Rethinking sperm: lessons from mitochondrial function

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Abstract

Biological nature of age-related declining fertility in males of any species, including stallions, has been elusive. In horses, economic costs to the breeding industry are frequently considered extensive. Mitochondrial function in ejaculated sperm, essential for sperm motility, is reflected by the dynamic processes of adenosine triphosphate production, mitochondrial oxidative efficiency and production of reactive oxygen species; this balance may become compromised in aging stallions and during cryopreservation process. This presentation will focus on mitochondrial integrity and function as an avenue for understanding the pathophysiology of sperm undergoing cryopreservation and male aging.

Keywords: Sperm, mitochondrial function, oxidative phosphorylation, reactive oxygen species

Introduction

Conventional thought in sperm biology holds that the sperm flagellum is powered by adenosine triphosphate (ATP) produced by a complex of mitochondrial enzyme systems including the electron transport chain (ETC). As such, the powerhouse of the cell, the mitochondrion, is likely the foundation of sperm pathophysiology and structure.[1-3] There are species differences in energy requirements for mitochondria and as optimal conditions for sperm function are becoming evident, individually tailored sperm handling and storage techniques[4-6] are likely to become clinical realities. Media modifications for sperm processing and storage may become necessary to provide appropriate energy substrates to minimize sperm damage and improve sperm longevity after cryopreservation.[5] Damage to sperm mitochondria is one of the most marked organelle changes during cryopreservation that is likely responsible for the majority of loss in motility and fertility after a freeze-thaw cycle.[7,8] An increased understanding of sperm mitochondrial bioenergetics provides strong rationale to make considerable improvements in preservation and assisted reproduction techniques. Mitochondria also have their own genome; therefore, investigations of mitochondrial DNA may be useful to identify currently unknown causes of sperm dysfunction.[9.10] The purpose of this review is to discuss current knowledge of sperm mitochondrial biology, focusing on appropriate sperm mitochondrial energy sources, mitochondrial production of reactive oxygen species and determination of future research directions that may improve sperm function, morphology and fertility.

Anatomy and physiology of sperm mitochondria

Equine sperm contain 40 - 50 helical mitochondrial gyres (turns) per cell in the midpiece composed of single mitochondria that line up end to end during spermiogenesis.[11-13] Functions of mitochondria in sperm include ATP production for motility, reactive oxygen species (ROS) production and redox signaling, and apoptosis.[12,14] There is also growing evidence that sperm mitochondria may also be involved in intracellular calcium signaling and homeostasis, well-established functions of somatic mitochondria. Equine sperm possess machinery for cellular processes of glycolysis, citric acid cycle, and oxidative phosphorylation (OxPhos) to meet large energy requirements for motility and capacitation-related events within the female reproductive tract, including hyperactivated motility, required for fertilization.

Further, sperm mitochondria are also known to be involved in lipid biogenesis, DNA replication of its own genome and protein and hormone production. During spermatogenesis, sperm undergo a reduction in cytoplasm and re-organization of cellular organelles (i.e. spermiogenesis). Mitochondria become organized end-to-end in a spiral formation around the sperm's midpiece flagellar machinery in a fashion that appears to prevent the dynamic functions of mitochondrial fusion and fission. These functions are inherent in somatic cell mitochondria in which this amazing organelle may adapt to

physiological changes and are thus capable of self-replication, reduction and consolidation. The so-called mitochondrial sheath is tethered and overlain by the sperm's fibrous sheath.

