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## REVIEW

Sperm Biology

# Oxidative phosphorylation versus glycolysis: what fuel do spermatozoa use?

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Spermatozoa are highly specialized cells. Adenosine triphosphate (ATP), which provides the energy for supporting the key functions of the spermatozoa, is formed by 2 metabolic pathways, namely glycolysis and oxidative phosphorylation (OXPHOS). It is produced in the mitochondria through OXPHOS as well as in the head and principal piece of the flagellum through glycolysis. However, there is a great discrepancy as to which method of ATP production is primarily utilized by the spermatozoa for successful fertilization. Mitochondrial respiration is considered to be a more efficient metabolic process for ATP synthesis in comparison to glycolysis. However, studies have shown that the diffusion potential of ATP from the mitochondria to the distal end of the flagellum is not sufficient to support sperm motility, suggesting that glycolysis in the tail region is the preferred pathway for energy production. It is suggested by many investigators that although glycolysis forms the major source of ATP along the flagellum, energy required for sperm motility is mainly produced during mitochondrial respiration. Nevertheless, some studies have shown that when glycolysis is inhibited, proper functioning and motility of spermatozoa remains intact although it is unclear whether such motility can be sustained for prolonged periods of time, or is sufficiently vigorous to achieve optimal fertilization. The purpose of this article is to provide an overview of mammalian sperm energy metabolism and identify the preferred metabolic pathway for ATP generation which forms the basis of energy production in human spermatozoa during fertilization.

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## INTRODUCTION

All living cells require energy for both development and function and male gametes, are no exception. Mammalian spermatozoa specifically use adenosine triphosphate (ATP) to maintain the intracellular milieu<sup>1</sup> and for cellular processes such as motility, capacitation, hyperactivation and the acrosome reaction,<sup>2,3</sup> all of which are needed for successful fertilization. ATP is formed from adenosine diphosphate (ADP), and therefore the addition and removal of phosphate groups to ADP molecules forms the basis of all life processes.

Throughout the years there has been ambiguity regarding the metabolic pathway that plays the most vital role in energy production in spermatozoa. According to Rees *et al.*<sup>4</sup> mammalian spermatozoa share a common problem in that ATP must be delivered along the entire length of the flagellum, which means they must adopt a variety of metabolic strategies to generate it. The purpose of the present article is to provide an overview on mammalian sperm energy metabolism and identify the preferred metabolic pathway for ATP generation in human spermatozoa.

## PATHWAYS OF ENERGY PRODUCTION IN SPERMATOZOA

Adenosine triphosphate is produced in the spermatozoon via two metabolic pathways – glycolysis and oxidative phosphorylation (OXPHOS) – with the former occurring in the head and principal piece of the flagellum and the latter occurring in the mitochondria. A glycolysis involves the breakdown of six carbon monosaccharides

during a series of enzyme-catalyzed reactions that yield two molecules of three carbon compound pyruvates. In the above mentioned metabolic process the net yield of ATP is two molecules per molecule of glucose oxidized. In subsequent steps, pyruvate is further oxidized and the carboxyl group is lost as CO<sub>2</sub> to yield the acetyl group of acetyl-coenzyme A. The acetyl group is then oxidized completely to CO<sub>2</sub> by the citric acid cycle. Electron donors, namely NADH and FADH<sub>2</sub>, are formed as a result of metabolic processes such as glycolysis, fatty acid oxidation, and the citric acid cycle, and they are energy-rich molecules with high electron transfer potential. In sequential steps the flow of electrons thus proceeds from NADH or FADH<sub>2</sub> to O<sub>2</sub> through protein complexes located in the inner mitochondrial membrane. An uneven distribution of protons that leads to a proton-motive force is created as the electron transfer results in the pumping of protons out of the mitochondrial matrix. ATP is synthesized when protons flow back into the mitochondrial matrix through an enzyme complex, namely ATP synthase.<sup>5,6</sup>

Oxidative phosphorylation is a more complicated process that transpires in a coordinated fashion and involves two components of the inner mitochondrial membrane – the respiratory chain and ATP synthase.<sup>7</sup> OXPHOS generates thirty molecules of ATP per molecule of oxidized glucose. The generation of ATP through OXPHOS is therefore 15 times more efficient than glycolysis. The most important factor that determines the rate of OXPHOS is the availability of ADP.<sup>6,8</sup> To summarize, the oxidation of fuels and the phosphorylation of

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ADP, coupled with a proton gradient across the inner mitochondrial membrane are key factors in the generation of ATP.

