VETERINÁRIA NOTÍCIAS

Review

Sperm energy metabolism

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Abstract: The development of techniques to increase sperm longevity demands knowledge about cell metabolism. Sperm requires a constant supply of energy to maintain its cell functions. Approximately 500 metabolic reactions take place in somatic cells, and several of them require energy. Most of the produced energy goes for sperm motility, which is an ATP-dependent specialized process. Spermatozoa possess the required mechanisms to produce energy through glycolysis, citric acid cycle (*Krebs cycle*) and oxidative phosphorylation. Understanding these pathways provides knowledge on the interactions between the semen extender substrates and sperm cells. The aim of this review is to approach the major energy producing pathways of the sperm, as well as the substrates available for this metabolism.
Keywords: spermatozoa, motility, ATP.

1 Introduction

During spermatogenesis, spermatozoa are reshuffled, causing compaction of organelles and loss of significant cytoplasm enzymes and energy reserve (Mann, 1964). Consequently, sperm is extremely dependent of the extracellular environment, using enzymes and substrates to supply energy and functional demands, from ejaculation to fertilization (Amann; Graham, 2011; Bucci et al., 2011).

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After the release in seminiferous tubules lumen, mammalian sperm is not capable to fertilize an oocyte, even though this capacity is acquired from the epididymal traffic until the passage through the female reproductive tract (Yanagimachi, 2005). The post-testicular maturations (i.e. sperm motility acquisition, capacitation and acrosome reaction) make the sperm able to fertilize (Gautier et al., 2016). This process demands an increased metabolism and, consequently, energy expenditure (Baldi et al., 2000).

Approximately 500 metabolic reactions occur in somatic cells, and several of them require energy (Alberts; Johnson; Lewis, 2002). Although the basal metabolism of the sperm cell needs low energy, several substrates are required to ensure its motility and fertilizer capacity after ejaculation. Therefore, knowledge on metabolic pathways to energy production enables comprehension of failures on the fertilization process, or implementation of sperm reproductive biotechnology.

The use of reproductive biotechnologies to increase livestock productivity or to genetic conservation of endangered species, encourages the funding for sperm research, as sperm cells preservation techniques are widely used in the animal reproduction market.

The use of artificial insemination (AI) is allowed in equine species (except Thoroughbred) and has been used in reproductive routines for years. Nonetheless, the reproductive program rates are under influence of several facts related to methods and subjects. Substances such as cholesterol, L-carnitine, and Q10-coenzyme are associated with semen extenders to protect sperm cell aim during the cryopreservation process (Hartwig et al., 2014; Lisboa et al., 2014; Carneiro at al., 2018).

In bovine, unlike equine, reproductive performance is used to select individuals, decreasing the differences between individual fertility in comparison with stallions. To improve the results, new sperm cryopreservation techniques (Dias et al., 2018) and semen extenders (Papa et al., 2015) have been constantly described in equine research.

There are no differences between husbandry procedures in this previously related species and porcine production. Similarly, most companies have used the AI in large-scale to genetic improvement of strains. In consequence, several modifications in semen extenders and new techniques of semen cryopreservation have been developed based on knowledge about sperm metabolism (Torres et al., 2018; Nesci et al., 2020).

This review gathers information on the major metabolic pathways of mammalian spermatozoa, by energy production and availability of substrates to metabolization.

2 Development

2.1 Sperm motility

The control of sperm motility occurs in three steps: in epididymis, motility is suppressed; in ejaculation, motility is activated; and in oviduct, motility is hyperactivated (Brito, 2007).

Sperm cells have intrinsic movement capacity in epididymis; however, they present motility after ejaculation (Morris; Tiplady; Allen, 2002). It is known that certain inhibitor proteins found in the epididymal cauda of rats allow motility activation when removed (Silva, et al., 2012). A pH-dependent inhibitor factor has been reported in bovine, demonstrating that acid pH can inhibit motility in epididymal environment (Varner; Johnson, 2007).

The epididymal cauda fluid pH in bovine is 5.5, while sperm cytoplasm pH after ejaculation is 6,5-6,6. Furthermore, epididymal cauda fluid in bovine, ovine, porcine, and equine have no expressive amount of bicarbonate (HCO_3), which is a key-point in sperm motility. Bicarbonate is found in largest concentrations on the seminal plasma, where the contact between the sperm cell and plasma can activate its motility (Luconi; Baldi, 2003).

Signaling pathways are not completely elucidated, as it is known that environment factors may activate sperm motility. However, studies have shown that the compounds HCO_3, calcium (Ca^{++}) and cyclic AMP (cAMP) are the producers of said signalization (Varner; Johnson, 2007). These substances are correlated to the increase in the sperm pH, that activates the regulation of cAMP in Na-K-ATPase, which acts on ensuring sperm motility (Demarco; Espinosa; Edwards, 2003).

The increase in sperm pH during its path to the uterine tube also activates the Ca^{++} channel, causing an augmentation of calcium influx to cytoplasm and resulting in motility hyperactivation, as Figure 1 shows (Ho; Suarez, 2001).

