

NON-TRADITIONAL METHODS OF DIAGNOSING ABNORMAL SPERM FUNCTION

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Introduction

Evaluation of male fertility in domestic livestock has been primarily limited to data involving female pregnancy rates, foaling, lambing, farrowing, or calving rates, and non-returns to estrus. In many herds and flocks, identification and culling of subfertile males that have been associated with lowered fertility serves as the most economic solution to problems of sire infertility. However, when the value of individual males is high, further investigations into the nature of the subfertility may be warranted. A test for prediction of fertility in unproven sires has been elusive since numerous factors contribute to the interaction between male and female that results in "good fertility."

In most cases of male subfertility, the underlying causes are largely unknown; reduced fertility occurs with a variety of etiologies and numerous contributory factors. Identifiable factors that can contribute to a male's decreased fertility include inadequate numbers of morphologically normal, progressively motile sperm, suboptimal management systems, testicular degeneration and genital tract trauma, and poor overall health and body condition. The role of other factors such as environmental toxic exposure, endocrine, autocrine, and paracrine control of spermatogenesis, epididymal maturation, and the function of sperm cells are less clearly defined. The objective of this presentation is to discuss several recently developed methods of assessing spermatozoal function and dysfunction as might occur during toxicologic exposure or subfertility. Our laboratory has focused on equine sperm function but other species will be discussed as pertinent.

Conventional Semen Evaluation

Evaluation of stallion, bull, boar, and ram fertility, defined as the ability to cause conception, pregnancy, and the birth of live young, has been performed traditionally by assessment of ejaculated semen. Conventional semen evaluation consists of measurements of sperm morphological and movement characteristics, seminal fluid characteristics including pH, turbidity, color, presence of immature germ cells or somatic cells, and testicular parameters such as size, shape, scrotal circumference, ultrasonographic appearance and volume, and total scrotal width (for stallions). Many of these parameters are relatively well correlated to fertility in most males evaluated, however, few have been able to account fully for decreased fertility in some males.

Clearly, males which have extremely low numbers of motile, morphologically normal sperm in their ejaculates are incapable of causing conceptions and are justifiably considered to be infertile. However, some males which have normal to moderately low numbers of morphologically normal, motile sperm in their ejaculates either fail to cause conceptions or cause

a decreased conception rate under good breeding management schemes. The subfertility in this latter population is not well-defined or diagnosable by conventional semen evaluation. Diagnostic tests, which probe sperm function, are needed to determine the potential etiologies and explore treatments of this type of subfertility. This would provide diagnostic information about sperm function that would otherwise not be available for males in which conventional semen evaluation fails to identify an etiology for lowered fertility.

Evaluation of Sperm Function

For sperm to fertilize oocytes and develop into embryos, sufficient numbers of motile sperm must ascend the female genital tract, become capacitated, acquire hyperactivated motility, undergo the acrosome reaction, penetrate the oocyte extracellular investing layers (which include the cumulus oophorus and zona pellucida), and initiate fusion with the oocyte's plasma membrane. Fertilization also includes sperm nuclear decondensation, male pronucleus formation, and syngamy. Many of these crucial steps in the fertilization process may be artificially isolated as *in vitro* fertilization in several model species demonstrate. In human medicine, the ability to undergo acrosome reactions, to bind and penetrate human ova, and to fuse with zona-free hamster ova have proven to be clinically useful as predictors of *in vitro* fertilization success and infertility in men. Such function tests have also been performed in livestock species but have not become routinely useful possibly because much infertility results from conventionally diagnosable sperm deficits such as total numbers of morphologically normal, progressively motile sperm in ejaculates.

Clinical evaluation of fertilization-based sperm function has been applied most widely in human medicine and has included endpoints such as measurements of total and progressive motility, hyposmotic swell test, cervical mucus penetration, hyperactivated motility, sperm fusion with zona-free hamster oocytes, or homologous *in vitro* fertilization. Measures of capacitation and acrosomal physiology have proven to be useful adjuncts to motility and morphologic endpoints for diagnosis and treatment of human male factor infertility.