Mitochondria are comprised of 4 sub compartments: outer mitochondrial membrane (OMM), intermembrane space (IMS), inner mitochondrial membrane (IMM) and mitochondrial matrix.[15] The IMS separates the OMM and IMM and is the site of proton accumulation from ETC activity, which ultimately generates the electrochemical gradient and transmembrane potential (mitochondrial membrane potential). The IMM, which is largely impermeable, houses the enzyme complexes of the electron transport chain (ETC) and is thus the site of ATP production by oxidative phosphorylation.[16] The ETC is a series of favorable sequential redox reactions, beginning with electron donors of low reduction potential. Enzyme complexes 1, 3, and 4 pump protons across their gradient to acidify the mitochondrial intermembrane space, establishing a proton motive force that powers the fifth complex, ATP Synthase, via chemiosmosis to generate ATP. Each ETC enzyme is itself composed of multiple subunits arranged dynamically and tethered through the mitochondrial membrane to the sperm's fibrous sheath. The OMM has nonspecific pores called porins, protein complexes that allow for passage of ions and most metabolites < 10 kDa into the mitochondria.[16,17] The IMM encloses the matrix, with many cristae or invaginations increasing the surface area available for energy production. The inner mitochondrial membrane must be intact and impermeable for sperm mitochondria to be fully functional.[18,19] The mitochondrial membrane potential describes a proton gradient formed by passage of electrons along the electron transport chain which is then used by ATP synthase to make ATP.[20] Decreased membrane potential, which occurs by membrane depolarization, indicates mitochondrial damage and the cell's subsequent inability to meet energy demands. Hyperpolarization, by contrast, can lead to increased cellular damage through reactive oxygen species production and lipid peroxidation.[21]

Function of sperm mitochondria

It was recently reported that production of ATP by equine sperm for motility is primarily driven by OxPhos rather than glycolysis.[5,22] This is different than metabolism in many other species, e.g. humans and nonhuman primates, which still have essential sperm functions such as flagellar motility when OxPhos is inhibited.[9,23] It is reasonable to consider that there may be a metabolic shift to glycolysis with aging and semen cryopreservation when mitochondria become less efficient, due to an altered physiological state of the sperm. Since glycolysis is less efficient than OxPhos (only producing 2 ATP per molecule of glucose, whereas OxPhos produces 30) this could at least partially explain decreased motility in both cryopreservation and aging. In the aging testis, there is decreased antioxidant buffering capacity, which includes decreased superoxide dismutase enzyme activity as well as less active intra- mitochondrial catalase and peroxidase.[24] Mature sperm are particularly susceptible to oxidative damage for several reasons, including: (i) diminished cytoplasm, which houses the majority of cellular antioxidant enzymes; (ii) membranes with a high content of polyunsaturated fatty acids (PUFAs), which are particularly susceptible to peroxidative damage; and (iii) lack of transcription and translation machinery leaves mature sperm without typical DNA repair mechanisms used by somatic cells to combat against oxidative damage. Ultimately, this leaves sperm more susceptible to oxidative cell and DNA damage which could lead to decreased OxPhos.

Cryopreservation significantly damages sperm after thawing, but exact causes and targets of cryoinjury have not been determined. Possible causes include ROS production and apoptosis, whereas potential targets include plasma and mitochondrial membranes, known to have high PUFA content. Post-thaw sperm motility is typically 50% of initial motility; this percentage may be lower and more variable for stallions than for other species.[14] Mitochondria are responsible for producing the majority of ROS that can cause oxidative cellular damage during cryopreservation.[14,22,25] The daily yield of ROS can reach 10^7 molecules of superoxide anion per mitochondrion and negative effects on sperm include decreased fertility, motility, morphology, viability, altered membrane potential and mtDNA damage.[14,26] The high variation among males, especially stallions, is an industry problem and may originate in sperm mitochondria.