Although a spermatozoon can survive purely on glycolytic energy,<sup>9</sup> it requires OXPHOS for differentiation and maturation.<sup>10-12</sup> The preferred metabolic pathway opted for by spermatozoa with regard to energy production is highly species specific.<sup>13</sup> For example, various studies<sup>14,15</sup> have shown that glycolysis is essential for fertilization in mice, rat, hamster and humans spermatozoa, but not so in bovine sperm.

## ADENOSINE TRIPHOSPHATE PRODUCTION SITES IN SPERMATOZOA

Spermatozoa are highly differentiated cells consisting of three distinct regions: the head, mid-piece and tail. The sperm head contains the nucleus, which houses the highly compacted paternal genome.<sup>16</sup> Extending posteriorly from the head is the mid-piece, which is followed by the flagellum. The flagellum comprises of the principal piece of the spermatozoa and inside it is the fibrous sheath - a characteristic feature of mammalian spermatozoa.<sup>17</sup> Similarly, energy production in spermatozoa is also compartmentalized as it is in all eukaryotic cells.<sup>18</sup> ATP generation through OXPHOS occur in the mitochondria whereas glycolysis occurs in the head and fibrous sheath of the flagellum (Figure 1).

### Site of adenosine triphosphate production via oxidative phosphorylation

Mitochondria play a key role in the production of ATP through the process of OXPHOS. Therefore, they are appropriately referred to as the “power house” of the cell. They are known to occupy a major portion of the cytoplasmic volume of eukaryotic cells, and play an essential role in the evolution of complex animals.<sup>1</sup>

Located in the mitochondrial sheath and confined to the mid-piece region of the spermatozoa,<sup>19</sup> mitochondria occupy a substantial portion of the total cellular volume (approximately 15%–22%).<sup>20</sup> A mature spermatozoon contains approximately 72–80 mitochondria, which are involved in major processes such as the acrosome reaction and oocyte penetration.<sup>21</sup> Although it consists of four distinct sub-compartments - the outer mitochondrial membrane, the inter-membrane space, an inner mitochondrial membrane and a matrix - a sperm mitochondrion varies from its somatic cell counterparts both morphologically and functionally.<sup>19,22</sup> These differences can be attributed to the tight wrapping of the mitochondria around the axoneme of the spermatozoon. This tight wrapping results

in a mitochondrial capsule consisting of selenoprotein and comprising of disulfide bridges,<sup>23</sup> rendering stability to the mitochondrial sheath.<sup>13,24</sup> The inner mitochondrial membrane is folded into structures called cristae, which are the principal sites for OXPHOS and ATP generation. Sperm mitochondria possess specific isoforms of proteins and isoenzymes such as cytochrome C,<sup>25-27</sup> hexokinase subunit VIb of the cytochrome C oxidase,<sup>28</sup> and lactate dehydrogenase (LDH)<sup>29</sup> all of which are absent in somatic cell mitochondria.<sup>30</sup>

### Site of adenosine triphosphate production via glycolysis

Glycolysis plays a key role in mammalian sperm energy production and thus, an understanding of the site of glycolytically produced ATP is therefore of utmost importance.<sup>31-33</sup> Because the head and principal piece of the flagellum are devoid of respiratory enzymes, ATP production can therefore only occur through the process of glycolysis in these areas.<sup>1</sup> Several glycolytic enzymes specific to the spermatogenic cell have been identified in the fibrous sheath of spermatozoa. These include hexokinase, phosphoglucokinase isomerase, phosphofructokinase, LDH and glyceraldehyde-3-phosphate dehydrogenase (GAPD).<sup>8,18,34-37</sup>

