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RESEARCH ARTICLE

Characterizing miRNA and mse-tsRNA in fertile and subfertile yak bull spermatozoa from Arunachal Pradesh

PARTHA PRATIM DAS[1], SAFEEDA SULTANA BEGUM[2], MANASEE CHOUDHURY[3], DINAMANI MEDHI[2], VIJAY PAUL[2] and PRANAB JYOTI DAS[2,3]*

[1]*Gauhati University, Guwahati 781 014, India*
[2]*Indian Council of Agricultural Research-National Research Centre on Yak, Dirang, West Kameng 790 101, India*
[3]*Indian Council of Agricultural Research-National Research Centre on Pig, Rani, Guwahati 790 101, India*
*For correspondence. E-mail: drpranabjyotidas@gmail.com.

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Abstract. Male fertility in farm animals is considered as an important economic trait. The phenomenon of spermatogenesis plays a dynamic functional role in determining the viability of sperm and thereby can impact on fertility-driven complications. The process of spermatogenesis is controlled by numerous molecular factors and requires a precisely regulated pattern of gene expression. The role of small noncoding RNAs in altering gene expression has been extensively studied. However, limited information is available apropos their role in yak spermatogenesis. The present study aimed to evaluate the assessment of some significant microRNAs and their expression pattern in the body tissues and sperm of fertile and subfertile yak from Arunachal Pradesh besides identified a novel class of sperm enriched small RNA 'mature-sperm-enriched small RNA' (mse-tsRNA) in Yak spermatozoa. The RNA was extracted from tissue and sperm using 27 gauge needles and subsequently reverse transcribed into small RNA cDNAs. The PCR positive sperm-predominant miRNAs were validated by quantitative reverse transcriptase PCR (qRT-PCR) for their expression in fertile and subfertile yak. Of the 22 microRNAs, the miRNA19a, miRNA142 and miRNA143 showed higher expression in the subfertile yak, whereas expression of miRNA7d, miRNA23a and miRNA23b were found elevated in the fertile animal. The presence of these small noncoding RNAs in yak sperm and testis indicated the legitimate involvement of their role in yak bull fertility.

Keywords. microRNA; quantitative polymerase chain reaction; subfertile yak; yak; mature-sperm-enriched small RNA.

Introduction

Reproductive fitness is critical for the existence of any species. Male infertility can cause major economic losses in terms of production for animal's farming. The primary cause of male infertility is prevalently due to anatomical dysfunction and endocrine disorder producing low sperm counts and poor sperm quality or might consider a genetic disorder (Tahmasbpour *et al.* 2014). However, from fertility data and progeny records it is seen that despite the acceptable motility numbers of high-merit males and morphology of their spermatozoa, not to produce successful fertilization with a healthy female (Parkinson 2004; Chenoweth 2007). This notion indicates the contribution of alternate molecular mechanisms in maintaining the stability of spermatozoa to fertilize and successful embryo development (De Jonge 1999; Lewis 2007; Simon *et al.* 2017). The fertilizing capacity of males predominantly depends on motility, membrane architecture, acrosome integrity and the ability to penetrate oocytes (Ward *et al.* 2003). Although, the quality of spermatozoa can be identified by advanced technologies, however, a comprehensive understanding of the molecular mechanisms that control sperm motility is still lacking (Huang *et al.* 2011). A detailed study of male derived factors for infertility in the animal along with the molecular approach can eventually enrich our understanding of sperm functions.

Past research revealed that during fertilization, coding and the noncoding RNAs (ncRNA) inclusively plays a significant role in the oocyte modification (Hua *et al.* 2019; Robles *et al.* 2019). The sperm RNAs are repertoire of information of the past activities of spermatogenesis as well as act as source of information of egg fertilization and development (Lalancette *et al.* 2009; Pacheco *et al.* 2012). The

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spermatozoa of mammals contain a complex repertoire of RNAs like mRNAs, rRNAs and snRNAs, largely derived from remnant active transcription (Dadoune *et al.* 2004). Among, the small noncoding RNA, microRNA (miRNA) and Piwi-interacting RNA (piRNA) play a central role in male reproduction and fertility as evidence suggests that lacing a piRNA generated mutant with fertility defects in different species (Das *et al.* 2008). Moreover, miRNA also plays a significant role in various biological functions and their presence in testis has been verified a decade ago (Hayashi *et al.* 2008). Characterization and identification of small RNAs in bovine spermatozoa revealed their involvement in the incapacitation and fertilization process as well as in early embryogenesis (Card *et al.* 2013). In yak, the knowledge of the miRNA repertoire and profiling from spermatozoa would open new vistas in understanding the fertility risk. Indeed, the comparative analysis of fertile and infertile males with sperm RNAs will be regarded as a good source of potent fertility biomarkers and likely to disclose the pool of RNAs for sperm functions. It is already established that the downregulation of miRNAs expression patterns severely affects the male reproductive functions (Govindaraju *et al.* 2012; Liu *et al.* 2012). Here, we report an integrated approach to illustrate the role of a few miRNA and a novel class of abundantly expressed small RNA in sperm, i.e. 'mature-sperm-enriched tRNAs' (mse-tsRNA) expression profiling in the fertile and subfertile Arunachali yak spermatozoa. The attempt has been made to study the profiling of 22 miRNAs with an objective of functional alteration of miRNAs associated with recognizing subfertility as well as to identify expression of four mse-tsRNAs in yak spermatozoa.