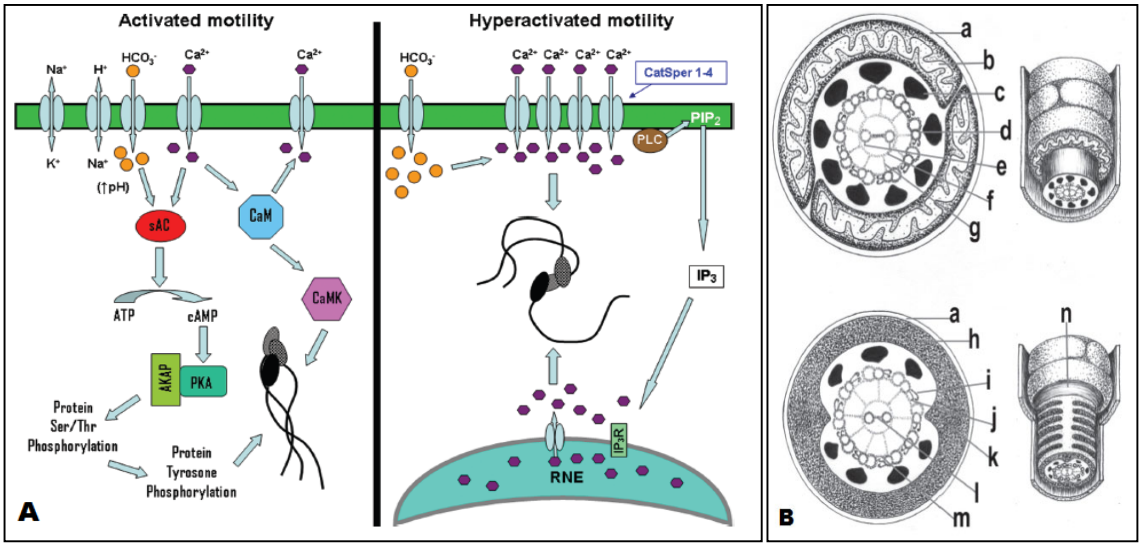

Figure 1. (A) Model of hypothetical spermatic events associated with sperm motility activation and hyperactivation. **(B)** Magnified illustration of transversal section of principal piece and midpiece of equine spermatozoa. (a) Plasma membrane. (b) Mitochondrion. (c) Outer dense fibers. (d) Pair of microtubules. (e) Pair of axonemes. (f) Sheath of central pair of axonemes. (g) Radial spoke. (h) Fibrous sheath. (i) Outer dynein arm. (j) Central dynein arm. (k) Longitudinal column of fibrous sheath. (l) Connection bridge of the central pair of microtubules. (m) Nexin connections. (n) Annulus (Luconi; Baldi, 2003).

The basic mechanism of tail sperm movement is similar to the myosin in muscle. However, the loss and rewiring of ATP products occur two or three times more quickly than spermatozoa (Ruiz-Pesini et al., 2007).

Progressive motility is the result of flagellar beating, which is caused by a series of coordinated waves propagated from the midpiece. The last form of flagellar movement results from active forces inside the sperm tail, working against restrictions produced for the nexin connections, radial spokes, dense fibers, and fibrous sheath (Amann; Graham, 2011). The extender viscosity is another factor to be considered, as it interferes in the normal frequency of flagellar beating (Marquez; Suarez, 2004).

Flagellar beating is produced by the sliding of microtubule pairs connected by the dynein arms. The sperm cell has nine pairs of microtubules, which contract and relax alternately. Dynein connection in the axoneme pairs is ATP dependent, as described in Figure 2 (Brito, 2007).

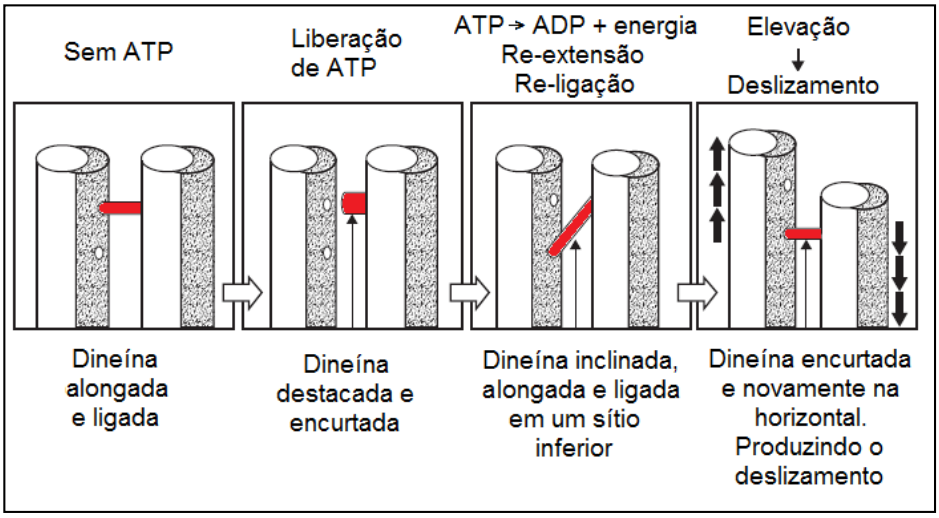

Figure 2. The slide mechanism of microtubule pairs resulted of separation, shortening, elongation and rewiring of dynein arms (Amann; Graham, 2011).