## ENERGY NEEDS OF SPERMATOZOA

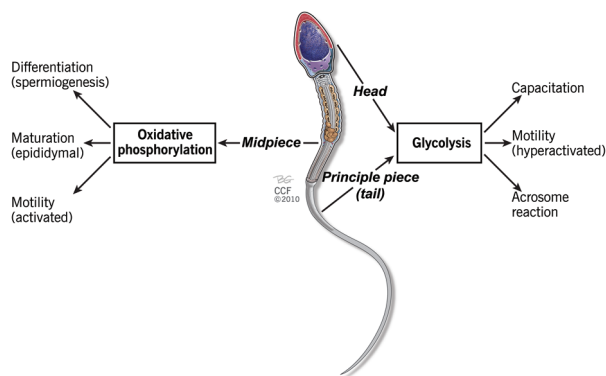
Mammalian fertilization is a multifaceted and complex process because the site of semen deposition is far from the site of fertilization in the female genital tract.<sup>38,39</sup> Therefore, cellular processes such as motility, capacitation, hyperactivation and acrosome reaction must occur in a synchronized fashion.<sup>40</sup> As all of these actions are energy dependent, the importance and role of energy in normal male gamete function cannot be overstated. The energy utilization of each of these processes will be discussed below.

### Adenosine triphosphate utilization during sperm motility

Spermatozoa compete with one another during their transit in the female reproductive tract in order to reach the female gamete first. Adequate motility is therefore required for successful fertilization. Movement is generated by the flagellum that constitutes more than 90% of the length of the mammalian spermatozoon.<sup>21</sup> Paoli *et al.*<sup>40</sup> define sperm motility as the “result of propagation of transverse waves along the flagellum in a proximal – distal direction, thus producing a hydrodynamic impulse that pushes the spermatozoon through the female genital tract toward the oocyte.” Spermatozoa display two types of motility – activated and hyperactivated.<sup>41</sup> Activated motility, as seen in the freshly ejaculated spermatozoa,<sup>20</sup> refers to the low amplitude, symmetric waves that propagate along the length of the flagellum and result in linear propulsion of the sperm cell.<sup>42</sup> Hyperactivated motility, another characteristic feature of the spermatozoa, occurs when flagellar movement becomes asymmetrical with higher amplitude, resulting in highly curved trajectories.<sup>43,44</sup> Spermatozoa is dependent on ATP-fueled motility,<sup>45</sup> yet how they are metabolically adapted to utilize ATP is less understood.<sup>46</sup> ATP is required to support the coordinated movement of the central axoneme and the surrounding flagellar structures.<sup>20</sup>

### The flagellar beat

The motile apparatus that propels a spermatozoon forward and enables it to penetrate an oocyte is contained in the flagellum.<sup>47</sup> In general, speaking, flagellar movement is accompanied by the bending of the eukaryotic flagellum produced by the sliding movement of microtubules caused by the activity of dynein “arms.”<sup>48-50</sup> Several experiments have been carried out to better understand the regulatory mechanism of flagellar beating of spermatozoa.<sup>51-54</sup> Axonemal dynein is a protein that powers the rhythmic beat of the flagellum.<sup>55</sup> Upon the activation of dynein ATPases, there is sliding of the outer axonemal



**Figure 1:** A proposed schematic representation of the compartmentalization of labor in the spermatozoon through different modes of adenosine triphosphate generation at varying sites.

doublet microtubules, causing the flagellum to bend.<sup>1</sup> A force is thus generated when the dynein protein transduces the chemical energy of ATP hydrolysis into mechanical energy.<sup>55,56</sup>

### ***Flagellum fuel***

Because mitochondria are bulky structures, they are localized in the mid-piece region of the sperm cell and not distributed along the flagellum, as they may cause mechanical problems during flagellar beating.<sup>46</sup> However, it is a known fact that efficient ATP synthesis occurs through mitochondrial respiration. The question then arises as to whether mitochondrial ATP can diffuse rapidly and sufficiently along the entire length of the flagellum to support the energy requirements needed for fast beating activity. Unfortunately, the answer is not clear.

