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Review

New flow cytometry approaches in equine andrology

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A B S T R A C T

Flow cytometry is currently recognized as a robust tool for the evaluation of sperm quality and function. However, within equine reproduction, this technique has not reached the sophistication of other areas of biology and medicine. In recent years, more sophisticated flow cytometers have been introduced in andrology laboratories, and the number of tests that can be potentially used in the evaluation of sperm physiology has increased accordingly. In this review, recent advances in the evaluation of stallion spermatozoa will be discussed. These new techniques in flow cytometry are able to simultaneously measure damage to different sperm regions and/or changes in functionality.

1. Introduction

The ultimate goal of semen analysis is to determine the potential fertility of a stallion or semen sample. Semen analysis may determine whether a stallion is potentially fertile, whether it needs specific management techniques to improve its fertility, or whether it requires specific assisted reproductive techniques to impregnate mares. The definitive proof of fertility is the capacity to induce conception, particularly in species such as swine and cattle; however, determination of pregnant mares after artificial insemination or natural mating (fertility trial) is expensive and time consuming. Therefore, robust laboratory tests can be useful. Understanding sperm function is an absolute prerequisite for *in vitro* sperm evaluation. Sperm are terminally differentiated cells with highly specialized functions that must be expressed at specific times in specific biological environments to achieve fertilization. Sperm also undergo dramatic changes in their environment during their journey from the tail of the epididymis to the oviduct. Moreover, sperm must have the capacity to readily respond to specific signals originating in the mare's reproductive tract. In a landmark article by Amann and Hammerstedt [1], the complexity of sperm evaluation was clearly underlined, and the authors addressed the need to evaluate the multiple functions that sperm must fulfill to reach and fertilize an oocyte. More recently, the discovery of the heterogeneous nature of sperm within the ejaculate [2,3] suggests that there is a need to develop methods to identify the fertilizing population within a given semen sample. Classical sperm evaluation relies solely on the evaluation of single attributes, i.e., morphology, sperm number, subjective motility. Techniques such as computer-assisted sperm analysis, evaluate multiple attributes including sperm velocity, which may serve as indicators of several sperm qualities, i.e., an adequate source of energy and fully functional metabolic pathways. To date, determination of multiple functional attributes of sperm with a single test is not yet common practice.

2. Flow cytometry in sperm evaluation

In sperm evaluation, it is important to bear in mind that a relatively small number of sperm are evaluated and considered representative to the whole ejaculate. In this respect, evaluating larger sampling populations (thousands of spermatozoa) are preferable for sperm analysis. Unfortunately, conventional semen analysis typically only evaluates a few hundred spermatozoa. This is true for most methods, including sophisticated analyses such as

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fluorescence microscopy and computer-assisted sperm analysis. Because flow cytometry allows the rapid evaluation of thousands of spermatozoa within a few seconds, the technique provides the potential for a more representative sample evaluation. The early use of flow cytometry for sperm evaluation was for DNA analysis in the 70s and 80s; however, multiple fluorescent probes have been introduced subsequently in the 90s to monitor membrane integrity, mitochondrial membrane potential, oxidative status, membrane fluidity and/or permeability, lipid peroxidation, and tyrosine phosphorylation of sperm proteins, among others. Recent reviews [4–7] have addressed flow cytometric techniques currently in use in veterinary andrology, and the reader is referred to them for more detail. The focus of this review will be stallion andrology and the advantages that multicolor flow cytometry may offer for the simultaneous evaluation of multiple functional compartments of sperm cells.