In veterinary medicine, bull fertility has been correlated to acrosome reaction inducibility by chondroitin sulfates and dilauroylphosphatidylcholine liposomes (Ax et al, 1985). Other than evaluation of sperm motility, which has not been highly correlated to stallion fertility, there are presently few clinically available indices of sperm function in equine reproduction. For assessment of environmental or toxic exposures, evaluation of sperm function at endpoints more finitely defined than 'fertility' may be appropriate.

Capacitation and Acrosome Reaction

It has been shown for several species that sperm must undergo the process of capacitation as a prerequisite for most events of fertilization including cumulus penetration, zona binding and penetration, the acrosome reaction (acrosomal exocytosis), and fusion with the oocyte. Sperm capacitation has been suggested to include increases in intracellular calcium, phosphorylation of protein tyrosine residues, and reversible changes in the sperm plasma membrane including removal of cholesterol and cholesteryl esters by cholesterol acceptor molecules in the female genital tract or in artificial media. Capacitation *in vivo* occurs within the female genital tract, but can be induced artificially *in vitro* for sperm of many species, including the horse. The acrosome reaction can occur only following completion of capacitation and can be induced by a variety of

chemical and biological agents. Cytological techniques for quantitation of acrosome reactions have been applied in several species including stallions, bulls, and boars. Sperm that are non-viable experience a "false" acrosome reaction, which must be differentiated from the true exocytotic acrosome reaction in order to reflect capacitational changes. Supravital stains, such as Hoechst 33258, accomplish this task efficiently. Consequently, the acrosome reaction in *live* sperm may be considered a rational endpoint for evaluation of sperm function as it can be used to evaluate a complex set of cell behaviors.

Progesterone has been evaluated for its ability to stimulate acrosome reactions because, in some species including horses, the preovulatory follicle has been reported to secrete progesterone prior to ovulation and luteinization. Therefore, progesterone is likely to be present in oviductal fluids and within the cumulus of ovulated oocytes. Differences have been reported for efficiency of progesterone-induced acrosome reactions in sperm of human fertile and subfertile patients and this has been attributed to the lack of progesterone receptor on sperm or to a nonfunctional receptor. It is not known whether these subfertile patients had a congenital absence of progesterone receptor, or whether an acquired condition resulted in the loss of functional receptor or receptor subunit. We, and others have reported similar results for fertile and subfertile stallions (Meyers et al, 1995). It has been determined that bull and stallion sperm have receptors for progesterone.

Several additional sperm function tests based on isolated steps in fertilization have been developed for livestock species. We have demonstrated that sperm - binding to the equine zona pellucida and subsequent acrosome reaction rates in bound sperm are higher for fertile stallions as compared to subfertile stallions (Meyers et al, 1995, 1996). Similar results have been reported for rams, boars, and bulls. The ability of motile sperm from subfertile men to bind to the zona is correlated with their ability to complete fertilization in vitro. Recent studies of stallion sperm capacitation have suggested that the ability to bind to and release from oviductal epithelial cells in culture is associated with stallion fertility or sperm function. Although the mechanisms are unclear concerning the cell biology of sperm-oviductal adhesion, these studies of sperm cell functions should eventually reveal cellular functions and molecular mechanisms that sperm must acquire to attain successful fertilizing capacity.

Sperm DNA and Chromatin Integrity

The nature of sperm DNA damage has not been clearly determined. It is known that a relationship exists between males with poor sperm parameters and spermatozoal DNA damage. Two broad theories have been advanced to explain the appearance of DNA anomalies in ejaculated sperm. The first theory suggests that endogenous nicks in DNA occur during specific stages of spermiogenesis. It has been demonstrated that the presence of nicks in the DNA is greatest during the transition from round to elongated spermatids in rodents. An alternative theory has been proposed in which the presence of endogenous DNA nicks is characteristic of programmed cell death, or apoptosis, followed by elimination of defective germ cells.

