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IMPORTANT FUNCTIONAL PARAMETERS OF SPERMATOZOA: A REVIEW

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Abstract: Selection of good quality semen is done usually based on conventional semen parameters. However, selection of good bulls requires tests more than the conventional tests. Various functional parameters, viz. reactive oxygen species generation, ATP concentration, Ca^{2+} ion signaling, mitochondrial membrane potential, leptin secretion and matrix metalloproteinase activity qualify the spermatozoa and influence its fertility potential. Present review discusses these important functional parameters of spermatozoa and stresses that these tests can be performed along with conventional tests to judge the semen quality of a bull.

Keywords: Sperm Quality, Functional Parameters, Fertility

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INTRODUCTION

In order to select sires with good sperm quality and fertility, *in vitro* assessment of the functional capacity of spermatozoa is generally preformed. Most of the research used *in vitro* technologies to measure the fertilizing ability of spermatozoa. Traditionally the quality evaluation of semen was limited to the measurement of volume, color, pH, sperm concentration, microscopic examination of progressive motility, viability and to the evaluation of morphology and membrane integrity by means of eosin/nigrosin staining[1,2] . A number of studies have been conducted in zebu, exotic, crossbred and buffalo bulls employing these conventional methods to evaluate the quality of semen [3,4,5,6]. Methods for sperm analysis have considerably increased in recent years. It is now possible to replace subjective motility assessment by Computer Assisted Sperm Analysis (CASA), [7] and the former eosin/nigrosin staining method is now being superseded by fluorescent dyes that bind to various regions of the cell to demonstrate particular functional characteristics of spermatozoa [8]. Moreover, other technological changes over the past decade have made it possible to apply either new techniques such as flow cytometry[9] or more specific functional *in vitro* tests. They help to determine sperm performance such as sperm migration [10], sperm binding to oviduct epithelial explants [11] or to the zona pellucida [12] and in vitro fertilization [13] for the assessment of sperm quality and fertility.

Functional properties of the spermatozoa

Various functional parameters qualify the spermatozoa and influence its fertility potential. Some of the important parameters, viz. reactive oxygen species generation, ATP concentration, Ca^{2+} ion signaling, mitochondrial membrane potential, leptin secretion and matrix metalloproteinase activity depict functional properties of the spermatozoa and influence their fertility potential.

Reactive oxygen species and spermatozoa

Oxidative stress is a condition associated with an increased rate of cellular damage induced by oxygen and oxygen-derived oxidants called reactive oxygen species (ROS). The susceptibility of human spermatozoa to oxidative stress has been suggested as a cause of male infertility [14]. The production of ROS by sperm is a normal physiological process, but an imbalance between ROS generation and scavenging activity is detrimental to the sperm and associated with male infertility [14]. Physiological levels of ROS influence and mediate the gametes function [15] and crucial reproductive processes, such as sperm-oocyte interactions [16], implantation and early embryo development [17]. At low levels, ROS mediates normal sperm functions such as capacitation, hyperactivation, acrosomal reaction, and sperm oocyte fusion, but at high levels,

the increased production of ROS can cause oxidative stress and induce pathophysiological changes in the spermatozoa [18].

Mammalian sperm cells present highly specific lipid composition, high content of polyunsaturated fatty acids, plasmalogens and sphingomyelins. This unusual structure of sperm membrane is responsible for its flexibility and the functional ability of sperm cells. However, spermatozoa's lipids are the main substrates for peroxidation, which may provoke severe functional disorder of sperm. The principal means of ROS-mediated injury to spermatozoa is peroxidative damage to the cell membrane, impairment of sperm motility, and oxidative damage to DNA [19]. On the other hand, low (physiological) levels of lipid peroxidation reflect the influence of reactive oxygen species (ROS) on sperm metabolism enhancing the ability of human spermatozoa to interact with zona pellucida [20]. A reason for higher, pathological lipid peroxidation of sperm membranes can be unbalanced oxidative stress. High levels of seminal ROS have been reported to cause 20%–40% of infertility [21]. Wang *et al.* [22] reported that semen samples with abnormal semen parameters had significantly higher ROS than normal semen samples.

ATP concentration in semen

Energy for flagellar action is metabolized by the mitochondrial dense mid-piece and these combine to propel the sperm head, carrying the male haplotype, to the ovum. Flagellar beating is the main energy-consuming process of live motile spermatozoa [23], with the hydrolysis of adenine triphosphate (ATP) to adenine di and mono phosphates as the major energy providing process [24]. The concentration of ATP in semen is related to the number of motile spermatozoa, which means that measurement of ATP concentration may provide an objective method of estimating sperm viability [25].

