



Evaluation of the semen swim-up method for bovine sperm RNA extraction

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ABSTRACT. Isolation of high-quality RNA is important for assessing sperm gene expression, and semen purification methods may affect the integrity of the isolated RNA. This study evaluated the effectiveness of the sperm swim-up method for seminal RNA isolation. Frozen semen samples in straws from three bulls of proven fertility were purified by the swim-up method. RNA extraction was carried out using the E.Z.N.A.[™] Total RNA kit II, with non-swim-up sperm as a control. Total sperm RNA was analyzed by UV spectrophotometry, reverse transcription polymerase chain reaction (RT-PCR), and agarose gel electrophoresis, and expression of the sex-determining region on the Y chromosome (*SRY*), leptin (*LEP*), and ribosomal protein subunit 23 (*RPS23*) genes, were determined. *18S RNA* was used as a positive control. Fewer somatic cells were found in sperm swim-up samples than in the non-swim-up counterparts (0×10^3 vs $17.33 \pm 2.52 \times 10^3$ sperm, $P < 0.05$). In addition, high-quality RNA was obtained in about 2 h, with no significant difference between groups. Interestingly, the yields of RNA fragments containing ≥ 200 nucleotides were significantly reduced in sperm swim-up samples ($0.92 \pm 0.41 \times 10^7$ sperm) compared with the non-swim-up samples ($1.36 \pm 0.33 \times 10^7$ sperm, $P < 0.05$). After RT-PCR, clear bands representing *SRY*, *LEP*, and *RPS23* in sperm cDNA were observed on agarose gel electrophoresis. Finally, no bands

corresponding to *18S RNA* were found in RNA samples from the sperm swim-up group. Our findings suggest that small amounts of sperm RNA can be efficiently extracted from frozen straw semen samples using the swim-up technique.

Key words: Bovine; Sperm; Total RNA extraction; Swim-up

INTRODUCTION

The existence of sperm RNA was originally questioned. Indeed, assuming that transcription ceases in the round spermatid stage, with the cytoplasm destined to be expunged and thus void of the components necessary for translational activity, any remaining male haploid RNA would be inconsequential (Jodar et al., 2013). This notion corroborated the observed heterogeneity of the ejaculate, the presence of somatic cell contaminants that accounted for the majority of large RNAs in most samples, and the absence of intact ribosomal RNAs (Jodar et al., 2013; Sandler et al., 2013). Most of the cytoplasm, including the RNA components, is depleted as a cytoplasmic droplet or residual body (Fischer et al., 2005), which is phagocytosed by the Sertoli cells. Evidence for the presence of RNA in sperm was provided when several research teams independently identified specific sperm RNAs in mammals, including rat (Morales et al., 1989), mouse (Koga et al., 2000), and human (Kumar et al., 1993; Miller, 2000; Wykes et al., 2000), through RT-PCR and *in situ* hybridization. To date, human sperm transcripts are the best characterized amongst all mammals. RNA profiling of human spermatozoa was initially attempted using cDNA cloning and sequencing (Miller et al., 1999), following which, specific RT-PCR methods were used (Paradisi et al., 2000; Motiei et al., 2013). However, the latter methods only survey a small fraction of all potential transcripts. The first comprehensive sperm RNA profiles obtained by microarrays suggested that human spermatozoa contain about 5000 different coding transcripts (Miller and Ostermeier, 2006).

Isolation of high-quality RNA is essential for assessing sperm gene expression. Therefore, nonviable sperm, cellular debris, and seminal plasma should ideally be removed from semen samples prior to their use or preservation. Indeed these components are sources of non-sperm RNA generation, which impairs sperm RNA purity. The removal of somatic cells ensures sperm RNA purity, as these can contribute to a substantial proportion of the isolated RNAs, and somatic cell lysis buffer containing both Sodium dodecyl sulfate (SDS) and Triton has been widely used for sperm cell purification (Johnson et al., 2011; Miller et al., 2005). This method effectively leaves the most robust sperm cells intact, but also tends to solubilize sperm-membrane structures. Another method used for sperm cell purification is gradient centrifugation using Percoll or clinical-grade reagents like PureSperm, which is an isotonic salt solution containing silane-coated silica particles (Mao et al., 2013). In fact, for sperm RNA extraction, it is very important that seminal round cells, such as immature sperm cells and white blood cells, are removed. There is a trend towards increased sperm count, motility, and pregnancy rate when the swim-up procedure is used (Somfai et al., 2002; Jameel, 2008). Preparation techniques, such as density gradient centrifugation and the simplified single layer centrifugation technique, have considerable potential for the separation of sperm from seminal plasma, which carries cells, cellular debris, and reactive oxygen species, as well as pathogens (Morrell, 2006; Parrish and Foote, 1987).