Materials and methods

Collection of samples

Fresh ejaculates from five reproductively fertile and five subfertile yaks were collected using an artificial vagina (Missouri model). Portioning of the fertile and subfertile animal was done by comparing adjusted conception rate which excluded female factors as well as environmental and nutritional factors. A yak bull was considered fully fertile when running with breeding groups of 40–50 cows, it should be able to achieve average pregnancy rate to each service of 60% meaning in nine weeks of breeding at least 94% of cows should be pregnant. A yak bull was considered as subfertile when achieving only 40% pregnancy rate to each service assuming a breeding group of 100 cows. Moreover, the ejaculates were first evaluated for sperm parameters like motility, progressive motility, head abnormality for portioning fertile and subfertile yak bull. This was followed by purification of sperm from somatic cells and immature sperm by BoviPureTM (Nidacon International, Sweden) discontinuous gradient centrifugation. Sperm characteristics were evaluated by light microscopy (x400), using a Neubauer Counting Chamber. Subsequently, the samples were evaluated for sperm concentration, motility and morphological features, and finally, good quality sperm were selected and stored for the downstream process. Testes and other body tissue sample, namely brain, heart, kidney, lung, skeletal muscles, and ovary were obtained from normally slaughtered yaks. Purified sperm and tissues were stored in RNAlater (Ambion) at −80°C until use. History of breeding and fertility records of the animals from which semen was collected were also recorded.

Background of primer design

MiRNA sequences were selected from the miRBase micro RNA database (http://www.mirbase.org/) where a miRNA itself acts as a forward primer. As most of the miRNAs are only about 22-bp nt long, their amplification with conventional PCR is less likely to give a distinctive product. Thus, the principle based on the elongation of the miRNA to produce a template long enough is allowed to design with two sets of primers, which also have an added advantage of using two specific oligos that allow high specificity and increased flexibility in primer design. In the present study, miRNAs are amplified by designing two miRNA-specific primers of 20–24 bp nt long forward primer (table 1, primers 1–24) and a universal reverse primer with 3–8 specific nucleotides at the 3′-end (table 1, primer 25) and an extension that is complementary to a universal tag, which is added to the template during cDNA synthesis. In a subsequent step, the polyadenylation and adaptor ligation adequately enhance the specificity and along with a universal reverse primer increases the product size to 70–80-bp long. Therefore, this becomes easier to isolate and visualize specific miRNA from the pool of small RNA cDNAs (srcDNA) on the agarose gel plate. To characterize the mature-sperm-enriched small RNAs (mse-tsRNA), two primer sets from two different extremely enriched RNA families of sperm RNA population, 'family I' and 'family II' that was described by Peng *et al.* (2012) were chosen to monitor the enrichment of mse-tsRNAs in yak spermatozoa. Mse-tsRNA-31 and mse-tsRNA-32 were derived from family I whereas mse-tRNA-30 and mse-tRNA-34 were derived from family II. Among the cluster of primers, only two sets were selected from each family based on the high percentage of enrichment on mouse spermatozoa.

Total RNA isolation from sperm and tissue samples

The total RNA was isolated from sperm by using TRIzol (Invitrogen) reagent by lysing mature sperm cells with a 27 gauge needle as described by Das *et al.* (2010) and from testes using RNeasy mini elute kit (Qiagen) and manufacturer's protocol. The pool of small RNAs was isolated from

Table 1. List of miRNA, mse-tsRNA and control primers used in the study.