2.2 Metabolic pathways to energy production

Monosaccharides like glucose and fructose are quickly metabolized by sperm, but not all sugar and carbohydrates are used. Substrates like lactate, pyruvate, fatty acids, and amino acids also are metabolized, in contrast, only for aerobic pathways. In equines, glucose is the primary source of energy,

considering that the sperm is not able to metabolize fructose in this species (Varner; Johnson, 2007).

Studies have found high concentration of fructose in bovine, ovine and human seminal plasma (40-1000 mg/ml), and extremely lower in stallions (<1 mg/100 ml), which demonstrates that fructose is its main energy source. Additionally, horses are extremely dependent of aerobic pathways like oxidative phosphorylation; in contrast, bovine, ovine, and porcine sperm uses mostly anaerobic pathways, such as glycolysis (Mann; Lutwak-Mann, 1981; Nesci et al., 2020).

Several monosaccharides have been considered species-specific, as the spermatozoa of each species present a greater amount of hexose carriers for these sugars (Rigau et al., 2002). However, the presence of these other monosaccharides in other seminal plasma is not completely elucidated (Rodríguez-Gil; Bonet, 2016). Comparative studies between porcine and canine have shown porcine sperm presents no specific sugar, that is, its functions remained unchanged in the presence of both glucose and fructose (Fernandez-Novel et al., 2011). Consequently, the presence or absence of specific sugar and proteins in porcine and canine semen, which control hexose metabolism, may be corelated with changes in these effects against different sugars (Rodríguez-Gil; Bonet, 2016).

In a research conducted by Martin-Cano et al. (2020), the proteomic profile of equine fresh and frozen semen was compared, and changes in mitochondrial proteins related to citric acid cycle and oxidative phosphorylation were found evident. Furthermore, ATP-synthase is another protein that is impacted by frozen process, elucidating the mechanisms that worsen frozen semen quality when compared with fresh semen regarding motility.

The transport of monosaccharides through the membrane is an ATP-dependent process, and requires carrier proteins (Varner; Johnson, 2007). In cattle, the transport of proteins that carry glucose have limited capacity to

deliver fructose, which is carried by an exclusive mechanism that allows its quick use (Varner; Johnson, 2007). Fructose transport mechanisms are probably absent in stallion's semen, which may explain the low ability to metabolize this substrate (Amann; Graham, 2011).

The metabolism of endogenous components corresponds to approximately 10% of sperm ATP production; the other 90% are produced by exogenous substrates. Among the endogenous substrates are, for instance, phospholipids oxidated by mitochondria. In a similar way, exogenous substrates are essential to energy production, proving the importance of environmental factors for the sperm longevity (Amann; Graham, 2011; Varner; Johnson, 2007; Hammerstedt, 1983).

Sperm has mechanisms to perform glycolysis, citric acid cycle (Krebs cycle), and oxidative phosphorylation. Nonetheless, glycogen, glycogen-synthase and glycogen-phosphorylase have been identified in ovine, porcine, and equine sperm; gluconeogenesis mechanism had only established in canine sperm (Ballester et al., 2000). In this study, canine semen incubation associated to an extender without energy substrates caused a decrease in the sperm glycogen level. In contrast, the addition of extenders with glucoses or fructose resulted in glycogen accumulation and deposition site changed with the supplied sugar (Palomo et al., 2003; Ballester et al., 2000). Nevertheless, the knowledge about gluconeogenesis occurrence in the sperm cell of another species is less convincing (Ford, 2006).

Equine sperm metabolization involves oxidative phosphorylation, carbon metabolism, glycolysis and gluconeogenesis, citric acid cycle, fatty acid degradation, valine metabolism, leucin, methionine and cysteine, as well as amino acid synthesis. In addition, the involvement between fatty acids oxidation and amino acid consumption had been recently reported as another energy source to these cells. Even mitochondrial inhibition studies have highlighted the oxidative phosphorylation contribution for sperm energy

production, acknowledging the importance of its glycolysis for the sperm function as slightly more complex (Gibb et al., 2014).

Studies suggest that porcine sperm have 95% of its energy demands supplied for glycolysis (Marin et al., 2003; Rodríguez-Gil, 2006), while equines are dependents of mitochondrial oxidative phosphorylation (Varner, Gibb, Aitken, 2015). The indicative of glycolysis as the main pathway for energy in some species, emphasizes the questions about the specific role of mitochondria in sperm metabolism (Rodríguez-Gil; Bonet, 2016).

