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Sperm Transcriptomics: An Emerging Technique to Assess Male Fertility

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A B S T R A C T

Semen sample has to be analyzed to determine the fertilization capacity of sperm. This helps in the efficient use of semen sample for cryopreservation and their further use in artificial insemination. The current methods used to analyze semen just indicate a predictive fertility status, not an actual fertilization capacity. Certain mRNA population / transcripts which are present in spermatozoa are found to have a potential role in fertilization capacity. The analysis of mRNA population in sperm by means of isolation of RNA from sperm and profiling of these mRNA transcripts may give the actual fertilization status of the sperm. This technique is non- invasive in nature and it can be used as promising technique to screen the fertility of male animals at an early stage.

Introduction

The fertility assessment of frozen-thawed semen is essential for the effective use of cryopreserved semen in animal breeding. The laboratory evaluation of semen from healthy male animals, before and after freezing for artificial insemination (AI), is largely based on subjective analysis of sperm motility and concentration. Hence, an accurate and efficient *in vitro* method is warranted to predict fertility, especially of buffalo sperm. This will help to select the future breeding male stock at an earlier age of maturity. However, the information pertaining to the fertility index in relation to sperm attributes is meager. The factors associated with high fertility in male animals help in designing better strategy for improving the field fertility of male animals following insemination with frozen-thawed semen.

Another important concern in animal breeding is sub-fertility, which even today remains as a non-predictable measure in case of many animals. Evaluation of fertility is primarily by

calculating the non-return rate (NRR). The male animals that are retained generally have an NRR that varies from 20-25% (Killian *et al.,* 1993). A number of methods are used to correlate the fertility potential of a sire, measured i*n vitro*, with the NRR in order to reduce the costs associated with subfertility. The selection of sires for species reproduction requires an *in vivo* evaluation of fertility that is carried out over a period that can run from several months to several years. During this period, the semen has already been treated and stored with a view of eventual marketing. The process involves the handling of thousands of ejaculates, their cryopreservation and breeding attempts that all incur significant costs. The new molecular detection tools that have been developed as a result of recent developments in the field of comparative genomics and global gene expression analysis, will allow a number of parameters to be effectively integrated into the evaluation of the fertilizing potential of semen.

The presence of RNA in spermatozoa is well established, yet little is known regarding its function and purpose (Dadoune *et al.,* 2005; Selvaraju *et al.,* 2017). A decade back, RNA in the sperm was believed to be non-functional. It is now known that RNA might perform active functions in the embryo development and fertilization (Ostermeier *et al.,* 2004); establishment and maintenance of a viable paternal genome (Miller *et al.,* 2005); activation of embryonic genome (Boerke *et al.,* 2007); fertilization (Yao *et al.,* 2014); regulate expression of their parent gene in the male germline (Jodar *et al.,* 2015), pregnancy outcome (Kasimanickam *et al.,* 2013) and may also influence the phenotype of the offspring (Rando, 2012). Rassoulzadegans *et al.,* (2006) provided a model for epigenetic inheritance by zygotic transfer of RNAs that dysregulate expression of c-Kit gene, which leads to the modification of phenotypic expression of the offspring. Sperm RNA could be available as a diagnostic tool for male infertility (Jodar *et al.,* 2012; Millar *et al.,* 2014) and also determine the quality of semen (Das *et al.,* 2013; Georgiadis *et al.,* 2015; Parthipan *et al.,* 2015). This finding sparked considerable interest in the field since such a potential contribution of the male gamete to embryonic development challenged the well accepted dogma, which restricted the male gamete to a DNA shuttling vector. To elucidate these functions of transcripts retained in sperm, approaches like microarrays, RNA-Seq, qRT-PCR have been applied (Carreau *et al.,* 2007; Platts *et al.,* 2007; Yang *et al.,* 2009; Jodar *et al.,* 2012; Lima-Souza *et al.,* 2012; Card *et al.,* 2013). Success of these technologies is dependent on the quality of the RNA obtained. Quality sperm RNA isolation depends on the purity of spermatozoa which should be free from somatic cells contamination.

