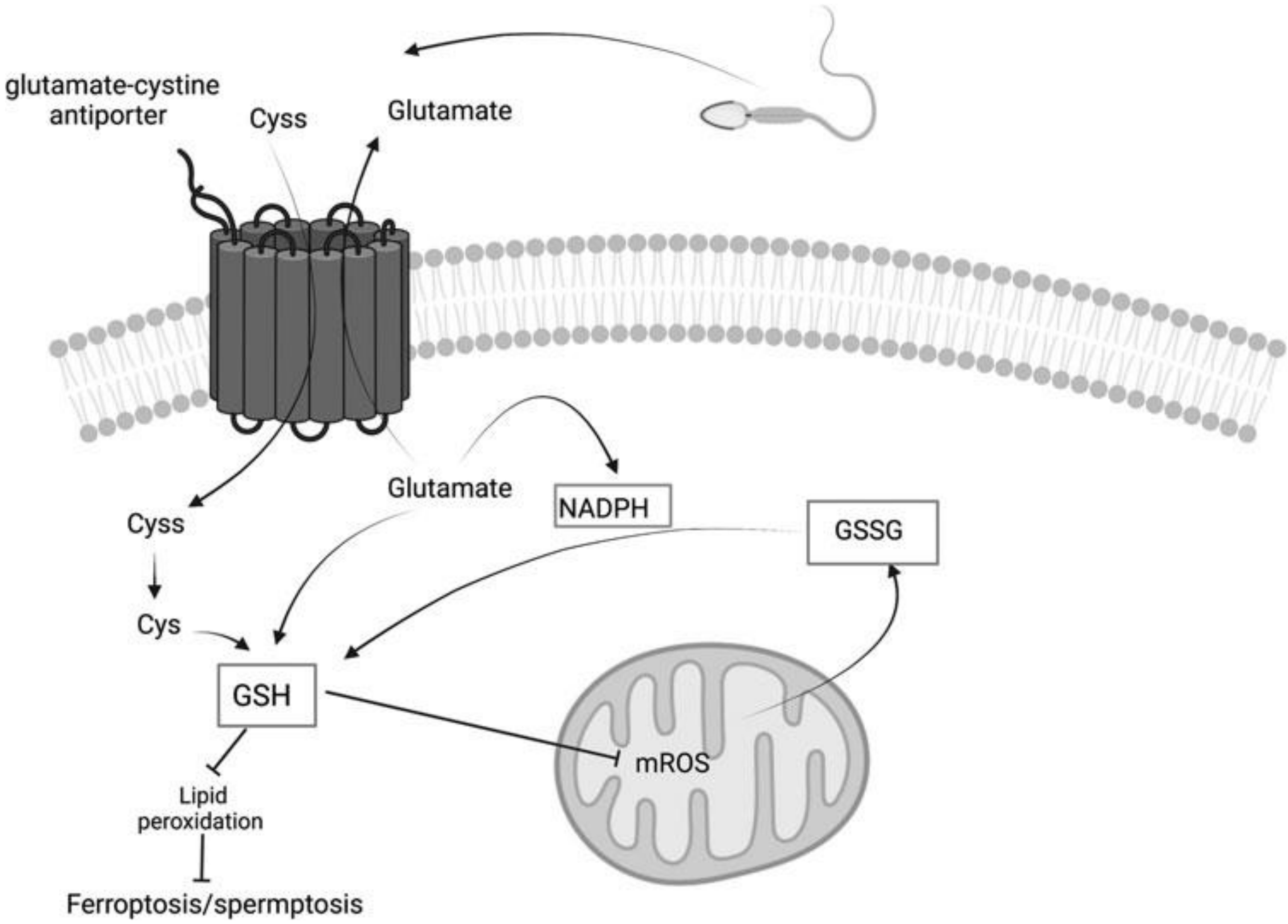

FIG. 4. The SLC7A11 antiporter contributes to redox regulation in stallion spermatozoa through the exchange of extracellular Cyss for intracellular Glut. Cyss is intracellularly reduced to Cys, which is used for GSH synthesis. Alteration of the SLC7A11 antiporter (e.g., after cryopreservation) leads to reduced intracellular Cys, and then reduction in intracellular GSH. This causes redox deregulation and mitochondrial damage. The deregulation of redox homeostasis may lead to increase of LPO and the induction of ferroptosis. Alternatively intracellular glutamate may be metabolized following an alternative pathway. This alternative pathway produces reducing power to recycle GSSG to GSH, and maintain redox homeostasis and mitochondrial function. Cyss, cystine; Cys, cysteine; Glut, glutamate; GSSG, oxidized glutathione; LPO, lipoperoxides.