The role of estrogens and its receptors in the sperm cell have been studied in several species (Gautier et al., 2016; Guido et al., 2011; Rago et al., 2014), as it can affect adiposity process, modulation of lipogenesis, lipolysis or adipogenesis (Pallottini et al., 2008). Additionally, estrogens play an important role in glucose homeostasis and modulation of insulin sensitivity (Bryzgalova et al., 2006). The mechanisms involved with estrogen-receptor actions and their relation to sperm have not yet been elucidated. However, it is known that these hormones are essential to high quality sperm production (Meccariello et al., 2014). Estrogen-receptors such as ERα and ERβ had been identified from spermatogonia to sperm cell (Hess et al., 1995; Aquila et al., 2004). Furthermore, animals with ESR1 deficiency had shown impaired spermatogenesis and decreased fertility (Joseph et al., 2010). Moreover, sperm can synthetize estrogen (Aquila et al., 2002), which confirms that they are exposed to hormones in the female reproductive tract as well and are also able to produce part of the demands (Guido et al., 2011).

2.2.1 Glycolysis

The glycolytic pathway is used in all tissues for lysis of glucose, as a means of providing energy, either in ATP form or intermediating other metabolic pathways (Champe; Harvey; Ferrier, 2006). In aerobic organisms or tissues in aerobic conditions, glycolysis only represents the first stage of

complete glucose degradation (Cox; Nelson, 2002). As previously described, most sperm of the studied species, including rodents, are dependent of glycolysis for ATP production (Storey, 2008).

As the plasma membrane is impervious to glucose, two systems are used for it to penetrate the cell and be metabolized: facilitated diffusion transport, independent of Na^+; and the monosaccharide-Na^+ co-transport system (Marzzoco; Torres, 1999).

Transport by facilitated diffusion is mediated by a family of 14 carriers designated as GLUT (*Glucose Transporters*). Glucose binds to the transporter, which undergoes a change in its conformation, transporting it across the membrane. Even so, the glucose movement follows the concentration gradient, in other words, it moves from the most concentrated medium to the least (Champe; Harvey; Ferrier, 2006).

Glucose transporters are expressed in tissue and cell-specific forms, with distinct kinetic and regulatory properties. The function of the same isoform can be different from one tissue to another because of the cell differentiation process (Machado; Shaan; Seraphin, 2006).

For instance, GLUT-3 is the main transporter of glucose in neurons, GLUT-1 in erythrocytes and GLUT-4 in adipose tissue (Champe; Harvey; Ferrier, 2006). Transporters 1, 3 and 4 are involved in the uptake of glucose from the bloodstream, while GLUT-2, which is found in the liver, kidneys and in the ß cells of the pancreas, can both capture and remove glucose from the cell according to glycemia (Cox; Nelson, 2002). The only transporter capable of binding to a substance other than glucose is GLUT-5, which is responsible for the capture of fructose (Bucci et al., 2011).

Research in cattle have shown the presence of GLUT-1, 2, 3, and 5 in sperm, changing its concentrations according to the gamete region, such as: acrosome, head, intermediate piece and tail (Angulo et al., 1998). Bucci et al. (2010) have shown the presence of GLUT-1,2,3,4 and 5 in porcine, canine, and equine sperm.

The monosaccharide-Na^+ co-transport system (SGLT) is a more specialized and energy-demanding process, as it transports glucose against the concentration gradient. This system is found in smaller number in sperm, when compared to GLUT (Gibb; Aitken, 2016). This process is mediated by a carrier, in which it is coupled to the Na^+ concentration gradient that is transported along with glucose into the cell (Bucci et al., 2011). SGLT-1 was found in the peri-acrosomal region and in the midpiece of canine sperm, enabling the hypothesis that canine sperm cell performs different metabolic pathways, each showing greater affinity with specific substrates (Rigau et al., 2002).

Cell membranes do not have specific carriers for phosphorylated molecules. In addition, these molecules are very polar and have difficulty diffusing through the membrane. As a consequence, glucose is immediately phosphorylated after entering the cell, which ensures its permanence within the cytoplasm for later oxidation. The enzyme that transforms glucose into glucose-6-phosphate is hexokinase, one of the three enzymes that regulate glycolysis (Murray et al., 2000). It has a high affinity to the glucose molecule, which guarantees efficient phosphorylation even with low glucose concentrations. However, this enzyme has a low V_{max},, in other words, it is not able to phosphorylate larger amounts of glycids or retain them for future metabolization, since in these cases all of its active centers remain occupied.

The next steps in glycolysis consist of isomeric reactions, oxidation, and phosphorylation, resulting in a balance of energy production of 2 ATP and 2 NADH for each glucose molecule. The end product of glucose metabolization is two molecules of pyruvate. In sperm, according to environmental conditions, pyruvate can take two different routes: under aerobic conditions it undergoes oxidation and forms acetyl-CoA, which is the main substrate of the Krebs cycle; and under anaerobic conditions it is reduced to lactate (Cox; Nelson, 2002).

