MOLECULAR ADVANCES IN SEMEN QUALITY ASSESSMENT AND IMPROVING FERTILITY IN BULLS - A REVIEW

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ABSTRACT

Fertility is declining in domestic animals across the world. The reduction in fertility could be attributed to male, female and managemental factors. Although artificial insemination (AI) using frozen semen emerged 50 years back, fertility rate with AI technology is less and unpredictable. In AI industry, one bull is used to produce at least three lakh insemination doses; hence, the selection of fertile bulls becomes very important. Since standard semen evaluation tests are not sufficient to predict bull fertility accurately, the researchers have sought laboratory assays / molecular tests that would accurately predict the fertilizing potential of a semen sample. In this context, biotechnological advances in "omic technologies" help to understand the events associated to spermatogenesis, sperm function, fertilization and embryonic development ending in birth of viable healthy offspring. Profiling fertility regulating biomolecules substances in spermatozoa or seminal plasma are considered to be helpful to predict fertility.

Keywords: Bull fertility, Metabolomics, Proteomics, Sperm quality, Transcriptomics

Reproductive efficiency of animals is declining globally over a period of time. It is well known that male, female and other managemental factors including the skill of the inseminators and time of insemination influences the fertility. Though male and female are equally responsible for fertility, more than 30% of the factors associated with infertility are still unknown. Fertility *in vivo* is a complex phenomenon consisting of sperm transport, capacitation, oocyte maturation, ovulation, female endocrine status, normalcy of fertilization and embryo development. Understanding the physiology of spermatozoa in their passage towards fertilization enhances our abilities to maximize results with assisted reproductive technology.

In order to predict bull fertility accurately, factors influencing molecular functions need to be evaluated in detail. In this regard, omic technologies are being employed to assess the bull fertility accurately. The present review focuses mainly on male fertility research, assessment and enhancement of semen quality and fertility conducted in domestic animals for the past 15 years in India.

Importance of semen evaluation

Artificial Insemination has many uses, from improving the genetic potential of farm livestock, through providing opportunities for sub-fertile animals to conceive and to maintain supply of diverse genetic material in endangered species. The routine semen analysis relies on assessing a number of parameters such as sperm concentration, motility and morphology for the prediction of fertility in the male (Selvaraju *et al*., 2008b). These tests provide the information about the status of spermatogenesis and cannot be used as reliable predictors of sperm fertilizing ability (Selvaraju *et al*., 2017).

Though the bulls are selected based on breeding

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soundness evaluation and the samples are processed based on standard semen evaluation tests, the field fertility rate varies among the bulls to the extent of 10-20% suggesting that these evaluations are not sufficient to predict fertility. It was reported that approximately 20% of the bulls are subfertile in nature under Indian conditions (Mukhopadyaya *et al*., 2010). The occurrence of sub-optimal fertility in bulls is a concern since one bull produces three lakh insemination doses. If a sub-fertile bull is selected for breeding, fertility of three lakhs cattle is compromised. A sub-fertile or infertile male is characterized by poor sperm motility, high number of abnormal spermatozoa or spermatozoa with inadequate freezability. However, a sub-fertile male with normal semen parameters remains an enigma. In these males, the factors contributing to their sub-fertility are largely unknown. In these situations, analyzing fertility regulating protein, if any, is considered to be helpful for improving fertility.

Progressive forward motility

The progressive forward motility reflects the physiological status of bull spermatozoa after semen collection or cryopreservation and is commonly used as an indicator of viability of sperm cell population. In addition to being a measure of spermatozoon viability, motility provides an indication that spermatozoa are able to navigate the barrier in the female reproductive tract to reach the oocyte. The fraction of motile sperm in semen is measured either manually or using computer assisted semen analyzer (CASA). Individual motility is assessed manually immediately after dilution, however, manual-counting estimates are based on subjective criteria and hence lack accuracy. A positive correlation exists among CASA parameters such as amplitude of lateral head displacement, curvilinear velocity, linearity and straight-line velocity and fertilization rates *in vitro*, but threshold levels were not established for selecting samples for freezing and/or AI programme (Selvaraju *et al*., 2009b). The higher percentage of progressive forward motility in fertile than in/sub-fertile bulls suggested that motility can be important attribute to classify fertile and infertile animals. However, the correlation of this parameter with fertility is questionable (Somashekar *et al*., 2015). The velocity parameters reported using CASA system such as post thaw straightline velocity, curvilinear velocity and progressive forward motility are correlated to field fertility in bovine (Perumal *et al*., 2011a).