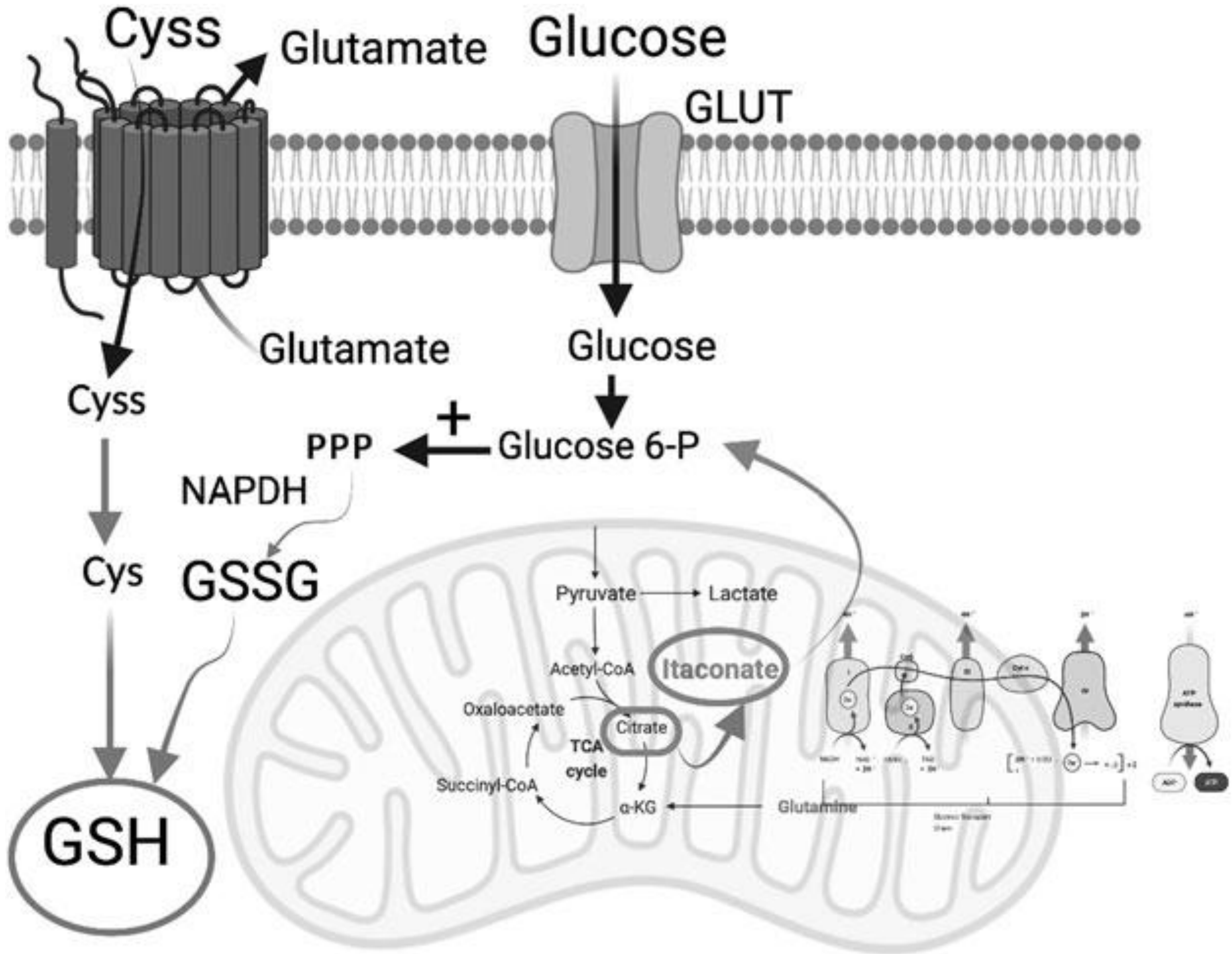

FIG. 5. Proposed mechanism of the interaction between metabolism and redox regulation in the spermatozoa (122, 124, 180). Glutathione plays a major role in the regulation of sperm redox status, spermatozoa incorporate cystine through the SLC7A11 antiporter in exchange for intracellular glutamate, cystine is reduced to cysteine and used for GSH synthesis. Oxidized glutathione is recycled using reducing power, provided by NADPH. The PPP acts as the main source of NADPH, and itaconate acts as regulator of the glucose metabolism inhibiting glycolysis and diverting the glucose metabolism to the PPP. The NADPH cooperates with GSH synthesized from the cysteine incorporated through the SLCTA11 to regulate REDOX homeostasis and increase ATP synthesis in the ETC improving sperm functionality. ETC, electron transport chain; PPP, pentose phosphate pathway.