Fructose is also metabolized through glycolysis, however, to enter the intermediate metabolism its phosphorylation is required. At first, it is phosphorylated by the enzyme *fructokinase*, forming fructose-1-phosphate. Unlike glucose oxidation, fructose-1-phosphate is not directly converted to fructose-6-phosphate (intermediate metabolite of glycolysis), but cleaved by *aldolase B*, producing dihydroxyacetone phosphate (DHAP) and glyceraldehyde. DHCP can directly enter glycolysis, whereas glyceraldehyde needs to be transformed into glyceraldehyde-3-phosphate to follow its oxidation (Marzzoco; Torres, 1999).

Lactate is formed by the action of *lactate dehydrogenase (LDH),* and the direction of this reaction depends on the intracellular concentration of pyruvate / lactate and the NADH / NAD + ratio in the cell (Champe; Harvey; Ferrier, 2006). In this reaction, a reduction of a NADH molecule formed during glycolysis occurs, generating 1 NAD +. In aerobic conditions, the NADH molecule enters the respiratory chain, generating approximately 3 ATP at the end (Cox; Nelson, 2002). However, under conditions of anaerobiosis, the NAD + molecules produced in the formation of lactate can be oxidized again at the beginning of glycolysis, guaranteeing the indirect production of energy in two ways (Marzzoco; Torres, 1999).

Pesch et al. (2006) found a positive correlation between LDH and total motility, progressive motility, and percentage of live sperm in stallions, which indicates that extracellular LDH ensures sperm metabolism. Corroborating this study, Guo et al. (2019) have shown that the amount of lactate and LDH in seminal samples of porcine increased with the use of extracellular ATP in seminal plasma, suggesting that this form of extracellular energy may stimulate lactate to produce intracellular ATP, enabling sperm motility.

In contrast, Dogan et al. (2009) have found only a correlation between ejaculation volume and activity of the enzymes *ALP (Alkaline Phosphatase)* and *AST (Aspartate Amino Transferase).* These results prove the sperm's

ability to produce energy from lactate both in the presence and in the absence of oxygen.

Despite the production of a certain amount of ATP during glycolysis, the final products, pyruvate or lactate, still retain the largest amount of energy originally contained in glucose. The citric acid cycle is necessary for the complete release of this energy (Marzzoco; Torres, 1999).

An alternative to the oxidation of glucose-6-phosphate by the citric acid cycle is the pentose-phosphate pathway, which is divided into an oxidative and a non-oxidative phase. This mechanism, in humans, has been described as supplying the needs of NADPH, being related to the expression of G6PD (Glucose-6-phosphate dehydrogenase) and sperm capacitation (Miraglia et al., 2010).

Humans with diabetes mellitus present changes in glucose transport through GLUTs, glucose metabolism, oxidative stress, nuclear fragmentation, and mitochondrial apoptosis, which is attributed to the action of insulin and has deleterious effects on the reproductive system of these individuals, through the different mechanisms presented (Dias et al., 2014).

2.2.2 Citric acid cycle

The citric acid cycle, also known as Krebs cycle, unlike glycolysis, is strictly aerobic. It is considered the final route to which the oxidative metabolism of carbohydrates, amino acids and fatty acids converges, being finally converted into CO_2 and H_2O (Murray et al., 2000).

Pyruvate, the final product of glycolysis, is converted to acetyl-CoA through a *pyruvate-dehydrogenase complex*. In strict sense, this complex is not part of the citric acid cycle, but it is an important source of acetyl-CoA, which is the main substrate of the cycle. For this reaction to occur, it is required to reduce a NAD + to NADH, and there is also the release of a CO_2 molecule. In addition to pyruvate, fatty acids and amino acids metabolized by

other metabolic pathways also provide substrates for energy production through this route (Champe; Harvey; Ferrier, 2006).

The molecular processes that involve the consumption of O_2 and the release of CO_2 by cells are also called cellular respiration (Murray et al., 2000), and can be divided into three stages: the first consists of glycolysis and includes the conversion of pyruvate to acetyl-CoA; in the second stage, the acetyl groups provided by acetyl-CoA, are introduced into the Krebs cycle and oxidized to CO_2, however, the energy released by oxidation is conserved by NADH and $FADH_2$ and reduced therein; third stage consists of the oxidation of these reduced coenzymes through electron transport chain, where the energy is released in the form of ATP (Delvin, 1997).

During the Krebs cycle, a great amount of energy is produced, but not directly in the form of ATP. From the introduction of acetyl-CoA to the release of CO_2, the balance of energy production in the cycle is three molecules of NADH, one of $FADH_2$ and one of GDP. The oxidation of these coenzymes is carried out by the electron transport chain, where for each NADH the production is of 2,5 ATP, while for each $FADH_2$ the production is of 1,5 ATP. GDP, like ADP, is phosphorylated to form one ATP. Thus, for each oxidized acetyl-CoA molecule, 10 molecules of ATP are produced (Nelson; Cox, 2014).