Several biophysicists<sup>57,58</sup> have calculated the required rate of diffusion of ATP to support motility in the spermatozoa of sea urchins and bulls and concluded that the diffusion rate of ATP is sufficient to keep pace with the beating frequency of the flagellum. Mitochondrial membrane potential is considered an indicator of sperm motility and a reduction in potential has resulted in reduced sperm motility and fertilization ability.<sup>59-62</sup> In the studies of Paoli *et al.*<sup>40</sup> which were conducted on asthenozoospermic patients, a positive correlation has been established between mitochondrial membrane potential and non-linear motility. Alterations in the mitochondrial respiratory chain enzyme activities can also have an effect on sperm motility.<sup>63</sup> The electron transfer chain is composed of two mobile carriers (coenzyme Q and cytochrome C) and four multimeric complexes (I, II, III, and IV). Based on their experiments on sperm motility and mitochondrial enzyme specific activities, Ruiz-Pesini *et al.*<sup>11</sup> suggested that mitochondrial dysfunction may lead to idiopathic asthenozoospermia.

This is in contradiction to the views of Tombes and Shapiro<sup>64</sup> whose findings suggest that when ATP is produced by the distant cell body or mitochondrion, whether by glycolysis or OXPHOS, it does not meet the energy demands needed for proper flagellar development due to the insufficient diffusion capacity of the ATP to the area where the energy demand is high. Whether this is applicable to human spermatozoa is still unclear.<sup>20</sup> Furthermore, the products of ATP hydrolysis such as ADP, inorganic phosphate Pi and H<sup>+</sup> must be removed efficiently to avoid kinetic and thermodynamic stress.<sup>65</sup> As such, several researchers have proposed an alternative pathway of energy production by the spermatozoa. They have suggested that there is a need for local ATP production closer to the site of ATP utilization, and that this is achieved by the glycolytic pathway of ATP production due to the presence of glycolytic enzymes in the fibrous sheath of the flagellum.<sup>18</sup> Moreover, despite species specific differences,<sup>66</sup> mammalian spermatozoa can use a variety of carbohydrates as substrate for ATP generation.<sup>67-70</sup> This allows for ATP generation in the cytoplasm independent of mitochondrial activity.<sup>18</sup>

Glucose is considered to induce a high beat frequency in the flagellum and in this context Mukai and Okuno, for example, used a glucose analogue, 2-deoxyglucose (DOG), to inhibit glycolysis in spermatozoa. They found that DOG had no effect on mitochondrial respiration as assessed by a fluorescent probe - 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1),<sup>32</sup> whereas it did inhibit the activity of pyruvate and lactate when provided as substrates. This further resulted in low motility and decreased ATP content.<sup>32</sup> The above evidence thus supports the fact that glycolysis acts as a key contributor to sperm motility and when suppressed, leads to decreased motility even though mitochondrial substrates are present.

The ATP content in the presence of DOG was also examined. Interestingly, the concentration of ATP in the presence and absence of pyruvate along with the DOG were found to be similar. Because DOG cannot inhibit ATP synthesis in mitochondria, this suggests that normal mitochondrial respiration is not sufficient to maintain the concentration of ATP required for sperm flagellar motility. Thus, the above facts strengthen the argument that glycolysis is the preferred energy source specifically for motility functions.