Sperm DNA has been analyzed by a number of laboratories using a variety of techniques. The nick translation assay directly assesses DNA nicks. High levels of nicks have been related to reduced fertility in men. The terminal deoxynucleotidyl transferase (TUNEL) assay has been used to evaluate DNA fragmentation and strand breaks and these breaks have been correlated

with abnormal chromatin packaging and protamination as well as abnormal sperm morphology and motility in human sperm. The sperm chromatin structure assay (SCSA), which measures DNA susceptibility to acid- or heat-induced denaturation, has been effectively used to identify males with poor fertility in a variety of species, including stallions (Kenney et al, 1995). The single cell gel electrophoresis method (SCGE, or Comet, assay) is one of the most sensitive methods of detecting DNA fragmentation/strand breaks and has been well-correlated to the SCSA and TUNEL assays. Of these techniques, the Comet assay can be used to detect DNA damage in individual sperm cells with the highest sensitivity; the SCSA cannot be used to evaluate individual cell DNA damage. In addition to factors that affect sperm survival, it is increasingly evident that “sublethal” damage to sperm is an important limitation to successful cryopreservation in the horse. For stallion sperm, there is little experimental or clinical data demonstrating any mechanisms for the described decrease in sperm fertility associated with cryopreservation of sperm. Recent research from our laboratory, suggests that equine sperm undergoing low-temperature storage experience characteristics of premature capacitation, osmotic damage, and DNA damage (Bedford et al, 2000, Linfor et al, 2002, Pommer et al, 2002, Linfor and Meyers, 2002). Our data suggest that the initial cooling of equine semen to 4°C that occurs prior to cryopreservation, as well as during cooling of transported semen, is capable of inducing significant fragmentation of sperm DNA. The observed degree of sperm DNA damage could influence the fertility of certain stallions, although sperm populations are significantly heterogeneous with regard to sperm morphology and biochemical function.

Cell Signaling Pathways: Intracellular Calcium and Tyrosine Phosphorylation

The initial event in sperm capacitation is a rise in intracellular calcium, bicarbonate, and hydrogen peroxide, which collectively activate adenylyl cyclase to produce cyclic AMP (cAMP). The stimulation of cAMP then activates protein kinase A (PKA) which begins to phosphorylate a number of proteins. Visconti and co-workers have correlated mouse, human, and bovine sperm capacitation with an increase in protein tyrosine phosphorylation of a variety of protein substrates of Mr 40,000–120,000 (Visconti and Kopf, 1998). The authors suggest that protein tyrosine phosphorylation mediates a variety of cellular functions such as growth regulation, cell cycle control, cytoskeleton assembly, ionic current regulation, and receptor regulation and is an essential downstream component of capacitation. Studies into sperm cell signaling as a consequence of cellular osmotic and oxidative stress have been ongoing in our laboratory. Our recent preliminary data suggest that reactive oxygen species (ROS) and osmotic stress may contribute to membrane leakiness and subsequent increases in intracellular calcium concentration and downstream phosphorylation cascades. The assays can be performed using fluorescence microscopy or flow cytometry using a variety of calcium-sensitive dyes including Fura-2AM and Fluo-3.

We, and others, have also demonstrated that one consequence of cryoinjury to sperm is that of excessive levels of protein tyrosine phosphorylation within the sperm tail region (Cormier et al, 1997, Bailey et al, 2000, Linfor et al, 2002, Pommer et al, 2002a). Mitochondrial membrane potential (MMP), particularly notable in the sperm flagellum, has also been reported as a consequence of osmotic and oxidative cellular stress (Ball and Vo, 2001). The assays used to determine MMP have been largely flow cytometric evaluations using several commercially-available cationic fluorescence dyes (Gravance et al, 2000). The dyes are excellent indicators of mitochondrial membrane depolarization and fluoresce at varying wavelengths dependent on high

or low potential. Individual cells (microscopy) or cell populations (flow cytometry) can be tracked for changes in red or green fluorescence relative to various cellular stressors. It can also be useful to combine multiple fluorophores and determine MMP, calcium status, cell viability/membrane permeability, and phosphorylation simultaneously in single cells using fluorescence microscopy or dual-laser flow cytometry.