Plasmalemma integrity

The vital staining of spermatozoa allows quantifying the fraction of living cells independently from their motility. The correlations of motility and live and dead spermatozoa did not provide consistently any predictive value with regard to fertility (Selvaraju *et al*., 2008b).

Acrosome integrity

The morphology of acrosome should be maintained for the sperm to undergo capacitation and acrosomal changes in the female reproductive tract to attain fertilizing ability. The maintenance of optimum fertility depends on the acrosome being structurally and biochemically intact. The physiological acrosome reactions can be differentiated from degenerative acrosomal loss that accompanies cell death. Although maximum limit for acrosome alterations is not fixed, it is reported that the samples should not contain more than 40% of spermatozoa with acrosomal alterations (Selvaraju *et al*., 2016a).

Functional membrane integrity (Hypo-osmotic swelling test, HOST)

During HOST, spermatozoa with intact and functionally active plasma membranes will undergo swelling due to influx of water inside the sperm membrane resulting in bent of the tail. The hairpin bend in the principal piece of spermatozoa tail when exposed to hypo-osmotic conditions is positively related to sperm fertility. The correlation co-efficient between HOST and progressive forward motility was positive and significant (r=0.81) indicating that HOST is of important in semen evaluation. The use of this inexpensive and simple assay was recommended

and validated as additional fertility indicator assay (Selvaraju *et al*., 2008b, 2012).

Sperm subpopulation positive for functional membrane and acrosomal integrities (HOS-G test)

The functional integrity of plasma membrane of spermatozoa is pre-requisite for maintaining fertility. The spermatozoa must maintain the functional integrity of cell membrane since gross physical indicators such as live and dead and acrosome integrity are not sufficient for effective fertilization. The sperm subpopulation positive for both functional membrane and acrosome integrities were assessed in semen samples by HOS-G test (Selvaraju *et al*., 2008b), developed at ICAR-NIANP. The HOS-G test was performed by incubating 50 µL of semen sample with 450 µL of 150 mOsm (hypo-osmotic) and 300 mOsm (iso-osmotic, contol) solutions at 37ºC for 30 min. After incubation, the samples were mixed gently, smeared onto a warm glass slide, dried and fixed by immersing the slides in buffered formal saline (BFS) for 30 min. The smears were stained for acrosomal integrity using Giemsa stain for overnight. These tests provided results for percentage of spermatozoa subpopulation positive for functional membrane and acrosome integrities, functional membrane positive, acrosome intact and osmotic resistant spermatozoa.

Mitochondrial membrane potential of sperm

Spermatozoa motility and metabolic activity are regulated by energy pockets localized in the form of mitochondria in the mid piece of spermatozoa. The evaluation of sperm mitochondrial membrane potential and its relevance with sperm functions and fertilization was focused over the past two decades (Selvaraju *et al*., 2008b). The effect of *in vitro* additives (Selvaraju *et al*., 2010) and *in vivo* supplements (Selvaraju *et al*., 2012b) on semen quality was also evaluated by assessing the mitochondrial membrane potential. The spermatozoa with high membrane potential had significant correlation with sperm functional attributes, sperm-oocyte binding, cleavage and also an indicator of cell death (Selvaraju *et al*., 2009a). The direct correlation of sperm mitochondrial membrane potential with sperm motility and male fertility suggests the importance of mitochondrial functioning as marker for assessing semen quality. The evaluation of mitochondrial membrane potential is being rationalized with the advancement of flow cytometry for accurate male fertility diagnosis.

Sperm nuclear morphology

Most sperm evaluation methods concern primarily the viability and functional status of spermatozoa, which influences the fertilizing ability of gamete. However, vital factors essential for sperm to produce a healthy and viable offspring primarily depend on genetic material content and quality. Sperm with non-compensable defects such as abnormal DNA distribution was associated with improper zygotic, embryonic and or fetal development. Since sperm DNA is uniquely condensed and organized, any abnormality associated with DNA, chromatin packing or sperm nuclear matrix should be reflected by a change in sperm nuclear shape. The sperm nuclear morphology is not correlating with sperm functional parameters (Selvaraju *et al*., 2008a). The nuclear morphology is affected during stress (like heat and vaccination) and feeding of animals with fungal contaminated feeds. Hence, the sperm nuclear morphology needs to be evaluated when the animals are exposed to stress. The evaluation of sperm DNA distribution should be useful for assessing male fertility.