As mentioned earlier, spermatogenic specific isoforms of proteins that are essential for glycolysis are found throughout the fibrous sheath of the sperm flagellum. One of the most notable enzymes is the GAPD enzyme. GAPD-S is a gene that is specific to mouse spermatogenic cells;<sup>14</sup> its human ortholog is GAPD-2. In fact, GAPD is the first catalytically active enzyme proven to be bound to the fibrous sheath of spermatozoa.<sup>18</sup> It is a key regulator of glycolysis during spermatogenesis and is also the target of various environmental compounds that adversely affect male fertility.<sup>71</sup> Miki *et al.*<sup>14</sup> studied GAPD-S gene expression in knock-out mice and concluded that the sperm produced by GAPD-S<sup>-/-</sup> mice have low motility and exhibit no forward progression.<sup>14</sup> Welch *et al.*<sup>72</sup> also sequenced and cloned the cDNA for the human homologue GAPD-2 and suggested that it may have similar roles in human spermatozoa.

Carbonyl cyanide m-chlorophenylhydrazine (CCCP) acts as an uncoupler to inhibit mitochondrial ATP production. The addition of CCCP to highly active mice spermatozoa had no effect on ATP content and motility parameters, suggesting that mitochondrial respiration does not play a vital role in sperm motility.<sup>32</sup> Similarly, LDH-C, a spermatogenic cell variant and a glycolytic enzyme,<sup>29,30</sup> catalyzes the conversion of pyruvate to lactate, which is essential for sperm motility during anaerobic respiration. Disruption of the targeted LDH-C gene in mice showed neither tyrosine phosphorylation nor hyperactive motility necessary for capacitation.<sup>73</sup> On the other hand, Hung *et al.*<sup>74</sup> proposed that OXPHOS is not necessary for hyperactivated sperm motility in rhesus macaque monkeys. Moreover, Hereng *et al.*<sup>75</sup> have also shown that during exogenous supplementation of pyruvate and glucose, the glycolytic pathway acted as the predominant source of energy to support progressive motility and capacitation through ATP generation.

### ***Adenosine triphosphate utilization during capacitation and acrosome reaction***

The process of fertilization includes several steps that occur in an orderly and synchronized fashion.<sup>76</sup> After spermatozoa are deposited in the female reproductive tract, a series of events, collectively referred to as capacitation, take place before fertilization is possible.<sup>21</sup> Capacitation can be defined as "the cellular and biochemical modifications that the spermatozoon undergoes in response to appropriate stimuli essential for acrosome reaction to occur."<sup>77</sup> When the human sperm are incubated in capacitation medium, an average of 10%–20% develops motility patterns that look like hyperactivation.<sup>78,79</sup> Efficient generation of ATP is required by mammalian spermatozoa to fuel these crucial events.<sup>80</sup> Modifications occur at both the surface of the head as well as throughout the flagellum of the spermatozoa.<sup>77</sup> The acrosome reaction is an important aspect of the fertilization process and refers to the release of hydrolytic enzymes from the acrosome vesicles, ultimately leading to successful sperm binding and penetration of the oocyte.

The acrosome reaction is initiated when signals produced by the oocyte activate G-proteins inside the sperm thereby increasing intracellular Ca<sup>2+</sup> levels, and finally activating several kinases and phosphorylation of various proteins. Ca<sup>2+</sup> can be derived either through the plasma membrane channels formed by proteins in the CatSper

family or from  $\text{Ca}^{2+}$  stored in organelles.<sup>81</sup> It should be noted that although the CatSper channels are confined to the principal piece of the flagellum, the redundant nuclear envelope (RNE), which is a cluster of membrane vesicles<sup>82,83</sup> that also serves as an important store for  $\text{Ca}^{2+}$  ions is localized to the base of the midpiece of the mitochondrial sheath. A rise in  $\text{Ca}^{2+}$  levels in acrosome-reacted spermatozoa has been observed to increase flagellar intensity leading to an increase in of hyperactivation.<sup>84</sup>

Adenosine triphosphate is required for ATPase activity, cyclic adenosine monophosphate formation and phosphorylation. Thus, it plays a crucial role in maintaining acrosomal integrity and inducing of the acrosome reaction.