Sperm swim-up is a simple and effective method used to remove seminal plasma and

cellular debris from sperm samples (Sieme and Oldenhof, 2015). Therefore, this study aimed to evaluate the effectiveness of the sperm swim-up method in seminal RNA isolation. Mature bovine sperm samples from fertile bulls were used to assess the qualitative and quantitative effects of the swim-up purification method on sperm RNA extraction. We found that small amounts of sperm RNA can be efficiently extracted from frozen straw semen samples using this method.

MATERIAL AND METHODS

Preparation of sperm

The present study was approved by the ethics committee of Tarim University, Alar, China. Semen samples were collected using artificial vaginas from three bulls with proven fertility, and transported to the laboratory where they were resuspended in semen media (TriXcell® extenders, IVM, France). Semen samples were packed in 0.25-mL straws (5×10^7 sperm per straw, from the same bull) and cooled to 4°C for 1.5 h, to -10°C for 40 min, to -100°C for 70 s, and to -140°C for 30 s using a control rate freezer (IVM, France). Next, straws were transferred to liquid nitrogen for storage until use. Post-thaw sperm motility, progressive forward motility per straw, and pregnancy rate following artificial insemination complied with commercial standards (Januskauskas et al., 1996). During thawing, straws were gently shaken in a water bath at 37°C, and semen samples were processed as described previously (Parrish and Foote, 1987; Somfai et al., 2002). Briefly, a total of six straws from three bulls were defrosted in a water bath at 38°C. Then, about 4×10^7 sperm (two straws, 0.5 mL) per bull were overlaid with pre-warmed 1.5 mL Dulbecco's Modified Eagle's Medium (Sigma), submitted to centrifugation at 360 g for 10 min, and incubated at 37°C for 45 min in the presence of 5% CO₂. The supernatants (about 1 mL) were centrifuged to obtain motile sperm. Sperm smears were made and contamination by leukocytes or other cellular materials was assessed under a microscope following Wright staining (Nikon, Japan). The number of round cells and sperm density were determined with a hemocytometer. The swim-up experiment was repeated six times. A sterile Pasteur pipette was used to remove the supernatant containing actively motile sperms post swim-up. The specimen was kept in liquid nitrogen until the time of RNA extraction.

Sperm RNA extraction

Total RNA was isolated from sperm using the E.Z.N.A.™ Total RNA Kit II (Omega, USA) as directed by the manufacturer. Briefly, sperm suspensions were centrifuged at 1500 g for 5 min, and 1 mL lysis buffer per 10^7 sperm was added to the resulting sperm sediment. After mixing by vortex for 2 min, samples were allowed to stand for 3 min at room temperature. Then, 200 µL chloroform per 1 mL mixture was added, and the samples were mixed by vortexing. Next, the sample was placed on ice for 10 min and then centrifuged at 12,000 g at 4°C for 15 min. The supernatant was then mixed with 0.5X volume pure ethanol, and vortexed for 15 s at room temperature. The mixture was loaded onto a HiBindRNA column. After centrifugation at 10,000 g at room temperature for 1 min, the column was washed with 600 µL RNA Wash buffer I and then treated with 75 µL DNase I digestion reaction mix at room temperature for 15 min. This was followed by a washing step with 600 µL RNA Wash buffer I. Finally, RNA

was collected in 20 μ L RNase-free water (70°C) following centrifugation at 12,000 g for 1 min, and the resultant samples were stored in liquid nitrogen. RNA from non-swim-up sperm samples was extracted and stored in parallel. The OD_{260/280} ratio and concentration of total RNA were measured using a NanoDrop 2000 (Thermo Scientific, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

All RNA samples were reverse transcribed using a commercial kit (Gibco BRL, USA). Briefly, a 20- μ L reaction mixture containing 2.5 mM random hexamers and 200 U superscript reverse transcriptase was incubated for 1 h at 42°C. Reverse transcriptase was denatured at 99°C for 5 min, and cDNA samples were stored at -20°C until use.