Assessing sperm mitochondria

Although one of the most popular methods of assessing mitochondrial function has been chemical probe-based measurements of mitochondrial membrane potential (MMP), conclusions have been complicated by issues of reliability and a lack of appropriate controls. The MMP refers to the electrochemical gradient across the inner mitochondrial membrane which is necessary to enable mitochondria to generate ATP by OxPhos. Probing the magnitude of MMP in motile and nonmotile sperm can provide insights into the extent of that specie's reliance on OxPhos, as reported for dog and stallion sperm. Volpe and coworkers reported on the relationship between MMP and sperm motility in dogs of known fertility, ranging from 16 months to 10 years of age.[27] Using fluorescent markers for viability and the fluorophore JC1 for MMP along with computer assisted sperm motility analysis, they reported that sperm of low motility were correlated with a high inner mitochondrial membrane potential, suggesting ATP production via oxidative phosphorylation is not essential for fertility in male dogs. While these results provide exciting prospects for development of extenders of ideal substrate composition for long term preservation of sperm from valuable working dogs and stallions, these and the results of other such studies are recently being questioned. MMP probes are cationic and their rate of accumulation within the mitochondria is inversely proportional to inner mitochondrial membrane potential. The popular MMP probe JC1 allows dual color high/low assessment of inner mitochondrial membrane potential. However, like the other MMP probes used in sperm, JC1, which is sensitive to factors of intracellular stress such as ROS, is not reliable as a quantitative measure of MMP without appropriate controls. Ideally, high/low MMP controls would include using an inhibitor of ATP Synthase (e.g. oligomycin) and an uncoupler of MMP, such as trifluorormethoxy carbonylcyanide phenylhydrazone (FCCP) or 2,4 Dinitrophenol (DNP); these are ionophores that dissipate the proton motive force by transporting hydrogen ions across the mitochondrial membrane. Davilla and coworkers[28] challenged the previous understanding that oxidative phosphorylation is the dominant metabolic pathway for sperm motility in the stallion. Similar to Volpe and coworkers,[27] Davilla and coworkers[28] used the MMP probe JC 1 and computer assisted sperm motility analysis, but also included DNP and oligomycin as controls in their analysis of ejaculates from Spanish stallions. These authors used 60 µm oligomycin, whereas 1 µm is the commonly used concentration in studies of human, boar and stallion sperm.[28] Consistent with other studies, inhibition of mitochondrial respiration by mitochondrial uncouplers dramatically reduces MMP and sperm motility. To explore whether glycolysis alone can support sperm motility, Davila and coworkers[28] aimed to inhibit glycolysis by incubating stallion sperm in the presence of 2 deoxy D Glucose (2 DG) rather than glucose. Although 2 DG is a weak competitive inhibitor of glycolysis through its inhibition of glucose 6 phosphate production, its metabolism to 2 deoxyglucose 6 phosphate results in accumulation of phosphate binding products that cannot undergo further substrate level phosphorylation. Consequently, most of the phosphate in the sperm is bound, making it unavailable for ATP Synthesis in oxidative phosphorylation. In the presence of 2 DG application, sperm have increased MMP and decreased ATP production via oxidative phosphorylation as well as any ATP lost due to the inhibition of glucose 6 phosphate production in glycolysis. Davila et al[28] reported that inhibition of glycolysis resulted in decreased sperm motility. However, their results described a reduction in total motility, but no change in progressively motile sperm. This distinction is important, as the significance of motility measures of sperm vary widely. Progressive motility describes an efficient consistent forward movement, whereas nonprogressive motility refers to sperm making tight circles or are moving but not making forward progression. Although the glycolytic pathway likely provides support for other sperm functions (e.g. capacitation), its role in the ability of stallion sperm to reach the egg is unlikely. That glycolysis can provide the energy necessary for motility of stallion sperm has been disproved by other more recently developed methods. The use of MMP as a method of defining reliance on oxidative phosphorylation, though valuable in enabling comparison with previous studies, can be unreliable.