Mitochondria play a key role in capacitation-dependent tyrosine phosphorylation in mammalian spermatozoa.<sup>85,86</sup> In human spermatozoa, a secretory pathway –  $\text{Ca}^{2+}$ -ATPase – has been immunolocalized to the midpiece and rear head and may play a role in clearance of  $\text{Ca}^{2+}$  released from the RNE store.<sup>87</sup> In addition, selenium-dependent phospholipid hydroperoxide glutathione peroxidase is abundantly expressed in spermatids and displays high activity in the post-pubertal testis.<sup>88</sup> In mature spermatozoa it is restricted to the mid-piece region<sup>23</sup> which embeds the helix of mitochondria and plays a key role in sperm maturation and mitochondrial function.<sup>89</sup> However, the exact mechanism of tyrosine phosphorylation during human sperm capacitation has not been described.

Mammalian spermatozoa rely on high concentrations of glucose, pyruvate and lactate,<sup>32,67,90</sup> all of which are present in the oviductal fluid.<sup>91</sup> The oviductal fluid of sheep and pigs is known to contain lactate, which serves as a substrate for spermatozoa activity whereas in mouse sperm, pyruvate and glucose could maintain vigorous motility for a longer duration.<sup>92,93</sup> In fact, the use of metabolic substrates for ATP production varies amongst species and this ultimately determines the preferred metabolic pathway of ATP production in the spermatozoa to support capacitation.<sup>13</sup> Depending on the presence of extracellular substrates, sperm metabolize those substrates by glycolysis to provide energy for flagellar movement. Alternatively, when there is a paucity of substrates for glycolysis, sperm metabolize respiratory substrates.<sup>32</sup> In this context, respiratory substrates function as substrates for gluconeogenesis in the midpiece, resulting in glucose that can diffuse to other regions of the sperm flagellum. Although bull spermatozoa depend on OXPHOS to support capacitation,<sup>94</sup> human spermatozoa seem to depend on glucose-derived ATP.<sup>67,95</sup> Although glucose-derived ATP serves as an immediate source for energy, mitochondrial functionality increases during sperm capacitation.<sup>96,97</sup>

In order to determine the functionality of the mitochondria during capacitation, a comparative analysis of spermatozoa was done by taking samples before and after swim-up treatment and incubating them for different time intervals. The results showed mitochondrial respiratory activity in the cells incubated under capacitating conditions was significantly higher than that in the cells before swim-up. This might be due to efficient ATP generation through OXPHOS.<sup>98</sup>

## CONCLUSIONS

A great deal of research has been conducted to understand the mechanisms of ATP utilization and identify the forms that act as the major source of ATP generation for human spermatozoa. Several authors have proposed that the cytosolic process of glycolysis forms the main source of ATP production in spermatozoa-even in the presence of oxygen. However, it is also believed that mitochondria significantly contribute to energy manufacturing based on their efficiency of ATP

production during aerobic respiration in comparison to glycolysis. Understanding the molecular processes relating to the development of a functional and motile spermatozoon will help us to answer some of the questions related to idiopathic asthenozoospermia and possibly even idiopathic teratozoospermia. Further knowledge of molecular mechanisms related to sperm function during fertilization may help to target specific signaling pathways, and the disruption of the activity of certain proteins could even lead to the development of a safe and effective male contraceptive.

Our research suggests that (1) even though glycolysis is the predominant ATP generation pathway during hyperactivation, the mitochondria's role in sperm maturation and differentiation must not be underestimated; (2) both pathways seem to be essential for human sperm function and successful fertilization; and (3) metabolic energy production in human spermatozoa may be restricted to either glycolysis or mitochondrial respiration, or could take place in combination, depending on the surrounding environment and the availability of substrates.

## AUTHOR CONTRIBUTIONS

SSDP conceived the idea and helped to write and edit the manuscript. GM helped to write and edit the manuscript. MVDL and AA helped to edit the manuscript. All authors discussed and commented on the manuscript at all stages.

## COMPETING INTERESTS

The authors declare that they have no competing interests.

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