2.1. Membranes and acrosome integrity

SYBR-14 and propidium iodide (PI) are widely used probes for the assessment of sperm membrane integrity. SYBR-14 is a membrane-permeant nucleic acid stain, and PI is a conventional (nonpermeant) dead cell stain. When used in combination, live cells fluoresce bright green (SYBR-14), whereas dead cells with damaged cell membranes fluoresce red (PI). This combination of probes allows the rapid discrimination of debris (because both are DNA binding probes), and both probes are excited with the blue laser (488 nm). However, spillover between emission wavelengths of both probes can occur (521 nm for SYBR-14 and 635 nm for PI), and proper fluorescence compensation has to be established to use this probe pair because SYBR-14 still has, on average, a 6% emission at 635 nm. Furthermore, staining with SYBR-14/PI discriminates only between live and dead sperm and does not expose states of membrane damage, which might reflect sublethal damage. Alternatively, Hoechst 33342 and PI [8] also permit the rapid discrimination of debris and have the advantage that spillover is unlikely due to the distinct excitation and emission spectra of these probes. However, H33342 needs a violet or ultraviolet laser for excitation. Detection of more subtle changes in the sperm membrane requires the use of other probes. Fluidity of sperm membranes can be assessed with merocyanine 540 [9], and subtle increases in the permeability of the sperm plasma membrane can be detected with YO-PRO-1 [10,11]. YO-PRO-1 is routinely used in the authors' laboratory in combination with PI, and H33342 is also incorporated to gate out debris. These combinations allow the detection of changes in sperm membranes at much earlier stages than SYBR-14/PI and correlate better with motility and sperm velocities [11].

Recently, new fixable fluorescent dyes have become available in multiple colors, which facilitate experiments with multiple spectra in fixed sperm. These probes are based on the reaction of a fluorescent reactive dye with cellular amines. These are proprietary dyes that can permeate the compromised membranes of the necrotic cells and react with free amines both in the cytoplasm and on the cell surface resulting in intense fluorescent staining. In contrast, only the cell surface amines of intact cells are available to react with the dye, resulting in relatively dim staining. The discrimination is maintained after formalin fixation of the sample under conditions that inactivate pathogens. Moreover, these assays use only one channel of the flow cytometer, leaving the other channels available for multicolor panels. The potential advantage of these dyes is processing and staining the sample at locations remote to the flow cytometer.

Evaluation of acrosome integrity is a popular technique to detect putatively functional spermatozoa; however, assessment of acrosome integrity is often misused and misinterpreted. Because the acrosome cannot be stained in membrane-intact (live) spermatozoa, assessment of acrosome integrity in nonpermeabilized, nonstimulated cells can be redundant [12]. Only the evaluation of the sperm's ability to undergo the acrosome reaction in response to an agonist challenge can provide meaningful results. This assay, the acrosomal responsiveness assay [13,14], evaluates the ability of the acrosome to react when challenged with the Ca^{2+} ionophore, A23187. Common probes to assess acrosomal status are those that recognize targets inside the acrosome, including specific lectins (*Pisum sativum* agglutinin and *Arachis hypogea* agglutinin); which bind to glycosidic residues in different parts of the acrosomal membrane. The acrosome has also been monitored in human sperm with anti-CD46 antibodies [15,16].

2.2. Mitochondria

The mitochondria of spermatozoa are increasingly studied in both basic and applied andrology [8,17,18]. Stallion spermatozoa are highly dependent on mitochondrial production of ATP, and mitochondrial malfunction leads rapidly to sperm senescence and death. Stallion spermatozoa have particularly active mitochondria, and as a result, equine sperm generate large amounts of reactive oxygen species (ROS) [8,17]. Sperm mitochondria are sensitive indicators of sperm stress during processes such as cooling and cryopreservation [19,20]. Two common probes are used to assess stallion mitochondrial function by flow cytometry. The probe 5,5′,6,6′–tetrachloro-1,1′,3,3′ tetraethylbenzymidazolyl carbocianyne iodine (JC-1), forms multimeric aggregates in mitochondria with high membrane potential (active mitochondria); these aggregates emit in the high orange wavelength of 590 nm when excited at 488 nm. In mitochondria with low membrane potential (inactive mitochondria), JC-1 forms monomers that emit in the green wavelength (525–530 nm) when excited at 488 nm [21,22]. Recently, MitoTracker dyes [23,24] have become available in multiple colors; which provide alternatives to be used in multicolor experiments. Both JC-1 and MitoTracker deep red have been used recently in our laboratory [10]. These probes measure different aspects of mitochondrial function; JC-1 reflects mitochondrial membrane potential, whereas MitoTracker deep red passively diffuses across the membrane and binds to thiols in active mitochondria. The JC-1 is considered more sensitive to subtle changes in mitochondrial membrane potential but is also more technically demanding