Nuclear material quality

The quality of genetic material carried by sperm plays an important role in deciding fertilization and embryo development. The quality can be detected at single cell level by single cell gel electrophoresis and comet assay. Since spermatozoal DNA are more susceptible to oxidative stress during processing, the quality can be maintained by addition of suitable antioxidant in the extender during cryo preservation. It was reported that IGF-1 is one such additive which reduces DNA fragmentation (Selvaraju *et al*., 2010)

Transcriptomics

The phenotypic and seminal characters provide basic information on fertility status but they are not sufficient to accurately assess bull fertility. The bulls are also genetically selected based on the set of QTLs and SNPs, and dam and daughter's milk yield. Spermatozoa carry transcripts, and such transcripts are known to influence sperm function, fertilization and embryo development upon deliver (Jodar *et al*., 2013). Spermatozoa also carry various classes of RNAs (mRNA, LncRNAs, miRNAs, tRNAs; Selvaraju *et al*., 2017). Although the presence of mRNA is being explored with the possible potential function and clinical application in humans, information is emerging recently in livestock.

The membrane based methods with cocktail of lysis buffer and additional phase separation are essential for isolation of good quality and sufficient quantity RNA from bull spermatozoa. Each spermatozoon contains approximately 20-30 fg of RNA (Parthipan *et al*., 2015). Although the complete spermatozoal mRNA profile has not been reported, the spermatozoa reportedly contain 3,000-7,000 mRNAs with predominantly short fragments. The study suggested that during spermiogenesis, large numbers of transcripts are incorporated into the spermatozoa and the purpose of these packed transcripts has not been yet studied in detail. Estimating the expression level of these transcripts may aid to select elite bulls for breeding. Spermatozoa also contain RNA elements, which are correlated with male infertility. Since sperm mRNAs are delivered to oocytes, the profiling and identifying fertility associated mRNA expression levels in spermatozoa could be a valuable diagnostic tool to predict the actual fertility status of a bull.

The apoptotic factors are mainly responsible for determining conception rate of semen sample. This study also indicated that in order to obtain >40% conception rate, the spermatozoa should have very low/negligible CASP3, TRADD, UBE2D3 and HSFY2 transcript expression. The spermatozoal transcripts such as BMP2 and TRADD might influence semen quality and fertility (unpublished data). The sperm transcripts can be used to assess past spermatogenic events and have potential functional role on sperm function, fertilization and embryonic development. The relative/absolute quantification of fertility-associated transcripts may lead to development of fertility diagnostic chip using panel of sperm transcripts for bull fertility prediction.

Cell free RNAs are promising non-invasive diagnostic markers of health and diseases. An experiment was conducted to establish and assess the effectiveness of seminal plasma mRNA as markers to assess reproductive performance of bulls. The study suggested that cfs-FASLG and cfs-UBE2D3 expressions could be used as a positive and negative marker, respectively for assessing semen quality. The cfs-RNA levels can be used to predict the sperm susceptibility to cryoinjury (unpublished data).

Proteomics

Seminal plasma proteins: The presence or absence of critical concentration of proteins in seminal plasma could be potentially responsible for the effects of seminal plasma on sperm fertility. Seminal plasma protein profiles were different between bulls of high and low fertility (Somashekar *et al*., 2016). In buffalo, heparin-binding proteins in seminal plasma were correlated with fertility (Arangasamy *et al.,* 2005).

Seminal plasma is mixed with epididymal sperm at ejaculation and serves as the carrier of sperm to the female genital tract and was described as both beneficial (Selvaraju *et al*., 2016a) and detrimental to sperm (Somashekar *et al*., 2015) fertility. The BSP proteins stabilize the sperm membrane and enhance capacitation. The BSP proteins may damage the sperm membrane and render the membrane very sensitive to storage in liquid or frozen state. PDC-109 was identified in buffalo seminal plasma (Hiron *et al*., 2009). For reducing the negative effect of excess PDC-109, sequestration of protein using specific antibodies

from ejaculates improved sperm quality and freezability of bull spermatozoa (Srivastava *et al*., 2012). The probable reason for cryodamage in poor freezable buffalo and bull spermatozoa is high concentration of heparin bound protein and PDC-109 in seminal plasma (Mahak *et al*., 2014; Somashekar *et al*., 2015). However, the heparin bound fraction of buffalo oviductal fluid protein protected sperm motility, viability and membrane integrity during cryopreservation (Imam *et al*., 2008). The optimal concentrations of these proteins in seminal plasma for sperm function were not reported. The exact components of seminal compounds that are responsible for fertility regulation are yet to be identified.