	Primer name	Sequences	No. of base pairs (bp)
1	MIR1248*	GCGGCGGTCCTTCTTGTATAAG	22
2	MIR34B*	GCGGCGGAGGCAGTGTAATTAG	22
3	MIR34C*	GCGGCGGAGGCAGTGTAGTTAG	22
4	MIR223	GCGGCGGTGTCAGTTTGTCAAA	22
5	MIR191*	GCGGCGGCAACGGAATCCCAAAAG	24
6	MIR1905C	GCGGCGGCACCACCAGCCCCAC	22
7	miR-34c-5p	AGGCAGTGTAGTTAGCTGATTGC	23
8	miR-495-3p	AAACAAACATGGTGCACTTCTT	22
9	miR-19a-3p	TGTGCAAATCTATGCAAAACTGA	23
10	miR-656-3p	AATATTATACAGTCAACCTCT	21
11	miR-142-3p	TGTAGTGTTTCCTACTTTATGG	22
12	miR-19b-3p	TGTGCAAATCCATGCAAAACTGA	23
13	miR-409-3p	GAATGTTGCTCGGTGAACCCCT	22
14	miR-23a-3p	ATCACATTGCCAGGGATTTCCA	22
15	miR-23b-3p	ATCACATTGCCAGGGATTACC	21
16	miR-197-3p	TTCACCACCTTCTCCACCCAGC	22
17	miR-148a-3p	TCAGTGCACTACAGAACTTTGT	22
18	miR-143-3p	TGAGATGAAGCACTGTAGCTCA	22
19	miR-1468-5p	CTCCGTTTGCCTGTTTTGCTGA	22
20	miR-502-5p	AATCCTTGCTATCTGGGTGCTAGT	24
21	miR-362-3p	AACACACCTATTCAAGGATTCA	22
22	miR-362-5p	AATCCTTGGAACCTAGGTGTGAGT	24
23	miR-103-5p	AGCTTCTTTACAGTGCTGCCTTG	23
24	miR-7d-3p	CTATACGACCTGCTGCCTTTCT	22
25	UniRev primer	ATCCAGTGCAGGGTCCGAGG	20
26	RT primer	GTCGTATCCAGTGCAGGGT CCGAGGTATTCGCACTGGATA CGACTTTAGCTTTTTTTTTTTTTTTTTTTTTT	75
27	U6 snRNA-H	CGCAAGGATGACACGCAAATTC	22
28	miR-16	TAGCAGCACGTAAATATTGGCG	22
29	U6 snRNA-M	TGGCCCCTGCGCAAGGATG	19
30	GAPDH-F	GGGTCATCATCTCTGCACCT	20
31	GAPDH-R	ATAAGTCCCTCCACGATGCC	20
32	AR-F	TAAGGCAGTGTCGGTGTCC	19
33	AR-R	GCCATCATCCAGCAAAGA	18
34	mse-tsRNA-F1-31(family I)	TCCCTGGTGGTCTAGTGGTTAGGATTCGGCG	31
35	mse-tsRNA-F1-32 (family I)	TCCCTGGTGGTCTAGTGGTTAGGATTCGGCGC	32
36	mse-tsRNA-F2-30 (family II)	GCATTGGTGGTTCAGTGGTAGAATTCTCGC	30
37	mse-tsRNA-F2-34 (family II)	GCATTGGTGGTTCAGTGGTAGAATTCTCGCCTGC	34

*Elite Genettancer-gene association.

the collected sperm and tissue panel using the mirVana miRNA isolation kit (Ambion). The quality of RNA samples was checked and validated using intron spanning *PTPRC* (*CD45*) and *PRM2* primers for sperm, testis and tissues for quality control of RNA.

Polyadenylation and adopter ligation miRNAs

The small RNAs extracted from the tissue of sperm and testis was polyadenylated and reverse transcribed into srcDNAs using adapter attached oligo-dT primers (RTQ) (table 1) to generate small RNA cDNAs (srcDNAs). For this purpose, the srcDNAs were PCR amplified using the miRNA-specific forward primer and adapter-specific universal reverse primer (table 1) following the methods described previously (Ro *et al*. 2006; Ro *et al*. 2007).

Expression profiling of miRNAs using semi-quantitative RT-PCR

The small RNAs were extracted from yak spermatozoa and tissue panel, and the concentration was measured. Subsequently, the polyadenylated RNA was reverse transcribed into srcDNAs using adapter attached oligo-dT primers (RTQ) (table 1). The remaining RNA was removed by RNase-H treatment from the synthesized srcDNA pool, and stored at −80°C until use.

A total of 22 miRNA were chosen for the initial study, selected from previous literature reports for a fertility-related response on humans, cattle and horses. A known small RNA family ‘family I’ and ‘family II’ of mouse mse-tsRNAs (three from each group) were also chosen for the study to identify their characterization on the yak. All the miRNAs were eventually amplified in seven body tissues of yak

including testis. Ubiquitous expression of miRNAs in all tissue panels was subjected to omit a few of them and those with the only testis and sperm-specific amplification were selected for subsequent analysis. Among them, four miRNA were found to show differential expression in the sperm of fertile and subfertile of yak bull.