2.2.3 Electron transport chain

Knowledge on energy production pathways requires the understanding of oxidative phosphorylation and electron transport chain, since they complement the energy production of the other metabolic routes.

During the metabolization of glucose, a small amount of energy is produced directly in the form of ATP, while most of the energy is produced in the form of reduced coenzymes (Murray et al., 2000).

The electron transport chain is located in the internal mitochondrial membrane and is the final route by which electrons from different fuels flow

to oxygen, which is why it is also called the respiratory chain (Pesch; Bergmann; Bostedt, 2006). It is composed of five complexes which, through oxidation-reduction reactions, are able to reduce oxygen to H_2O (Dogan; Polat; Nur, 2009).

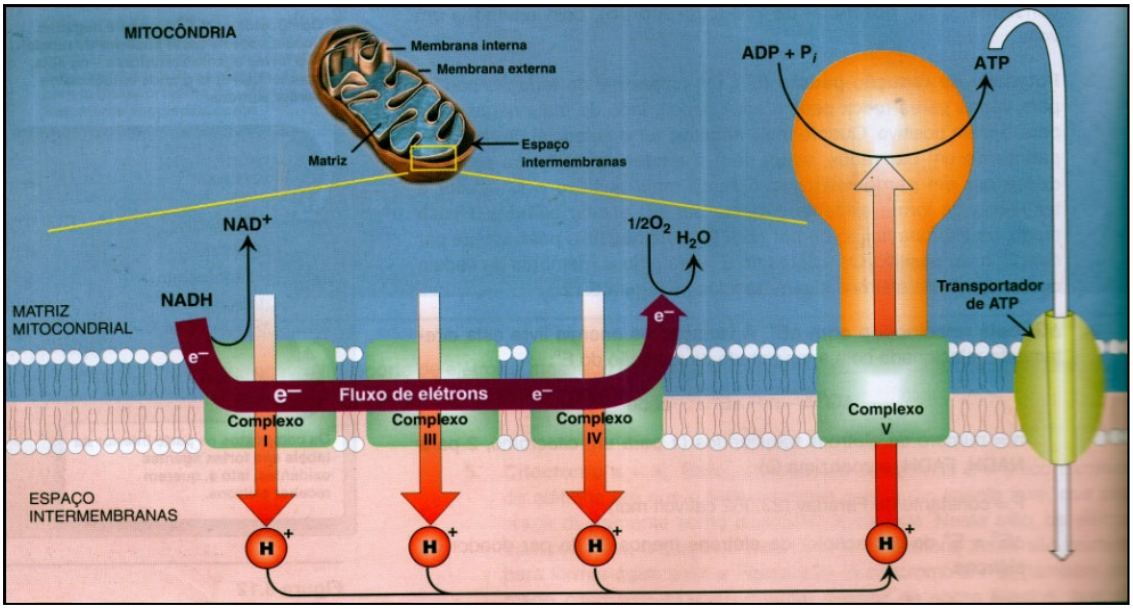

Figure 3. Electron transport chain, shown attached to the proton transport. (Pesch; Bergmann; Bostedt, 2006).

During this process, metabolic intermediates donate electrons to specific coenzymes, such as NAD^+ and FAD, forming reduced energy-rich coenzymes, NADH and $FADH_2$. These coenzymes interact with the chain through the donation of a pair of electrons, which, as they are carried, lose a great amount of free energy (Delvin, 1997). Part of this energy can be used for the production of ATP through ADP and inorganic phosphate (P_i), while the rest is released in the form of heat. This process is called oxidative phosphorylation.

The importance of the electron transport chain to energy metabolism is thus related to the use of energy of reduced coenzymes, as well as for the release of oxidized coenzymes, so they return to new nutrient degradation processes (Murray et al., 2000).

2.2.4 Oxidative phosphorylation

Oxidative phosphorylation is the final stage of the energy-producing metabolism in aerobic organisms. This pathway may complement the electron transport chain, since it uses the proton gradient formed in the previous steps for the production of ATP (Nelson; Cox, 2014).

The transfer of electrons along the electron transport chain is energetically favored, since NADH is a strong electron donor, and molecular oxygen is an avid electron receptor (Champe; Harvey; Ferrier, 2006).

During electron transfer, proton transport occurs simultaneously through the internal mitochondrial membrane, from the matrix to the inter-membrane space (FIGURE 3). This process creates an electric gradient and a pH gradient in the inner membrane (Marzzoco; Torres, 1999).

The inner mitochondrial membrane is impervious to most hydrophilic or charged substances. There are numerous transport proteins that enable the passage of substances from the inter-membrane space to the mitochondrial matrix. Among these proteins, the ATP-synthetase complex enables the passage of H^+ ions, which is responsible for the electrical gradient created during electron transport to the mitochondrial matrix. This passage of protons (H^+) results in the synthesis of ATP through ADP+P_i and, at the same time, dissipates the electrical and pH gradients (Nelson; Cox, 2014).