Sperm Cell Membrane Function

LIPID COMPOSITION. The plasma membrane of sperm is unique within species with regard to phospholipid composition (approximately 70% phospholipids, 25% neutral lipids, and 5% glycolipids (Parks and Lynch, 1992). Cold shock resistance is generally greater for species with sperm membranes characterized by high sterol to phospholipid ratio and a high degree of saturation of phospholipid-bound acyl moieties. Resistance to toxic insult could also be reflected by plasma membrane integrity and phospholipids composition. Two reports have illustrated that desmosterol and docosahexanoic acid (DHA) are unique lipids in monkey and human sperm. Desmosterol is a cholesterol precursor and is a unique sterol in primates and is localized only to sperm. DHA is otherwise observed only in primate retina and brain. This study also reported that most (99%) of the desmosterol and DHA in sperm is located in the sperm tail and may be responsible for the different functions of head and tail components of sperm. Double-bond characteristics of DHA may contribute to the membrane fluidity necessary for the motility of sperm tails. In one study, patients with retinitis pigmentosa, in whom there is degeneration of photoreceptors, demonstrated low DHA content in their sperm and also poor sperm motility which positively correlated with DHA concentration. Membrane fluidity, assessed by measuring the fluorescence polarization anisotropy of sperm is also indicative of ability to withstand freezing-thawing. Fluidity is largely dependent on cholesterol:phospholipid molar ratio and ratio of saturated to unsaturated lipids. A marked increase in these two indices of fluidity indicates a significant decrease in fluidity of plasma membrane measured at physiological temperature. In species in which the cholesterol to phospholipid molar ratio and the degree of saturated fatty acids in the phospholipid fraction were high, there was greater resistance of spermatozoa to cold shock and osmotic stress.

THERMOTROPIC LIPID PHASE TRANSITIONS. The sperm of most mammalian species undergo thermotropic lipid phase transitions in the temperature range of 17-36°C (Parks and Lynch, 1992, Drobnis et al, 1993). Plasma membranes respond to temperature changes through lipid phase transitions and liquid, crystalline and gel phases can coexist in individual cells during freezing and thawing. Evidence that cold shock damage to mammalian and invertebrate sperm cells is mediated by lipid phase transitions has been reported and toxic exposures could conceivably result in similar damage to sperm membranes. Boar sperm exhibits a cooperative phase transition compared to human sperm, which displays a minor transition, an indication that sperm susceptible to cold-shock display distinct phase transition behavior. Sperm from most species are susceptible to varying degrees of cold shock in which rapid cooling above 0°C confers major structural and functional damage to sperm. In addition to physical membrane disruption, cooling sperm below the lipid phase transition temperature may disrupt membrane enzyme systems including ATPases. Several studies have demonstrated that thawed sperm display increased intracellular calcium concentration $[Ca^{2+}]_i$ and this is indicative of poor control of calcium regulation. Lipid membrane phase transitions of equine and non-human primate sperm have been studied in our laboratory using Fourier Transform Infrared Spectroscopy (FTIR). This method provides a sensitive, non-invasive probe of lipid packing within the cell membrane and is detected by melting trends and -CH₂ band stretching of membrane lipids.

CELL RESPONSE TO OSMOTIC CHANGES. Osmotic volume responsiveness could also be used as an adjunctive test for sperm toxicity. The hyposmotic swelling test (HOS) has been used for sperm membrane responsiveness from a variety of species including boar, human, bull, and stallion and has been related to sperm function and subfertility. The rate at which osmotic volume regulation, and hence, cooling, warming, or response to stressors, may take place is highly dependent upon the cell's hydraulic conductivity (water permeability), L_p . Water permeability has been shown to be dependent on temperature, cryopreservative, and ice crystal formation in boar sperm and there is evidence that plasma membrane permeability is regionally variable in sperm. Lipid packing structure was suggested to be altered by intramembranous cryoprotectants and may be a mechanism whereby L_p and signal transduction processing could be altered during cooling. Studies in our laboratory have suggested that exposure of sperm to variations in temperature and medium resulted in differences in the ability of sperm to volume-regulate for sperm of stallions and non-human primates (Pommer et al, 2002b).

Conclusions

In this presentation, I have attempted to outline some recent advances in sperm function assessment. In recent years, advances in cell function have become increasingly applicable to sperm and methods to assess functional capacity of sperm are in various stages of development and application. Much of the recent literature regarding sperm has focused on male subfertility and cryoinjury as avenues for improvement and further understanding of sperm function. As cells that may represent an endpoint for toxic exposure or environmental toxicity, sperm offer an external cell type that could reflect toxicant exposure on male subfertility in addition to the ability to withstand cryoinjury during sperm preservation.

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