Amplification of selected srcDNA and mse-tsRNA

The targeted miRNAs were amplified using specific forward primer and a universal reverse primer (table 1) with PCR condition 95°C for 10 min, followed by 28 cycles at 95°C for 15 s, and 60°C for 1 min carried out in a thermal cycler (ABI, Veriti 96-well) and finally confirmed in 2% agarose gel.

The mse-tsRNA sequence was also amplified using mse-tsRNA specific forward primer and a universal reverse primer (table 1) using the same reaction condition.

Quantitative reverse transcriptase PCR (qRT-PCR)

The PCR positive sperm-predominant miRNAs were further validated by qRT-PCR, where the reaction was performed using 1x SYBR Green PCR Master Mix (Roche), 200 ng of srcDNA, 10 pmol forward and reverse primers and required amount of nuclease-free water in a 20 μL total reaction volume. The reactions of PCR condition were carried out for 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, primer annealing at 54°C for 30 s, and strand elongation at 60°C for 30 s. Four housekeeping genes U6 snRNA-H, miR-16, U6 snRNA-M and *GAPDH* were utilized as the reference. A P value less than 0.05 (typically $\leq$ 0.05) is statistically significant. It indicates strong evidence against the null hypothesis, as there is less than a 5% probability the null is correct. A P value greater than 0.05 means that no effect was observed. These whole experimentations were repeated thrice from sample collection to qRT-PCR analysis to eastblish specific miRNAs that are usefull to differentiate fertile and subfertile yak bull.

The procurement of semen and tissue samples was done following the approval of the Institute Animal Ethics Committee of the Indian Council of Agricultural Research-National Research Centre on Yak, India. The approved animal use protocol number is 4(17)/NRCY/IAEC-02.

Results

Parameters of sperm preparations

All sperm samples had a normal morphologic profile with acceptable fertility and motility above 95%. In this study, sperm purification with a one-layer Bovipure gradient was found to be optimal and repetitively confirmed by examining each sperm sample under a light microscope before and after purification.

Assessment of sperm RNA quality

With a concentration of 90 μg/mL, all RNA samples had spectrophotometry values between 1.97$\pm$0.5 for absorbance ratios 260/280, which indicated that the RNA was free from proteins and other reagent contamination. Further validation by a reverse transcriptase PCR by tissue-specific primer, protamine (*PRM2*) and CD45 (*PTPRC*) genes showed an accurate expression of amplification in a specific tissue (figure 1, a&b).

Expression profiling of miRNA

RT-PCR amplification of the selective assessment of all 22 miRNA revealed differential expression in various tissues and spermatozoa in the yak. All the 22 miRNA were highly expressed in both sperm and testis (figure 2, a&b). Among them, the miR7d, miR23a, miR23b, miR19a, miR142 and miR143 clearly amplified in yak spermatozoa and testis with varied band intensity in fertile and subfertile yak sperm (figure 3, a&b). Although all 22 miRNAs were expressed in sperm and testis, some of these miRNA was ubiquitously expressed in other body tissues, so eventually; they were excluded for further analysis. Only the six miRNA that were specifically expressed in both fertile and subfertile sperm and testis were considered for the expression pattern with copy number analysis. To distinguish the pattern of expression, quantitative real-time PCR study was conducted to know the copy number variation of miRNA in fertile and subfertile yak spermatozoa.

The differences in expressions are observed with quantitative real-time PCR of six miRNA, namely miR7d, miR23a, miR23b, miR19a, miR142 and miR143 in fertile and subfertile. Comparatively, miR19a, miR142 and miR143 showed more expression in subfertile animal whereas miR7d, miR23a and miR23b showed high expression in fertile animal relative to subfertile one (figure 4)

Expression profiling of mse-tsRNA

Initially, two each of the mse-tsRNAs, namely mse-tsRNA-31, mse-tsRNA-32 of family I and mse-tsRNA-30, mse-tsRNA-34 of family II were used to observe their expression in normal yak sperm. All these mse-tsRNAs show an amplification pattern ($\sim$69–74-bp long) in fertile yak bull sperm (figure 5a). This finding is considered as first such report of expression in higher eukaryotes of distinct lineage other than rodents working with mse-tsRNAs. Subsequently, all these four mse-tsRNAs are used to testify expression