The majority of ATP produced by spermatozoa is used to support motility [26]. Bohnensack and Halangk [27] determined that 75% of the ATP produced by bovine spermatozoa was used in this way. Two processes can form ATP: oxidative respiration and glycolysis, both of which occur in bovine spermatozoa [28]. Oxidative respiration occurs within the mitochondria, located in the midpiece of the spermatozoan, and results, in the case of glucose, in 36 ATPs being formed per molecule. Since the distance between the mitochondria and the distal tip of the flagellum is approximately 40 to 50 µm, it is unlikely that ATP generated in the midpiece could diffuse the length of the flagellum (while being consumed en route) and supply enough energy to support motility [29]. It is more likely that ATP used by the distal end of the flagellum is produced locally by glycolysis. Hexokinase (the first enzyme of glycolysis) has been localized to the membranes of the spermatozoon head, the flagellum, and the mitochondria [30], suggesting that glycolysis could occur in these regions. The discovery that glyceraldehyde 3-phosphate dehydrogenase

(GAPDH) [31] and the other glycolytic enzymes downstream from GAPDH [32] are bound to the fibrous sheath of the flagellum, provides further evidence that glycolysis occurs in this region. For each molecule of glucose metabolized by glycolysis there is a net yield of 2 ATP. In the mitochondria, electrons are pumped across the inner mitochondrial membrane and then return via ATP synthase, forming ATP from adenosine diphosphate (ADP) and inorganic phosphate. Garrett *et al*. [33] reported a strong positive correlation between total ATP formation by the spermatozoa of a bull and the NRR (Non-Return Rate, as a measure of fertility) of the same bull, and suggested that the same could form the basis of a test for bull fertilizing ability after freeze/thawing.

Calcium signaling in spermatozoa

Regulation of cellular activity, in response to signals from other cells or from the extracellular environment, can occur at number of levels. Various pathways have been characterized, by which the actions of extracellular signals such as hormones, growth factors and transmitters are transduced, leading to appropriate modification of protein function. One such mechanism is through changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Regulation of protein function through Ca^{2+} signaling is central to a range of activities that are pivotal to sperm function, including hyper activation, chemotaxis and acrosome reaction [34]. Impairment of Ca^{2+} signaling in sperm is associated with male sub-fertility [35].

A number of voltage-gated calcium ion channels have been identified in testis, spermatocytes and spermatozoa [36,37,38,39,40]. Although several prototypical voltage-gated calcium channels are thought to be present in spermatozoa, probably not all of them are essentially required for motility [41].

The presence of Ca^{2+} channels in the plasma membrane of sperm cells, and their significance in the key activities of sperm is well established. Various types of Ca^{2+} ion channels are distributed in the membrane of sperm cells [42,43,44,45]. Cation channel of sperm (CatSper) is a sperm-specific, weakly voltage-dependent, Ca^{2+} selective, pH-sensitive ion channel that controls the entry of positively charged calcium ions into sperm cells, which is essential for sperm hyperactivation and male fertility [46]. Under normal physiological conditions, sperm membrane potential and intracellular pH are at values such that CatSper channels remain active at a minimal level, which are potentially activated only in the presence of agonists or physiological stimuli[47].

Signaling through $[Ca^{2+}]_i$ is achieved by permitting Ca^{2+} to enter the cytoplasm (where concentration is maintained very low) from the extracellular space and/or from intracellular organelles, where the Ca^{2+} concentration is up to four times higher. Signal initiation requires merely opening up of the permeable membrane channels allowing the ions to flow down their electrochemical gradient.

In somatic cells, the endoplasmic reticulum is the primary Ca^{2+} storage organelle. A mature sperm has no recognizable ER but does have a nuclear membrane, an acrosome, mitochondria (which are concentrated in the midpiece) and some poorly defined, irregular membranous structures in the region of the sperm neck from where the cytoplasmic droplet has been shed. Since organelles other than the ER can participate in storage and release of Ca^{2+} in somatic cells, any or several of the membranous structures of sperm may act as releasable Ca^{2+} stores. The first clear evidence that intracellular organelles in mature mammalian sperm might act as Ca^{2+} stores was the finding of Walensky & Snyder[48] who showed that components of the phosphoinositol signaling system are present in mammalian sperm. De Blas *et al*. [49] used human sperm, permeabilised with streptolysin O and labeled with fluo3, directly to visualize Ca^{2+} stores. Fluorescence (indicating the presence of Ca^{2+} containing organelles) was again observed in the acrosomal region and at the midpiece. The evidence regarding expression of ryanodine receptors (RyRs) calcium channels in mammalian sperm is less clear but the presence has been established [50].

Mitochondrial membrane potential (ΔΨm)

During the past few years, a number of tests have been proposed to investigate the integrity of different cell compartments of spermatozoa such as acrosome reaction [51], expression of phosphatidyl serine [52], DNA damage [53], and viability [54]. Such assays have the advantage of being more statistically robust, accurate, and quicker. Evaluation of the mitochondrial membrane potential is one such approach. Measuring ΔΨm in spermatozoa provides useful information about fertility potential.