Ribosomal protein subunit 23 (*RPS23*) (Gur and Breitbart, 2006), sex-determining region on the Y chromosome (*SRY*) (Modi et al., 2005), *18S RNA* (Ostermeier et al., 2005), and leptin (*LEP*) (Nikbakht et al., 2010) gene-specific primers were designed using Oligo 6 software based on the cDNA and oligo mRNA sequences (*Bos taurus 18S RNA* gene, cDNA; *B. taurus RPS23*, mRNA; *B. taurus SRY*, mRNA; *B. taurus LEP*, mRNA), and were commercially synthesized (Table 1).

Table 1. Specific primers used to amplify the *RPS23*, *SRY*, *18S RNA*, and *LEP* genes.

Gene	Sequence (5'-3')	T _m (°C)	Length (bp)	Accession No.
<i>18S RNA</i>	5'-CGCAGGCTCCACTCTGGTGG-3'	62	388	BG467091
	5'-CGAGCCGCCTGGATACCGCAG-3'			
<i>RPS23</i>	5'-AGTGTCCGGTCTTCGTA-3'	54	258	NM_001034690
	5'-CATCATTGGGAACAAAAGCA-3'			
<i>SRY</i>	5'-CTCAGACATCAGCAAGCAGC-3'	60	405	NM_001014385.1
	5'-TTGTTACAGGAAAGTCCGC-3'			
<i>LEP</i>	5'-ACAGAGGGTCACTGGTTGG-3'	60	627	NM_173928.2
	5'-CAGTGAGAGGGAGCTGGAAC-3'			

18S RNA, *RPS23*, *SRY*, and *LEP* transcripts were amplified by PCR using cDNA as a template. The primer pairs used spanned 405, 388, 258, and 627 bp *SRY*, *18S RNA*, *RPS23*, and *LEP* gene sequences, respectively. For PCR, 4 μ L cDNA was amplified in a final volume of 25 μ L containing 10 mM Tris/HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 30 pmol each primer, and 2 U Taq polymerase (Gibco BRL). Amplifications were carried out using a PCR instrument (MJ Research, MA, USA), with the following cycling conditions: 30 cycles of 95°C for 1 min; 62°C (*18S RNA*), 60°C (*SRY* and *LEP*) or 54°C (*RPS23*) for 1 min; 72°C for 1 min. Final extension was performed at 72°C for 5 min. A 5- μ L aliquot of each PCR product was separated by 2% agarose gel electrophoresis, stained with ethidium bromide, and observed under a UV transilluminator. The specificity of the bands was also assessed by sequencing the PCR products. Amplification of *18S RNA* using the cDNA template from non-swim-up samples served as a positive control, while that from swim-up samples served as a negative control.

Statistical analysis

Statistical analyses were carried out using the SPSS software (Version 18, SPSS Inc., USA). Means were compared by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

Sperm and RNA isolation

The sperm count, number of round cells, total RNA concentration, and $OD_{260/280}$ ratio of each sample are shown in Table 2. Except value of OD_{260}/OD_{280} , a significant difference was observed between non-swim-up and swim-up sperm groups. Significantly fewer sperm were found in swim-up samples ($31.49 \pm 4.14 \times 10^7$ sperm) compared with non-swim-up samples ($5.12 \pm 0.48 \times 10^7$ sperm) ($P < 0.05$). As shown in Figures 1 and 2, Wright staining revealed a significant difference in the number of round cells in sperm from non-swim-up as compared with sperm from swim-up groups ($17.33 \pm 2.52 \times 10^3$ sperm vs 0×10^3 sperm) ($P < 0.05$). In addition, significantly higher levels of total RNA were obtained from sperm in non-swim-up samples ($1.36 \pm 0.33 \times 10^7$ sperm) as compared with sperm in swim-up samples ($0.92 \pm 0.41 \times 10^7$ sperm). However, sperm swim-up status had no effect on the $OD_{260/280}$ ratio of RNA (non-swim-up: 1.82 ± 0.07 , swim-up: 1.80 ± 0.09 , $P > 0.05$). These data indicated that RNA from both sample types contained very low levels of protein, phenol, and guanidine salt contaminants. In addition, they suggest that RNA from bovine sperm obtained using both methods was intact.

Table 2 Sperm counts, round cell amounts, total RNA quantities, and $OD_{260/280}$ ratios pre- and post- sperm swim-up.

Type	Sperm count (10^7 sperm)	Round cell count (10^3 sperm)	Total RNA ($\times 10^7$ sperm)	OD_{260}/OD_{280}
Sperm non-swim-up (N = 6)	31.49 ± 4.14^a	17.33 ± 2.52^a	1.36 ± 0.33^a	1.82 ± 0.07^a
Sperm swim-up (N = 6)	5.12 ± 0.48^b	0^b	0.92 ± 0.41^b	1.80 ± 0.09^a

^{a,b}Values within columns with different superscripts are significantly different ($P < 0.05$).