In porcine, even though glycolysis is commonly cited as a sperm energy mechanism, the importance of mitochondrial oxidative phosphorylation in the total and progressive sperm motility has been proven, as this energy derives from the production of ATP by the ATP-synthase process, while ATP produced by respiratory complexes of NADH-O_2-oxidases are indirectly impacted by the production of ATP through the mitochondrial electrochemical gradient (Nesci et al., 2020).

2.3 Role of the extender in sperm metabolism

The components and functions of seminal plasma have not been completely elucidated. It is known that plasma is a source of substrates for sperm metabolism, maintaining the energy status of sperm cells. However, proportions greater than 20% of plasma have been reported as harmful to stallion semen during refrigeration (Loomis, 2006). Studies have shown that proportions between 0.6 and 20% of seminal plasma are beneficial to seminal parameters and fertility in stallions (Moore; Squires; Graham, 2005; Arruda et al., 2008).

In porcine, the exosomes present in large quantities in seminal plasma are able to produce ATP in extracellular form by glycolytic pathway, increasing progressive motility and decreasing the rate of seminal apoptosis (Guo et al., 2019).

For the maintenance of sperm viability for long periods, the use of semen diluents is essential. In addition to diluting the seminal plasma, its positive effects are based on the control of pH, osmolarity, and energy supply (Aurich, 2011; Brinsko, 2011).

Different classes of seminal additives have been identified, including: antioxidants such as vitamin E and ascorbic acid; methylxanthines such as pentoxifylline and caffeine; trace elements such as copper, zinc, and selenium; enzymes such as superoxide dismutase and glutathione peroxidase; amino acids and proteins such as cysteine, taurine, hypotaurine, bovine serum albumin, L-carnitine, pyruvate, and platelet activating factors; and also polysaccharides such as trehalose and hyaluronic acid (Lisboa et al., 2014; Gibb et al., 2015; Thangamani et al., 2018).

Storage for long periods requires cryopreservation of the semen, which can be refrigerated or frozen. Cooling is aimed at reducing the sperm metabolic rate, allowing it to be transported at 5º for up to 48 hours (Hartwig et al., 2014).

As equine sperm is dependent on oxidative phosphorylation to obtain energy, and this consequently produces reactive oxygen species (ROS), cooling causes the rate of energy production to decrease, as well as the production of ROS (Maxwell; Welsch ; Johnson, 1997). However, this reduction in metabolic rate is also responsible for reducing ATP-production, causing many energy-dependent sperm functions to be compromised during storage, impairing cell homeostasis and causing premature sperm death (Moore; Squires; Graham, 2005). Thus, Gibb et al. (2016) developed an extender for storage of semen at room temperature, aimed at maintaining sperm functions and reducing damage caused by thermal shock during storage.

Most equine semen centrifugation and refrigeration extenders are composed of milk or egg yolk (Pagl; Aurich, 2006). However, the positive effects of milk and egg yolk have not been completely disclosed (Amann; Graham, 2011). These diluents are a source of lipoproteins that protect sperm from damage caused by thermal shock (Aurich, 2011).

One of the main problems related to milk based or egg yolk based extenders is the lack of standardization between batches of raw material (milk and egg). In addition, only a certain fraction may be necessary to obtain beneficial effects on sperm, while the other components may have harmful effects. Due to difficulties in obtaining quality certificates, information on risks of biological contamination, and individual variability, many studies have been carried out to define the beneficial substances and possible replacement of unnecessary components (Battelier et al., 2001; Gil et al., 2003; Ricker et al., 2006; Gibb et al., 2015).

Among the milk components, phosphocaseinate and ß-lactoglobulin are considered the most effective at maintaining sperm longevity during refrigeration (Battelier et al., 2001). Pagl et al. (2006) have shown that the substitution of skimmed milk with caseinates and whey proteins did not impair the maintenance of seminal quality during refrigeration between 5

and 8° C, when compared to the extenders based on skimmed milk and glucose.

In both bovine and equine, certain seminal plasma proteins bind to the surface of the sperm and stimulate the efflux of cholesterol and phospholipids from the membrane, which is important for the training process (Bergeron et al., 2004). During semen processing and storage, this efflux can result in damage to the sperm membrane (Pagl; Aurich, 2006). Bergeron et al. (2004) suggested that the sequestration of these seminal proteins may act as a protection mechanism provided by egg yolk in bovine sperm.

In clots, the addition of bovine serum albumin and skim milk to the semen was tested, and these two agents acted as protectors of membrane integrity, increasing the production of intracellular ATP and sperm motility (Fu et al., 2017).

As all living cells in aerobic conditions, sperm produces reactive-oxygen species (ROS) as a result of energy metabolism. Most ROS are neutralized by elements contained in the seminal plasma, maintaining a balance between production and neutralization. However, when there is an excess of ROS, the sperm suffers oxidative stress, which can cause damage to membrane and DNA (Maia; Bicudo, 2009; Rajender; Rahul; Mahdi, 2010).