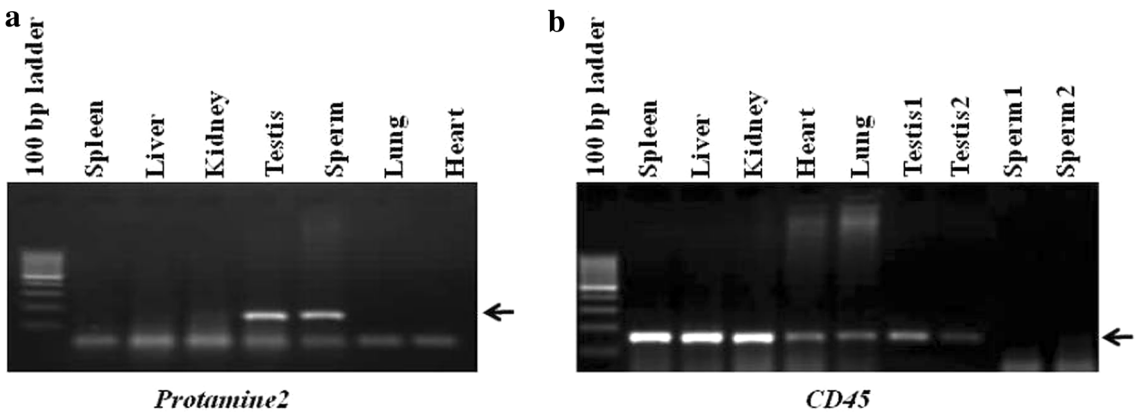

Figure 1. (a) Amplification of *Protamine2* gene (167 bp). (b) Amplification of *CD45* gene (147 bp).

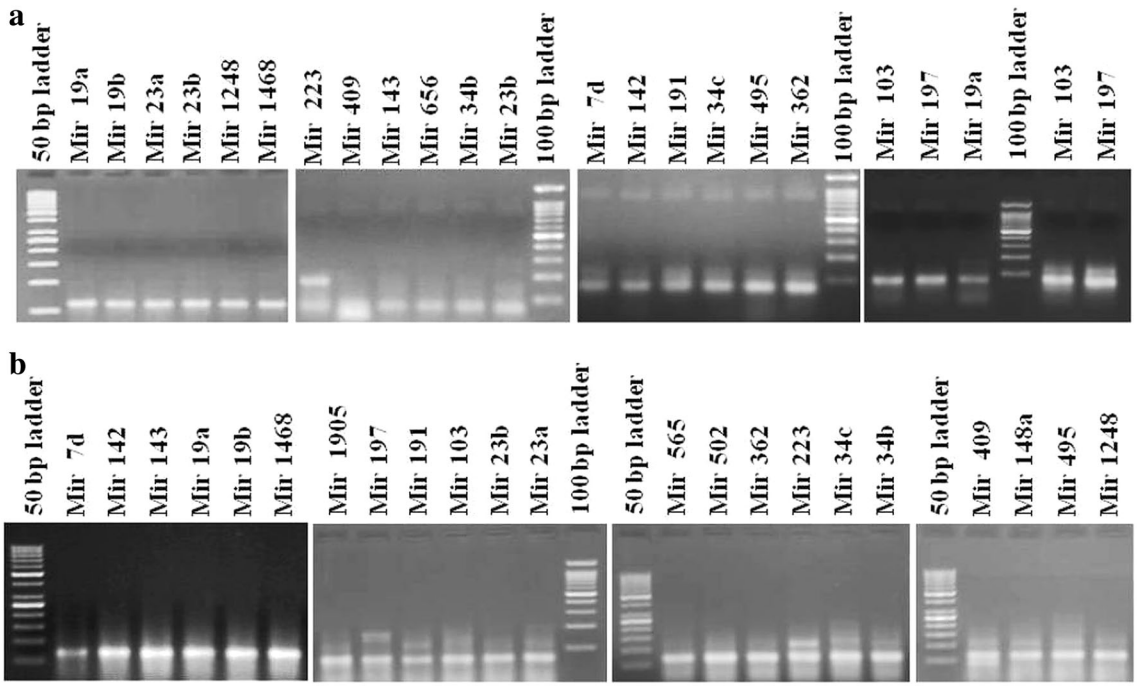

Figure 2. (a) Amplification of miRNA in yak sperm of size about 70 bp. (b) Amplification of miRNA in yak testis of size about 70 bp.

pattern in sperm RNA of fertile and subfertile yak. However, multiple amplification was also observed with the primer set apart from a requisite band size of around 69–74 bp.