In aerobic organisms, oxygen serves as the terminal electron acceptor; its reduction to water results in depletion of oxygen. Measuring this oxygen consumption provides an indirect measure of ATP produced by oxidative phosphorylation in sperm and reliably indicates mitochondrial function when employed alongside adequate mitochondrial effector drug treatments. To facilitate measurement of

oxygen consumption in a variety of cell types, an oxygen biosensor system has been developed using a microplate system with an oxygen impermeable matrix and oxygen-sensitive, ruthenium-based fluorophore. As oxygen is consumed from the media by the cells, the ruthenium-based probe fluoresces in proportion to oxygen depletion. In contrast to the approach used by Davila,[28] this method was used by Darr et al.[5] to determine major substrates for motility and mitochondrial function in stallion sperm. Production of low levels of ROS is a normal byproduct of mitochondrial function, resulting from low levels of electron leakage during normal ETC activity. These free radicals are highly reactive and can quickly oxidize lipids, cause DNA mutations and lead to apoptosis. To minimize damage caused by free radicals such as peroxides and superoxide anions, sperm produce antioxidant substances to scavenge free radicals. Reducing agents such as glutathione peroxidase and superoxide dismutase, which are produced during normal metabolism, exert antioxidant effects by converting free radicals into nonradical forms. Although antioxidants are an efficient protective mechanism, excessive reactive oxygen species generation can overwhelm protective effects provided by these reducing agents. In a system primarily relying on glycolysis for progressive motility in sperm, supplementation with substrates supportive of oxidative phosphorylation would result in increased ROS production. As a result, inadequate substrate composition of storage media can lead to mitochondrial dysfunction and oxidative damage; therefore, it is crucial to provide optimum storage conditions. Since oxidative phosphorylation provides the energetic support for stallion sperm motility, the oxygen biosensor assay was utilized to determine the preferred substrate for sperm storage.[3,5] Consumption of oxygen was highest in groups provided with the substrates lactate or pyruvate; additional testing indicated a dose-dependent response of increasing mitochondrial activity with increasing substrate concentrations. Because glucose can only be utilized through glycolysis, it was expected that lactate and pyruvate would better support sperm motility and mitochondrial function. Progressive motility was highest in lactate- and pyruvate-supplemented groups. Understanding the preferred metabolic pathways of sperm is important, as being able to control metabolism is the basis of developing extenders and media. In sperm, slowing metabolism during preservation and increasing metabolism just before insemination is the ultimate goal. Measurement of oxygen consumption is a reliable, high-throughput method of investigating dependency on oxidative phosphorylation for sperm motility, despite limitations to the oxygen biosensor assay.

There are several methods to analyze preferred metabolic pathways underlying motility of sperm from various species. However, many of these methods suffer from a variety of pitfalls that limit interpretability of the resultant data. Although the underlying metabolic preference for sperm motility has been well established in some species, there is an ongoing quest to identify ideal substrates for long term storage and artificial insemination

Calcium and sperm motility

Our recent studies demonstrated that stallion sperm motility is fueled primarily by mitochondrial-produced ATP and that both mitochondrial oxidative function and motility decrease significantly with cryopreservation.[5,30] Additionally, cryopreservation alters stallion sperm Ca^{2+}_{i} signaling and homeostasis,[31] known to have important roles in sperm motility.[32,33] Because mature sperm lack an intact ER, the predominant intracellular Ca^{2+} store in most cell types, it was previously believed that influx of extracellular Ca^{2+} is the sole Ca^{2+} source and regulator of Ca^{2+}_{i} homeostasis.[34] However, there is now evidence that intracellular Ca^{2+} stores are present in sperm of several species, including humans, mice and cattle,[35,36] but Ca^{2+}_{i} stores have not yet been identified or characterized in stallion sperm. In other cell types, mitochondria act as Ca^{2+}_{i} stores, in addition to the ER, with an important role in maintenance of Ca^{2+}_{i} homeostasis.[37] Specifically, increased OxPhos activity likely triggers mitochondrial Ca^{2+} uptake through the mitochondrial Ca^{2+} uniporter, resulting in increased Ca^{2+}_{mt} stores, which increase mitochondrial oxidative function and ATP production.[38] Investigation of Ca^{2+}_{mt} uptake in sperm are limited, but considerable evidence suggests maintenance of Ca^{2+}_{mt} homeostasis is essential for motility regulation in human[39] and bovine sperm.[40] We demonstrated that mitochondrial oxygen consumption (MITOX) is an indicator of mitochondrial oxidative balance and is likely involved mechanistically in calcium function. A new understanding of Ca^{2+} homeostasis in context with sperm oxidative function will