Spermatozoal membrane protein: These are directly or indirectly involved in the fertilization process, therefore, the composition and functional role of these proteins on fertilization is being explored in our laboratory (Karunakaran *et al*., 2012). Sperm membrane proteins could be potential receptors for zona binding. These proteins are acquired either during the process of spermatogenesis or during ejaculation. Analyses of male fertility have revealed that several sperm surface proteins are associated with sperm function (Karunakaran *et al*., 2015). Sperm surface molecules would be useful in understanding some of the factors associated with male infertility.

The development of 2D electrophoresis has aided the identification of sperm surface molecules (Somashekar *et al*., 2015). Membrane proteins can be extracted from spermatozoa either by exposure to high ionic strength or by detergent. However, the detergent was efficient in extracting integral membrane proteins. Sperm membrane proteins extracted with sodium deoxycholate were significantly higher followed by sodium dodecyl sulphate and Triton X-100. The deoxycholate is an anionic detergent, whereas, Triton X-100 is a neutral detergent. It was observed that a detergent with a charge is extracting proteins more efficiently than a strictly neutral one.

The presence or absence of the critical concentration of seminal proteins could potentially affect sperm fertility but studies from our laboratory indicated that relative composition of seminal proteins influence the fertility outcome. The sources of those proteins, control of their synthesis, secretion, and mechanisms for binding to sperm to affect differences in fertility of males will be important topics for future research.

Metabolomics

The infertility in males is rapidly increasing and thus the use of genomic and proteomic approaches were not sufficient in elucidating the reason behind the case. Though spermatozoa lack transcription and translation, but are metabolically active. Hence, the metabolites involved in sperm during maturational events and its metabolic activity are vital for sperm function and fertilization. The semen as compared to peripheral blood contains higher concentrations of biomolecules and byproducts, which are secreted from excurrent duct system of male reproductive tract. In India, the study of seminal metabolites in domestic animals is in its infancy. The recent advancement in metabolite profiling of infertile individuals provides better insights into the development of novel fertility markers.

The profiling of seminal metabolite fingerprints such as reducing sugars (fructose and mannitol), fatty acids (palmitic acid and pantothenic acid), amino acids (tyrosine and glutathione), phospholipids (phosphatidylcholin and phosphatidyl ethanolamine), glycoproteins (mucins), hormones (IGF1 and prostaglandins) and key minerals (calcium, boron, zinc, copper and selenium) is gaining importance in domestic animals (Selvaraju *et al*., 2009b; 2012a). Analyses of metabolites provide clinical relevance in search for male fertility biomarkers by analyzing oxidative stress, lipid peroxidation and sperm energy metabolism (Selvaraju *et al*., 2009b). The metabolic evaluation of semen along with traditional sperm attributes, genomics, and proteomics could be a valuable approach to study novel biochemical

pathways associated to male infertility.

***In vitro* fertilization tests**

Assays based on sperm-oocyte interactions, *in vitro* fertilization tests are used to predict bull fertility. These differences in the number of spermatozoa bound per oocyte suggest that there is a relationship between the sperm zona pellucida binding capacity and the fertility of bulls (Selvaraju *et al*., 2008b). In our laboratory, for buffalo fertility prediction, homologous zona pellucida binding and oocyte penetration test have been employed to achieve better accuracy. For assessing the number of sperm bound per oocyte, after *in vitro* incubation of sperm with oocyte, the oocytes are vortexed to remove loosely bound spermatozoa and so that the numbers of spermatozoa have real intention to bind and penetrate are calculated. In order to assess the penetration ability of spermatozoa through oocyte, immature oocyte penetration test was employed. Since immature oocyte does not have block to polyspermy either at zona-pellucida or vitelline membrane, the number of spermatozoa penetrated through zona pellucida is directly proportional to the fertility of the bull (Selvaraju *et al*., 2009a).