Discussion

Fertility is a critical selection factor that determines the fate of the existence of any species. The rising incidence of fertility-related concerns in both humans and animals has drawn the attention for sperm derived studies in recent times. However, such a study possesses several challenges that started with the extraction of cellular components from tiny sperm cells. The high density, compactness and condensed structure of sperm attribute a major hindrance in isolation of nucleic acid. Moreover, the quantity of RNA in sperm usually resides low compared to other tissues (Goodrich *et al.* 2007). Therefore purity of mature sperm after ejaculation is a key to derive maximum RNA content. Here we made the first-ever successful attempt of RNA isolation from purified mature sperm of Arunachali yak by the conventional method, that has been corroborated by molecular biomarker *Protamine2* and *CD45* (figure 1, a&b). The transcriptional profiling of sperm RNAs previously been reported in many mammalian species such as human, bovine, swine, mouse, stallion and chicken, showed resemblance to our result

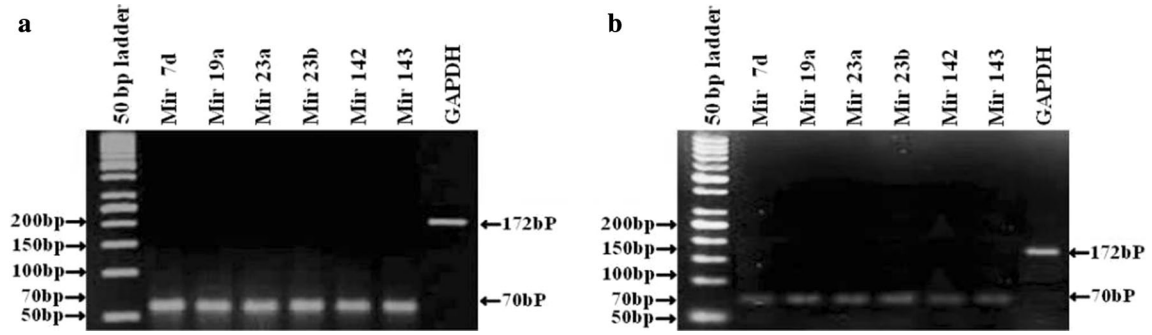

Figure 3. (a) miRNA expressed fertile yak sperm along with control GAPDH. (b) miRNA expressed subfertile yak sperm along with control GAPDH.

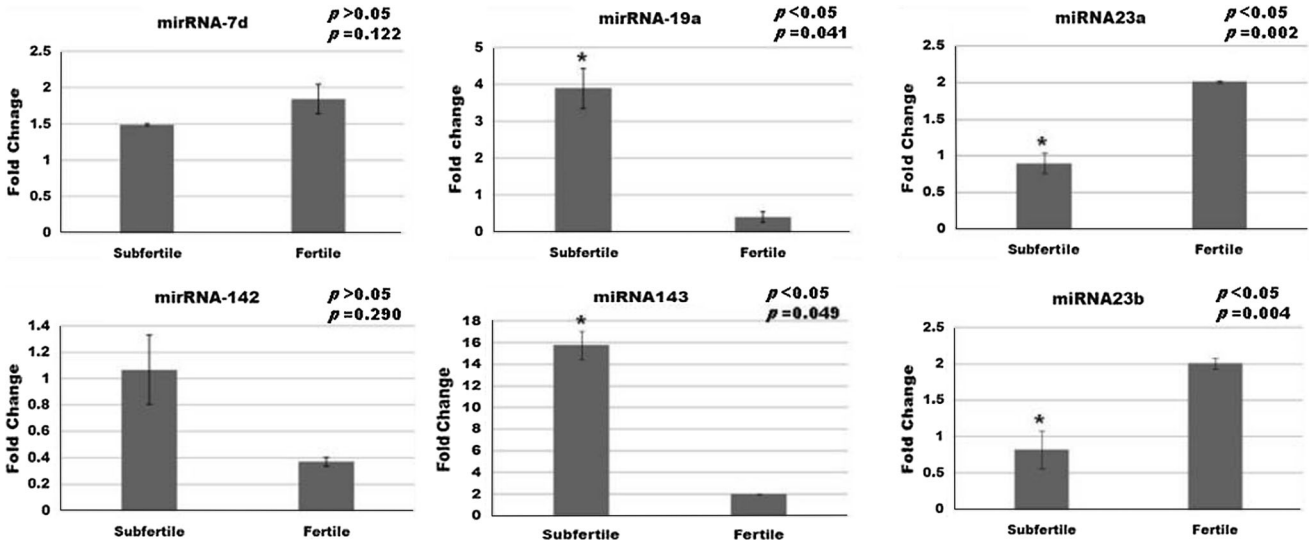

Figure 4. Expression pattern of six sperm enriched miRNA in fertile and subfertile animal.