Though the male animals form an integral part of livestock agriculture but lower conception rate of AI with frozen semen, is mainly due to the susceptibility of spermatozoa to hazards during freezing-thawing process. It is well documented that cryopreservation induces sperm damage owing to the rapid change in low temperature which leads to mechanical damage to plasma membrane, metabolic alteration, oxidative stress to phospholipid membrane, DNA damage and eventually reduced fertilizing capacity. Traditionally a number of methods are available for evaluation of semen quality in laboratory like assessment of the integrity of genomic DNA, acrosome and plasma membrane and mitochondrial as well as sperm-oocyte interactions. But, the development of a reliable method for routine isolation of high quality RNA from sperm will be an important step to develop novel non-invasive approach to evaluate male fertility. The RNA transcript may have important roles in sperm development, chromatin repackaging, genomic imprinting, capacitation, acrosome

reaction and early embryonic development. Understanding the make-up of sperm RNA transcripts may prove to be important in understanding the events surrounding capacitation, motility, fertilization and ultimately in explaining the male fertility. The development of new investigative method such as analysis of mRNA profile in sperm and understanding the significance of the transcripts would be helpful as additional diagnostic tool and be of prognostic value to fertilization and establishment of pregnancy.

Historical perspective of sperm RNA

The existence of spermatozoal RNAs was formerly examined based on the postulation that termination of transcription process takes place in round spermatid stage, where there is removal of cytoplasm and the components which are necessary for translational activity is absent. Hence, the remaining male haploid RNA which is present would be insignificant. This view was supported by the observed heterogeneity of the ejaculate, the presence of somatic cell contaminants which accounted for the majority of large RNAs in most samples and the absence of intact ribosomal RNAs. These caveats partly reflected the inadequacy of the methods that were available to purify spermatozoa and to detect low abundance RNAs (Krawetz, 2005). The controversy was resolved when several laboratories independently identified specific sperm RNAs in plants (Rejon *et al.,* 1988) and in mammals, including rat (Pessot *et al.,* 1989), mouse (Wykes *et al.,* 2000) and human (Kumar *et al.,* 1993; Miller *et al.,* 1994; Wykes *et al.,* 1997) using RT–PCR and *in situ* hybridization. Above mentioned studies demarcated the existence of RNA in mature sperm nucleus and later studied the paternal contribution in fertilization and embryo development. Persistence of a low but detectable level of transcription in mature sperm cells has also been reported by Miteva *et al.,* (1995). Up to now, human spermatozoal transcripts are regarded as the best detectable amongst all mammals.

The RNA profile of human spermatozoa was initially attempted using a cDNA cloning and sequencing strategy (Miller *et al.,* 1999) which was followed by select RT-PCR (Lambard *et al.,* 2004). But, these methods were able to investigate only a small fraction of all potential transcripts. The first general spermatozoal RNA profiles were obtained using microarrays, which suggested that human spermatozoa contain ~3000–7000 different coding transcripts (Ostermeier *et al.,* 2002). This was subsequently protracted to the clinic with the assessment of specific transcripts in cases of asthenozoospermia in humans (Jodar *et al.,* 2012), teratozoospermia in humans (Platts *et al.,* 2007), oligozoospermia in humans (Montjean *et al.,* 2012) and idiopathic infertile males in humans (Garrido *et al.,* 2009). Their ability to serve as potential biomarkers of fertility was highlighted. Transcript profiling of coding RNAs using microarrays in conjunction with RT-PCR has nowadays broadly defined the abundance of known sperm transcripts in other mammals (Gilbert *et al.,* 2007; Bissonnette *et al.,* 2009; Yang *et al.,* 2010) and non-mammalian species like plants (Borges *et al.,* 2008) and *Drosophila Melanogaster* (Fischer *et al.,* 2012). In comparison to the above-mentioned statement, RNA-Seq has provided a complete picture of the population of sperm transcripts, allowing for the identification, quantification and characterization of both known and previously unknown RNAs (Sendler *et al.,* 2013; Selvaraju *et al.,* 2017). These studies highlighted the selective retention of a cadre of both coding RNAs and small non-coding RNAs in all individuals studied. In recent times, many scientists have initiated in employing RNA-Seq to examine the distribution of sperm RNAs in bovine (Card *et al.,* 2013; Selvaraju *et al.,* 2017),

stallion (Das *et al.,* 2013) and the small RNA population of mouse (Kawano *et al.,* 2012; Peng *et al.,* 2012).