Lipid peroxidation alters the fluidity and functional integrity of the membrane, which compromises the fertilization capacity and sperm motility (Catala, 2006). Unsaturated fatty acids are more susceptible to peroxidative damage caused by ROS, and equine spermatozoa are especially more susceptible due to the high concentrations of these fatty acids in their membrane (Varner; Johnson, 2007).

To minimize the negative effects of ROS generation and improve seminal parameters, substances such as pyruvate have been associated with semen diluents, in order to preserve membrane integrity and sperm motility after the cooling process (Webb ; Arns, 2006; Bruemmer et al., 2002).

Pyruvate is an effective energy substrate, as it can move freely across the sperm membrane. It has recently been recognized as a neutralizer of hydrogen peroxide (H_2O_2), which is one of the free radicals responsible for the oxidative stress of sperm (Varma; Devamanoharan, 1990; Salahudeen; Clark; Nath, 1991). Fabroccini et al. (2000) suggested that pyruvate can be used as an energy source, to aid sperm to overcome the stress of the freezing / thawing process when small amount of pre-freezing time is given.

It has been reported that the addition of 10mM of pyruvate in stallion semen has caused a decrease in sperm motility and speed when compared to the control group; the same has been reported in humans (Bruemmer et al., 2002; Bilodeau et al., 2002). However, concentrations between 2 and 5 mM have increased progressive motility in equine sperm incubated at 25°C for 24 hours (Bruemmer et al., 2002).

Bilodeau et al. (2002) were able to reduce the damage caused by lipid peroxidation through the use of pyruvate in frozen semen extender for cattle. The addition of pyruvate in equine cooling extenders has resulted in improved sperm motility and speed after 48 hours of refrigeration (Webb; Arns, 2006).

In goats, glucose and pyruvate had been more efficient in maintaining sperm motility during the storage of semen for a prolonged period, when compared with lactate. Furthermore, it have been shown that the pentose phosphate cycle is more compelling than glycolysis in maintaining sperm motility (Qiu et al., 2016).

Glucose is commonly added to semen extenders as an energetic substrate, being beneficial to sperm metabolisms initially dependent on glycolysis in long-term storage of samples. Thus, the addition of glucose reduces oxidative stress resulting from normal mitochondrial energy production (Koppers et al., 2008). A study by Hernández-Aviléz et al. (2020) has shown that the absence of glucose in the extender impaired kinetic parameters, but there was no reduction in the plasma and acrosomal membrane of equine spermatozoa refrigerated for up to 5 (five) days.

Glycerol is widely used as a cryoprotectant in freezing extenders, however, in addition to providing protection, it is also used as an energetic substrate for sperm (Ford, 1980). Glycerol-3-phosphate is a substrate for intermediate metabolism oxidized by the glycolytic pathway. It is rapidly metabolized by cattle, sheep, rats, pigs, and bird sperm (Mann, 1964; Frenkel; Peterson; Freund, 1975). In contrast, it is not oxidized by rabbit, human, dog and stallion sperm (Kevhani; Storey, 1973).

High concentrations of glycerophosphocholine are present in the semen of several species (Mann, 1964), and can be hydrolyzed to glyceraldehyde-3-phosphate by diesterases in the reproductive tract of females (Ford, 1980), thus being a significant source of energy for the sperm after ejaculation.

The concentration of calcium, chlorine, and potassium also seems to influence sperm hypermotility, because a decrease in sperm motility was evident when added to extenders for rat semen containing low concentrations of Ca_2^+. The inhibition of energy metabolism caused by this extender was associated with a decrease in Cl^- and K^+ in the sample (Cordero-Martinez et al., 2019).

3 Conclusion

Sperm preservation biotechnologies aim to increase the viability period. Thus, knowledge on sperm metabolism is required to the understanding of the physicochemical changes that are responsible for energy production. Therefore, the use of a suitable extender containing the necessary substrates for the energetic metabolism of the sperm of each species is a determining factor for biotechnologies aimed at maintaining the physical and functional integrity of the male gamete.

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Resumo: Para o desenvolvimento de técnicas que visam o aumento da longevidade espermática, é necessário o entendimento metabólico desta célula. O espermatozoide exige um fornecimento constante de energia para a manutenção das funções celulares. Aproximadamente 500 reações metabólicas são conhecidas por ocorrer nas células somáticas, sendo que grande parte delas requer energia. Para atingir seu objetivo, grande parte da produção energética do espermatozoide é destinada à motilidade, o qual é um processo especializado e dependente de ATP. O espermatozoide possui os mecanismos necessários para produzir energia através da glicólise, Ciclo do Ácido Cítrico (*Ciclo de Krebs*) e Fosforilação Oxidativa. O entendimento destas vias torna-se indispensável para a compreensão das interações entre os substratos do meio diluente e o espermatozoide. O objetivo desta revisão é abordar as principais vias de produção energética do espermatozoide, assim como os substratos disponíveis para metabolização.
Palavras-chave: Espermatozoide, motilidade, ATP.

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