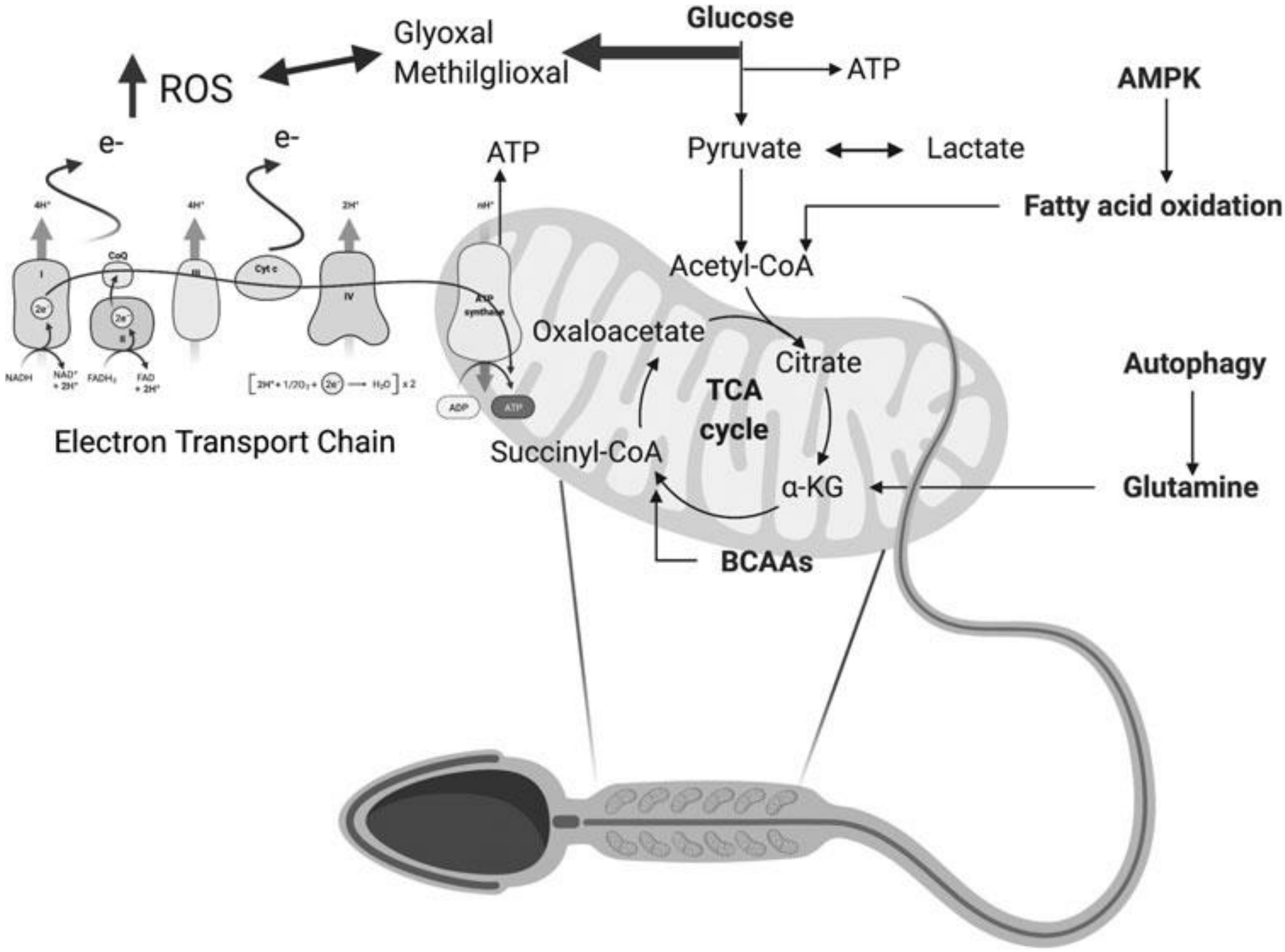

FIG. 6. Summary of the interactions between the energetic metabolism and ROS in the spermatozoa. The main source of ROS in the spermatozoa is the mitochondrion, mainly the ETC at the complexes I and III. Other sources of ROS are the tricarboxylic acid cycle and the b-oxidation of fatty acids. Mitochondrial dysfunction increases the production of ROS. In addition, during the glycolysis MG and G are continuously produced; 2-oxoaldehydes due to their adjacent carbonyl groups are potent electrophiles that readily react with proteins, DNA, and fatty acids leading to the formation of AGEs. Glutathione plays amajor role in the detoxification of these products. High glucose can directly increase the production of mitochondrial ROS. AGEs, advanced glycation end products.