Derivation of sperm RNA during spermatogenesis

Spermatogenesis is the process where there is development of spermatozoa from spermatogonium in the seminiferous tubules. This process is more or less similar in all mammalian species. The process of spermatogenesis is mainly classified into three phases depending upon their functional considerations:

Spermatocytogenesis

Mitotic phase / Proliferative phase (Equational division): Mitosis of undifferentiated spermatogonium occurs.

Meiotic phase (Reductional division): Primary spermatocyte yields two secondary spermatocytes (A1-A4; B Spermatogonia), by means of undergoing meiosis I, whereas secondary spermatocytes (B-spermatocytes) again go for meiosis II to yield four haploid round spermatids.

A- Spermatogonia have the potential to go back to its preceding stage of undifferentiated Spermatogonia, by means of renewal of stem cell. The round spermatids enter into a final phase of spermatogenesis called differentiation phase.

Spermiogenic phase (Differentiation / Metamorphosis): Here the actual differentiation of spermatid to become complete spermatozoa, which is equipped to cause fertilization, occurs. The series of changes include the morphological transformations where in the fully functional head and tail is formed to become a complete spermatozoon.

During proliferation and meiosis, expression is chiefly under transcriptional control. Transcriptional control also applies to the early haploid stages of spermatogenesis, and indeed, Meiosis I prophase is when many of the RNAs that will be translated post-meiotically are transcribed. Their translation corresponds with nuclear shutdown. The fate of these transcripts is suggested, with most of the pre-meiotic and early meiotic RNAs going into the residual bodies. Selected pre-meiotically transcribed RNAs and haploid-expressed transcripts are then retained by the spermatozoa. This relationship has not been experimentally proved.

However, mRNA in sperm may be residual, which reflects the transcription shutdown during the process of spermiogenesis. Developing spermatids solely depends on mRNA stores for long time, because they require translational control of gene expression, whereas spermatids may mislay the ability to exclude it from maturing cell (Miller and Ostermeier, 2006). The equal sharing in case of gene products by means of developing spermatids is preferred by a common block on exclusion pathways of RNA. This process is maintained with the help of incessant presence of cytoplasmic bridges which interconnects spermatids (Caldwell and Handel, 1991). Further, absence of sufficient 28S or 18S rRNAs (80s ribosomal complexes) in mature spermatozoa for supporting transition, signifies their breakdown or elimination during the course of spermatozoal evolution (Miller *et al.,* 1999).

Sperm and its types of RNA

Sperm is a remarkably differentiated cell whose prime function is to deliver the paternal haplotype to the ovum. Despite this, sperms are extremely variable in form, with all aspects of the sperm phenotype showing high levels of variation (Pitnick *et al.,* 2009). The

fact that sperm has until now been presumed to only contribute DNA (plus structures such as centromeres) to eggs, makes the recent discovery of a complex sperm RNA population (Miller *et al.,* 2005; Boerke *et al.,* 2007; Fischer *et al.,* 2012), surprising and hard to explain. The RNA population carried by sperm is large and varied. It includes messenger RNA (mRNA), micro RNA (miRNA), interference RNA (iRNA), and antisense RNA (Dadoune, 2009). These include transcripts for heat shock proteins, cytochrome P450 aromatase, and a range of receptors, including odour receptors (Dadoune, 2009). Sperm RNA is unlikely to be transcribed from sperm nuclear DNA because of the changes in chromatin structure that occur when protamine replaces histones during sperm DNA compaction. Hence, it was originally thought that sperm RNAs were simply remnants of spermatogenesis (Curry *et al.,* 2011). However, various evidences suggests that sperm RNAs are not merely discards from the sperm-building process. First, there are indications of translational activity in the sperm cells, using sperm RNA as the substrate (Fischer *et al.,* 2012). Second, there is evidence that sperm RNA contributes to fertilization and to embryo development (Liu *et al.,* 2012). All this infers that the presence of sperm RNA has fitness consequences for both males and females, and is there because of its adaptive value. However, unequivocal evidence of precise sperm–RNA contains thousands of transcripts which function is rare or completely unknown.

Location and nature of sperm RNA

It has long been apprehended that the tightly packaged chromatin within mature spermatozoa is transcriptionally inert. Despite this, RNA was observed in the mature sperm nucleus of fern *Scolopendrium* (Rejon *et al.,* 1988) and in rodents and other species (Pessot *et al.,* 1989; Concha *et al.,* 1993). When subjected to acrylamide electrophoresis, the RNA described by Pessot *et al.,* (1989) was found to resolve into discrete bands that included 5.8S and 5S RNAs as well as tRNAs.