Development of semen extender for buffalo semen

In the seminal plasma, the concentration IGF-I influences fertility of bull. In fact, IGF-I acting as antioxidant, protects the spermatozoa from reactive oxygen species produced by highly metabolically active spermatozoa. The addition of physiological level of 150 ng IGF1/ml of semen/diluents protects sperm membrane integrity and sperm fertilizing ability during cryopreservation (Selvaraju *et al*., 2010). Addition of IGF-I also protects spermatozoal membrane proteins such as spermadhesin, dermicidin and sperm acrosomal binding protein during the cryopreservation process, thus preserving sperm fertility (Selvaraju *et al*., 2016b). Furthermore, the prefreeze addition of reduced glutathione to the semen diluents improved post-thaw semen quality and improved the field fertility (Perumal *et al*., 2011a; 2011b). The addition of Zn to semen extender improved velocity parameters and reduced oxidative stress at refrigeration temperature (Unpublished data). The addition of amino acids such as L-glutamine and L- proline as additives in semen extender improved sperm motility, plasma membrane and acrosomal integrity in ram semen (Sangeeta *et al*., 2015).

Improvement of semen quality through nutritional interventions

In vitro study revealed that *in vivo* manipulation of IGF-I levels in the testis and seminal plasma might improve spermatogenic process and thus the competent spermatozoa can be produced. Hence, an experiment was undertaken to assess the impact of different levels of dietary energy and different source of energy on reproductive behavior of ram. The experiment revealed that improving energy level more than the requirement failed to improve semen quality suggesting that optimum level of dietary energy is sufficient to elicit optimal fertility (Selvaraju *et al*., 2012b). Further studies on different source of dietary energy studies revealed that PUFA rather than maize as a energy source produces better semen quality and fertility. Feeding optimum level of PUFA, 4.5% dry matter in the feed reduces lipid peroxidation levels of spermatozoa during *in vitro* storage and fertility (Selvaraju *et al*., 2012a). Non-conventional feeds, like detoxified karanja and neem seed cakes were tried as a replacement to conventional protein supplements in rams. Detoxified karanja cake reduced the expression of IGF-1 and luteinizing hormone (LH) receptor in testes in a dose dependent manner and long-term feeding may affect testicular function (Dineshkumar *et al*., 2013). Neem seed cake in replacement of soybean up-regulated the testicular expression of LH receptor and IGF-1 by 3-4 folds without affecting the digestibility and metabolism of lambs (Rao *et al*., 2016). Such manipulations might improve overall sperm function and fertility.

Spermtogonial stem cell technology: Promising potentials in livestock research

The spermatogonial stem cells (SSCs) are unique

cells of the adult body having the capability for self-renewal and differentiation to form sperm cells. It has various applications in livestock like preservation of the genetic material of valuable males, improving the fertility of low fertile animals, production of elite animals, production of transgenic animals, understanding the process and the pathways of self-renewal and differentiation, and mining factors regulating male fertility. The information regarding the isolation, purification, characterization, culture and transplantation of SSCs in livestock is very limited, however, attempts were made in buffalo (Mahla *et al*., 2012; Kadam *et al*., 2013), sheep (Binsila *et al*., 2015) and goat (Pramod and Mitra, 2014) from India. The numbers of SSCs/tubule were higher in adult ram testis compared to neonatal (Binsila *et al*., 2015). The combinations of collagenase and trypsin could effectively digest testicular tissue and release SSCs. In rams, the number of SSCs was less than 1% of the total cell isolate (Binsila *et al*., 2016). However, the intensive study in livestock must be undertaken for the successful culture, cryopreservation and transplantation of SSCs.

Analysis of testicular microenvironment for predicting bull fertility

Ultrasonography is a noninvasive technique for the evaluation of the internal structure of the scrotum and a recently developed water bath based sonographic examination enables more distinct visualization of testicular architecture (Jeyakumar *et al*., 2013). The testicular cytology from fine needle aspiration technique revealed that higher Sertoli cell counts correlated with better sperm quality and cryo-tolerance capacity (Rajak *et al*., 2015). The researchers suggested that the testicular fine needle aspiration technique might be used to detect sub-fertility and infertility in bulls without affecting their reproductive health (Rajak *et al*., 2013). To understand the reasons for variations in fertility status among adult bulls, the knowledge on the testicular microenvironment, molecular associations among the testicular cells and events that occur for the derivation of fertile sperm can be obtained from the testes of prepubertal animals and researches are being carried out recently on these aspects. Spermatogenic and Sertoli cells protein profiling yielded variation in expression between pre-pubertal crossbred, indigenous and exotic breeds of bulls (Tripathi *et al*., 2014). However, the association between the fertility status to a particular protein has not been fully explored.

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