(Gilbert *et al.* 2007; Goodrich *et al.* 2007; Yang *et al.* 2009; Das *et al.* 2010; Kawano *et al.* 2012; Shafeeque *et al.* 2014). This notion, in turn, reflects the conservative nature and diversity of mammalian RNA pool and their potential role in fertilization that hits plausible control in well-programmed spermatogenesis processes. Thus progressively admit that sperm RNAs that serve as noninvasive fertility biomarkers. However, downstream regulation of the genes for mapping the signalling pathway that controls male fertility in yak needs further study.

Our initial attempt of RNA isolation via both conventional trizol method and spin column-based method resulted in difference in RNA quality, reproduce better result with trizol. All the 22 miRNA were reportedly represented and linked with vivid function in various mammals including humans. Indeed, all the miRNA selected for this study has its link with fertility and subfertility in a higher eukaryote (Curry *et al.* 2011; Abu-Halima *et al.* 2014; Khazaie and Esfahani 2014; Salas-Huetos *et al.* 2016; Balasubramanian *et al.* 2019). The past study revealed the differential expression of miRNA-23 family (miR23a and miR 23b) in fertile and subfertile men found upregulated in oligoasthenozoospermic and asthenozoospermic spermatozoa happen to be involved in posttranslational modification of targeted genes. (Abu-Halima *et al.* 2013; Abu-Halima *et al.* 2019). However, the replication process and mode of functioning in an animal are not yet been determined. Our study on the yak is the first report on mir23 and their differential expression pattern in fertile and subfertile animals. The IL-5, a key allergic cytokine in asthmatic patients was reported to be regulated by miR-1248 (Panganiban *et al.* 2012) shown distinct expression in yak sperm and testis. The miRNA mir-19a-3p inhibits breast cancer progression and metastasis by inducing macrophage polarization through the downregulated expression of Fra-1 proto-oncogene (Yang *et al.* 2014). The involvement of this miRNA in altering the functioning of several gastric cancer-associated genes such as *PTEN*, *TNF-a*, *STAT3*, *VEGFA*, *E2F1*, *RB1*, *p21*, *MMP16*, *BCL2*, *CCND1*, *CDK6*, *CyclinD1* has also been reported via cell cycle progression and apoptosis (Xia *et al.* 2014). Whereas