support development of rational semen and stallion therapeutic intervention and is needed in the AI industry. Cryopreservation-induced alterations in Ca^{2+}_i dynamics may significantly contribute to reductions in mitochondrial oxidative function and motility that we previously reported for cryopreserved stallion sperm. However, the role of sperm mitochondria in Ca^{2+}_i regulation and its subsequent impact on ATP production and motility have never been investigated in stallions.

Sperm morphology and reactive oxygen species

Despite being widely accepted as a negative indicator of sperm health in most species, implications of increased ROS in stallion sperm have been highly debated.[41] Stallion sperm fuel motility almost exclusively via mitochondrial-produced ATP, in contrast to most species, which use primarily glycolytic[42,43] pathways to produce ATP. Increased mitochondrial function and ETC electron flow results in increased passive electron leakage, increases in ROS production and oxidative stress accompany increases in mitochondrial activity. It has been proposed that the sperm in an ejaculate that generate high ROS concentrations are not defective, but actually may be the highly motile, more robust sperm and that biomarkers of oxidative stress are a positive indicator of stallion fertility.[44] Although not validated, this concept has been challenged by studies that have correlated increased ROS with decreased motility, viability and mitochondrial function.[3,5] ROS evaluation is a promising prospective addition to diagnostic semen evaluation for stallion infertility if it can be definitively established as a positive or negative biomarker of stallion sperm function. In stallions, a positive correlation between pregnancy rates and percentage of morphologically normal sperm and a negative correlation between pregnancy rates and midpiece or tail abnormalities has been reported.[45] ROS production in human sperm has been positively correlated with abnormal sperm morphologies including abnormal heads, cytoplasmic droplets, midpiece, and tail abnormalities.[46,47] If morphologic abnormalities are correlated with elevated ROS concentrations in stallion sperm, this will strongly implicate ROS as a negative biomarker of sperm function and fertility in stallions.

Recent technological advances in imaging flow cytometry demonstrates strong promise for evaluation of stallion semen.[48] The ImageStream® instrument has been successfully used for evaluation of human[49] and bull[50] semen. ImageStream® technology is unique in that it enables generation of high-throughput flow cytometry data with parallel fluorescence and brightfield microscopy, which has tremendous potential for uses in both clinical and research settings. Within a research setting, ImageStream® technology can be used to generate morphologic and fluorescence data with sample sizes large enough for statistical significance. Further, the ability of the ImageStream® to analyze fixed *or* live cells allows a wider range of applications in semen assessment than fluorescence microscopy alone, which requires nonmotile cells for accurate morphologic assessment. Although the cost and size of the ImageStream® system limits clinical applications, it is possible that fixable assays for negative biomarkers can be performed by clinicians and sent to a laboratory for further analysis.

Conclusion

This review has focused on the function and structure of sperm mitochondria and their role in sperm motility and morphology, primarily in stallions and other livestock. Greater understanding of negative and positive influences on sperm motility and abnormal morphology, which are highly variable in stallions, would have great benefit to the breeding industry, particularly for stallion management. Improved precision and accuracy of sperm measurements may be gained without additional samples taken during a breeding soundness exam or when shipping chilled or frozen semen. In this review, we also highlighted the translational nature of basic cell biology and its application to sperm and ultimately to various breeding industries.

Conflict of interest

Authors claim no direct or indirect affiliation with any manufacturer listed in text. Any information regarding equipment and various manufacturers is for readers' reference only.

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