This and the advanced report by Concha *et al.,* (1993) localizing U1 and U2 small nuclear RNAs were the first to provide visual information on the localization of spermatozoal RNA to the nucleus. Consequently, Kumar *et al.,* (1993) reported the presence of *c-myc* mRNA in the principal piece of human spermatozoa, and a few years later, two independent reports indicated the presence of protamine 2 mRNA by RT–PCR and *in situ* hybridization, with the later also localizing the RNA to the nucleus (Miller, 1997; Wykes *et al.,* 1997). These reports support the localization of spermatozoal RNA in or around the nucleus.

However, spermatozoa contain a complex range of RNAs, but meager information is available to indicate storage position of RNA within the cells. There are four main segments of a mature spermatozoon. As suggested by various studies, the perinuclear theca and the post-acrosomal sheath are the possible areas for spermatozoal RNA repositories. Additionally, Kumar *et al.,* (1993) have localized transcripts to present within the midpiece. Again, the fibrous sheath and axoneme may also tend to carry spermatozoal RNA.

Amount of RNA available in spermatozoa

The RNA quantity in a sperm cell is very low; one haploid spermatozoon contains ~10-20 fg of RNA, compared to ~450 fg of RNA in a haploid spermatid and ~ 10-20 pg of RNA in one diploid somatic cell (Krawetz, 2005; Carreau *et al.,* 2007; Galeraud-Denis *et al.,* 2007). The amount of RNA in spermatozoon is varying between individual species; which is indicated in the Table 1.

Table.1 RNA yield/spermatozoon in various species

Species	RNA yield (fg)/spermatozoon	Reference(s)
Human	10–20	Ostermeier *et al.*, 2005
Human	50–100	Goodrich *et al.*, 2007
Cattle	20-31	Card *et al.*, 2013, Selvaraju *et al.*, 2017
Cattle	180	Gilbert *et al.*, 2007
Horse	20	Das *et al.*, 2013
Pig	5-10	Boerke *et al.*, 2007
Rat	100	Pessot *et al.*, 1989

Table.2 List of mRNA transcripts present in spermatozoa of various species

S. No	Species	mRNA Transcripts	Cell Type	References
	Bovine			
1		Cadherin 15, EST, PABPC4, PABPN1	Spermatozoal cells	Lalancette at al., 2008
2		PRM1, PRM2	Spermatozoal cells	Bissonnette *et al.*, 2009
3		PRM1, PRM2, PRM3, Tnp1 and Tnp2	Testicular cells	Ferraz *et al.*, 2010
4		CRISP2, PEBP1, CCT8, BRP	Spermatozoal cells	Arangasamy *et al.*, 2011
5		PRM1, LOC783058, HMGB4, LOC404073, KIF5C, TMSB4X, GSTM3	Spermatozoal cells	Card *et al.*, 2013
6		Protamine 1, Protamine 2	Spermatozoal cells	Ganguly *et al.*, 2013
7		CCT5, GUK1, CTRB1, SRMS, ISCU, PJA1	Spermatozoal cells	Yathish *et al.*, 2016
8		MIR708, VSNL1, SQRDL, CD28	Spermatozoal cells	Selvaraju *et al.*, 2017
	Equine			
1		PAD16, DNAJC16B, DCDC2, CTTN, REEP6, ARID5B, ATG12	Spermatozoal cells	Das *et al.*, 2013
	Human			
1		Protamine1, Protamine 2, CD45, C-Kit, C-myc, E-cadherin, NOS, eNOS	Spermatozoal cells	Lambard *et al.*, 2004
2		TCP11, TESK1, TSPYL1, ADAD1	Spermatozoal cells	Bansal *et al.*, 2015

Relation of sperm RNA with oogenesis

Retention of RNA in spermatozoa seems to have an equivalent comparison with oogenesis, where mRNA is stored in large quantities until the termination of transcription (Briggs *et al.,* 1999). To support protein synthesis, maturing oocytes depends upon the translational stocks of maternal mRNA.