Mitochondria contain a double membrane. The outer membrane allows large molecules to flow into the mitochondrial intermembrane space while the highly invaginated inner membrane, which has a large surface area, is responsible for oxidative phosphorylation. During the process of oxidative phosphorylation, the protons are pumped from inside the mitochondria to the outside, creating an electrochemical gradient called the inner ΔΨm.[54] The ability to distinguish between mitochondria exhibiting higher mitochondrial membrane potential from those having low ΔΨm provides an estimate of the metabolic function of the cells. Several different fluorescent probes, such as rhodamine ^{123} (Rh-123) [55], lipophilic cationic probe JC1 [56] and carbocyanines dye DiOC6(3) [57] have been used to assess the ΔΨm of spermatozoa. There is positive correlation between ΔΨm and sperm motility and viability [58,56]. Rh^{123}-positive spermatozoa sorted by flow cytometric cell sorting have significantly improved quality of movement [59]. An increased proportion of spermatozoa with depolarized mitochondria have been reported in asthenozoospermic semen samples [56]. This suggests that ΔΨm is an important indicator of functional integrity of the spermatozoa.

Wang *et al.* [22] reported that the semen samples having abnormal semen parameters had a significantly lower ΔΨm as compared to normal semen. The ΔΨm was positively correlated with sperm concentration (r = 0.62) and negatively correlated with the ROS (reactive oxygen species) produced (r = -0.45). Thus, increased ROS production by spermatozoa was associated with a decreased ΔΨm. Artificially induced oxidative stress by incubation with H_2O_2 inhibited sperm motility, decrease ATP levels, and dissipated the ΔΨm [57].

Spermatozoa are the major source of ROS generation in oligozoospermic patients [60]. It has been reported that there is increased G6PD-mediated NADPH generation in infertile patients, which in turn promotes the NADPH oxidase mediated ROS generation in spermatozoa [61,21]. However, studies on other cell types raise a possibility that ROS generation by these spermatozoa may be due to damaged mitochondria [62,63]. Ehlers *et al.,* [64] demonstrated mitochondrial DNA damage and altered membrane potential by ROS in pancreatic acinar cells.

Leptin secretion and spermatozoa

Leptin in various cell types has a range of roles, but the principal role is as a lipostat, signaling to other systems the energy reserves available to the body, mediating fuel use, and consequently energy expenditure. Recently, a new target for leptin in the male genital tract was evidenced because leptin receptor was found to be present in human spermatozoa [65]. Various evidences have pointed to a direct role of leptin in the control of testicular function [66]. However, in contrast to its well-proven effects in female fertility, the actual role of the hormone in the regulatory network controlling male reproductive function has been a matter of debate. The ob/ob mice (lacking of functional leptin) or OB-R/OB-R mice (lacking of functional leptin receptor) were found to be infertile and failed to undergo normal sexual maturation [67]. The male mice (*ob/ob*) had small testes, azoospermia, and multinucleated spermatids. Fertility of ob/ob mice was restored by leptin and not by simply reducing body weight, indicating an effect of the hormone on reproductive function. Aquila *et al*. [68] showed that leptin is expressed in, and secreted from, human ejaculated spermatozoa, thus, providing evidence for a role of the hormone in sperm physiology.

It has long been recognized that capacitated sperm display an increased metabolic rate and overall energy expenditure, presumably to affect the changes in sperm signaling and function during capacitation [69]. However, the relationship between the signaling events associated with capacitation and the changes in sperm energy metabolism is poorly understood. Overall, there is a lack of information regarding how mammalian spermatozoa manage their energy status. In somatic cells, both leptin and insulin play a central role in regulation of energy homeostasis [70]. Particularly, in vitro and in vivo evidence supports the hypothesis that leptin may mimic insulin

action on glycogen synthesis [71]. Sperm glycogen metabolism seems to be regulated by modulation of glycogen synthase in a manner similar to that observed in other tissues [72].

Matrix metalloproteinase activity

Seminal plasma contains many proteinases originating either from testicular cells or prostate and other accessory sex glands. The matrix metalloproteinase (MMP) family is a group of calcium and zinc-dependent proteases expressed as zymogens (proenzyme), which are subsequently processed into active forms by other proteolytic enzymes [73]. These degrade protein components of the extracellular matrix [74].

MMPs activity detected in seminal plasma from numerous species have been documented for a variety of physiological manifestations. [75,76] MMPs presence in seminal plasma have been described for humans [76] and other mammals. [77] MMPs have been proposed to be involved in sperm maturation, [77] and have been associated with the regulation of male fertility.[74] The necessity of breakdown of physical barriers in the fertilization process suggests that MMPs along with tissue inhibitors of MMPs (TIMPs) are involved in this process. Buchman-Shaked *et al.* [74] showed MMP activity and TIMP presence in both normal and abnormal human sperm samples. MMP profile differed between normal and abnormal sperm samples, with a higher MMP activity in normal relative to abnormal samples.

Immunofluorescent staining study further revealed MMP-2 localization along the plasma membrane over the acrosomal region, whereas MMP-9 was almost undetected [74]. Others reported that MMP-2 was highly associated with sperm motility, while MMP-9 was correlated with unsatisfactory canine semen quality. [78,79]

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