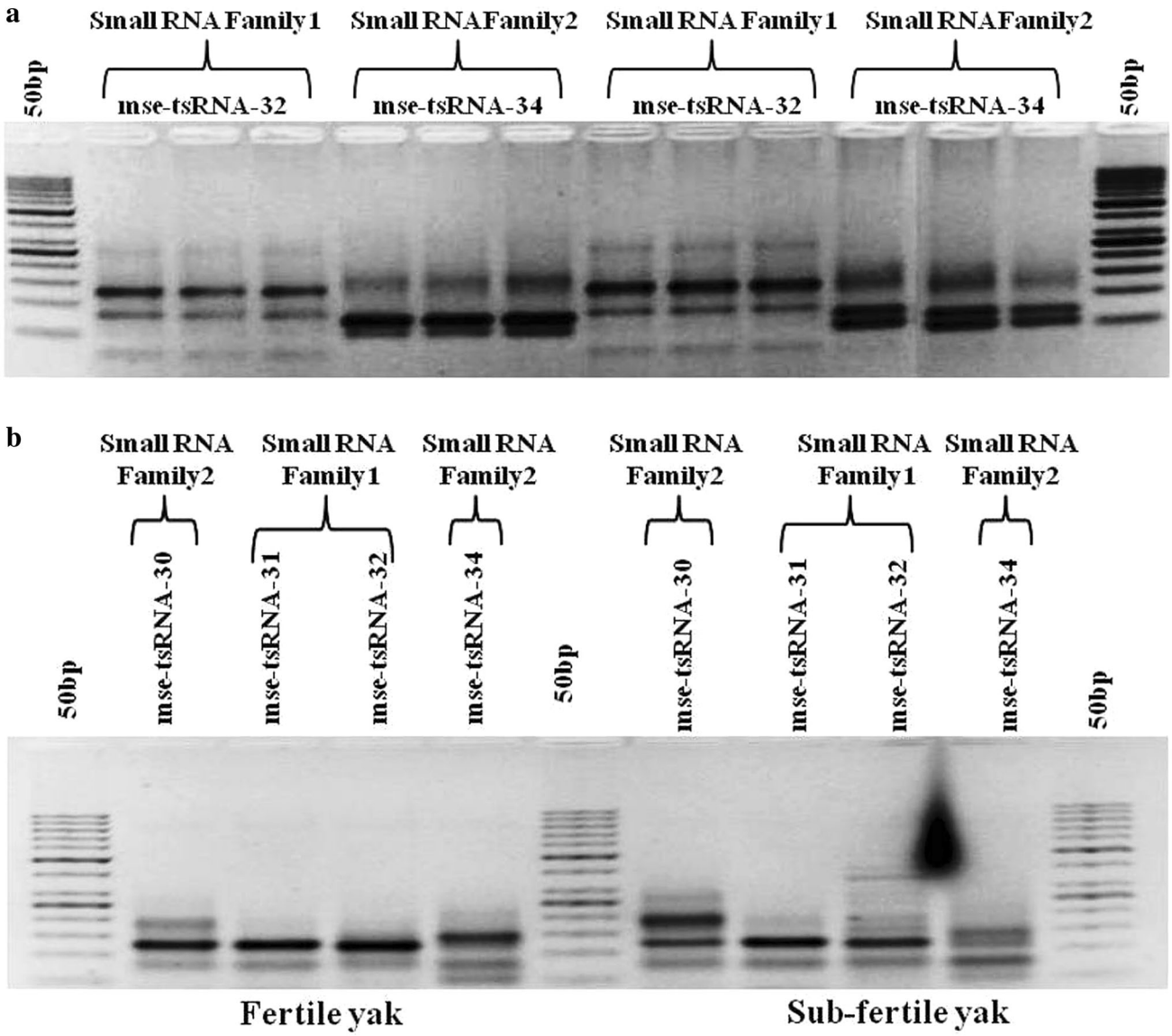

Figure 5. (a) Amplification of mes-tsRNA in sperm RNA of normal adult yak bull. (b) Amplification of different mes-tsRNA in sperm RNA of fertile and subfertile yak.

miR-19b along with miR-7a has shown elevated expression in idiopathic nonobstructive azoospermia infertile men compared to fertile control (Wu *et al.* 2012).

The miR23 family is enormously studied in the past for their indispensable role in male fertility. Here, in this study almost double the expression of both miR23a and miR23b has been found in fertile yak compared to that of subfertile one. A previous *in silico* study prediction stated the anonymous correlation of overexpression of miR23a/b and suppression of testicular active gene like *SPATA6* and *TEX15* in human (Abu-Halima *et al.* 2013). The *TEX15* genes encode a protein required for meiotic recombination in spermatocytes, whereas *SPATA6* is required for the formation of the sperm connecting piece during spermatogenesis. Low expression of miR23 family in subfertile yak might support the hypothesis that suppressing the essential crosstalk link gene required for healthy sperm production. On the other hand, miR-19a, miR-142 and miR-143 found highly expressed in subfertile yak compared to a fertile animal, which unlikely might suppress the fertility linked genes during the different stages of the life cycle. However, the precise pathway of monitoring and mechanism remains to be clarified for the development of subfertility in yak via target gene silencing.

The present finding revealed that the mse-tsRNA is biologically active in yak spermatozoa. The expression of mse-tsRNAs in the reproductive tissue of higher vertebrate like a yak indicates its dynamic nature. The previous studies on mse-tsRNA expression reported only on mouse reproductive tissue (Peng *et al.* 2012). Here we found a relatively constant

level of expression of family I and family II mse-tsRNAs in fertile and subfertile yak. The presence of these small tRNAs fragments in yak sperm that is supposed to be derived from tRNA cleavage indicates the production of diverse protein synthesis during the active stage of spermatogenesis. Indeed, a previous study on mice showed that mse-tsRNA is comprised of 67% of the total sperm small RNA reads (Peng *et al.* 2012). The result from the present study also revealed a unique similarity in the evolution of tRNA from lower to higher eukaryotes. It additionally supports the notions of conserved tRNA processing mechanisms in various classes of eukaryotic species from mouse to yak. However, since RT-PCR expression for fold change analysis could not be verified in the present study, it cannot conclusively indicate the actual number of genes expressed in the fertile and subfertile yak. From the present study, it is difficult to correlate the expression of mse-tsRNA in the sperm with subfertility of yak. Further quantitative analysis of this expression profile between fertile and subfertile may be elucidated the relative cause.

In conclusion, sperm-based miRNAs expression coherently indicates their correlation with spermatogenesis or factors positively impact on male fertility. However, the complete crosstalk mechanism among the molecular and genetic factors involving miRNA and male fertility needs profound study. Reportedly high expression of miR19a and miR143 in subfertile yak may indicate their suppressive role in the mechanism of maintaining sperm viability. The present study also revealed that a subset of miRNA is upregulated in the subfertile yak, whereas a few others are underexpressed in yak. Thus, indicates multiple roles of miRNA targeting the interrelated pathway and influences the controlling mechanism that categorically influences fertility and subfertility. The underlying mechanism of many of the miRNA is still largely unknown. Further analysis of these selected miRNA can unveil the substantial basis information in fertility-related dilemmas in the yak.

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