Translation modification in case of both the gametes is same, where there should be equal changes in case of poly-A-tail which is having synchronization with active associations between appropriate binding proteins of RNA and untranslated regions of RNA (Kleene, 1996; Hecht, 1998; DeJong, 2006). Spermatozoal ejaculate contains two populations of mRNA which have their own functions:

A centriolar population which maintainsthe embryo and also has some role in development and

A nuclear population which helps in supporting either repacking of chromatin of 'spent' proteins transitional replacement.

Relation of sperm RNA with embryonic development

By analyzing a group of RNAs which are specific to sperm and which got delivered into the oocyte, the role of these RNAs in the fertilization process in eggs is not clear (Ostermeier *et al.,* 2004).

Different sets of mRNAs have been found to have relationship with various functions like repackaging of chromatin, genome imprinting and development of spermatozoa. However, the particular sets of genes for predicting differences in fertility need to be analyzed (Miller *et al.,* 2005).

Association of sperm RNA with fertility

Active miRNAs are present in massive amount in spermatozoa, and it has been evident that precise sperm borne miRNAs have correlation with fertility. Hence, the potency of spermatozoa to promote and sustain the development of zygote, embryo and fetus and also for considering male fertility, spermatozoal expression profiling could be helpful. The differences in amounts of different miRNAs between their ejaculates, helps to identify the animals with sub-fertile to high-fertile nature (Fagerlind *et al.,* 2015).

Earlier it was presumed that the mature spermatozoal RNA undergoes certain cellular modifications during spermiogenesis; hence RNAs are either degraded or lost. In older days, spermatozoal RNA is considered as non-functional in nature, but present scenario is that the spermatozoal RNA have active functions when it enters into oocyte, during fertilization and also within the cell itself (Miller *et al.,* 2005; Miller, 2011). Spermatozoa have an active translation of stored mRNAs (Gur and Breitbart, 2006), and also have the capacity to take up a foreign RNA and DNA, this activity can be used for the production of transgenic animals (Spadafora, 1998).

This phenomenon of transgenic production arises due to the process of apoptosis which leads to spermatozoal chromatin auto-digestion (Maione *et al.,* 1997; Sotolongo *et al.,* 2003). Competent DNA templates transcribe to fresh RNA which appears to be histone bound. This happens because of three reasons,

Presence of enormous transcription factors (Pittoggi *et al.,* 2001)

Spermatozoa having RNA polymerase (Hecht and Williams, 1978) and

By means of enzyme mediated process in case of cells, RNA can be converted to DNA and there should be a possibility that DNA can also be converted to RNA (Sciamanna *et al.*, 2003).

Spermatozoal RNA as a marker for fertility research

Spermatozoal RNA reflects the spermatogenic perspective of testis, so it can be used as a probable indicator for various infertility studies (Ostermeier *et al.*, 2005). But prediction of infertility issues with the help of advanced molecular level studies is deficit (Balen and Jacobs, 2003). However, techniques like SAGE, microarray and various other techniques can also be used for characterization of sperm RNAs and also to study the comparison between sub-fertile and high-fertile males. (Wang *et al.*, 2004).

Potential mRNA markers for sperm quality

Spermatozoal motility can be used as a good indicator of male fertility, and it appears to have certain molecular associations which can be determined using real time PCR based studies. Protamine 1 (PRM1) expression was noticed in spermatozoal population harvested from Percoll gradient, which was having poor motility. Same expression pattern was observed in case of both neuronal mRNAs (nNOS) and endothelial mRNAs (eNOS) (Lambard *et al.*, 2004). Around 5000 transcripts present in spermatozoa (Ostermeier *et al.*, 2002), can give a considerable information (Table 2) about the fertility by means of understanding molecular events, necessary for spermatogenesis.

The analysis of spermatozoal RNA (a non-invasive technique) can be used as an efficient technique to analyze male fertility, with the help of only the semen samples. The collection of semen sample to study spermatozoal RNA profiling is of great ease when compared to other techniques, which requires testes tissue biopsy. The collection of testis tissue for examination may sometimes cause a permanent damage to the testis. Hence, analyzing the spermatozoal mRNA transcripts (sperm transcriptomics) is found to be an emerging and promising technique for analyzing male fertility. With the advent of sperm transcriptomics, screening and selection of male breeding stock can be done at an early stage. Sperm transcriptomics can serve as an ideal and efficient technique, in the future, for selection of efficient male breeding stock.

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