Figure 1. Wright staining of semen from the non-swim-up group (400X).

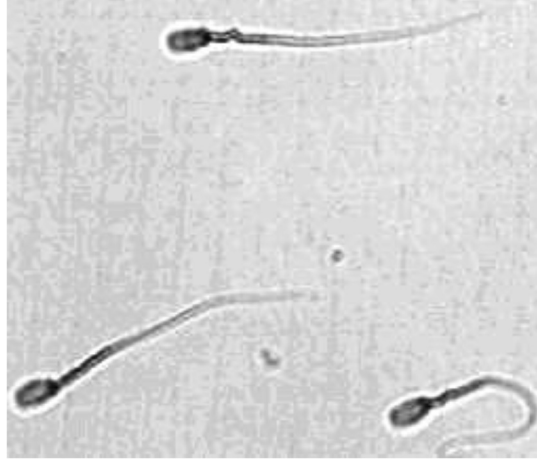


Figure 2. Wright staining of semen from the swim-up group (400X).

Integrity of bovine sperm RNA

Electrophoretic analysis of the amplified RT-PCR products is shown in Figure 3. Sequencing data revealed that *RPS23*, *SRY*, and *LEP* were present in RNA extracted from swim-up sperm, while *18S RNA* was not detected; conversely, *18S RNA* was detected in RNA non-swim-up sperm. The identity of the bands was demonstrated by sequencing, which revealed that the PCR products shared 100% homology with the indicated bovine genes (data not shown). These data suggest that RT-PCR based on these genes is reliable for the analysis of RNA from purified bull sperm.

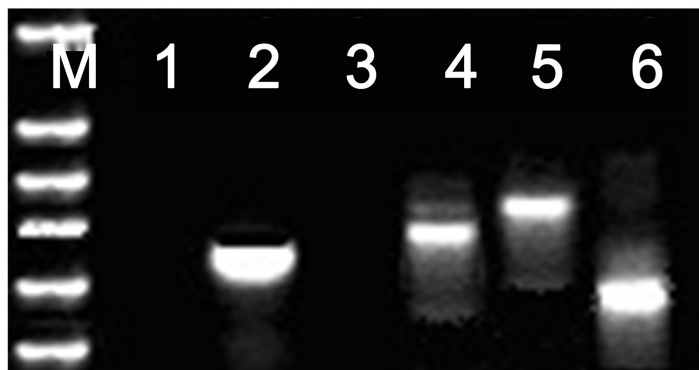


Figure 3. Agarose gel electropherogram after amplification of the *RPS23* *18S rRNA*, *SRY*, and *LEP* genes from bovine sperm. Lane M = TaKaRa DL-2000 DNA marker; lane 1 = *18S rRNA* negative control for a sample contaminated by somatic cells; lane 2 = *18S rRNA* (positive control sample contaminated by somatic cells); lane 3 = negative control (no template of the sperm non-swim-up cDNA); lane 4 = *SRY*; lane 5 = *LEP*; lane 6 = *RPS23*.

DISCUSSION

To optimize the extraction of sperm RNA from bull specimens, it may be necessary to modify the extraction method to improve efficiency. Here, a yield of 0.92×10^7 total sperm RNA and an $OD_{260/280}$ ratio of 1.80 were obtained, indicating that this was an efficient RNA extraction system using bovine frozen straw sperm swim-up samples.

In this study sperm cells from low density somatic cells were obtained during swim-up combined with a simplified single layer centrifugation technique: 360 g centrifugation for 10 min was adopted in the standard swim-up technique, and a significant difference in round cell counts was observed between non-swim-up and swim-up groups ($17.33 \pm 2.52 \times 10^3$ sperm vs 0×10^3 sperm, respectively). In addition, no contamination by somatic cells was found in swim-up sperm samples. The results obtained using frozen bull semen indicates that the swim-up technique can yield high purity sperm cells that are suitable for total RNA extraction. These findings suggest that the sperm swim-up technique is an easy, reliable, and effective method of processing sperm for subsequent RNA extraction.

Interestingly, electrophoresis revealed that *18S RNA* was absent from sperm RNA samples, which is consistent with previous reports (Miller and Ostermeier, 2006; Ostermeier et al., 2002). Indeed, it is generally accepted that mature spermatozoa are not translationally active, and rRNAs that are essential for ribosome assembly may not be available. Accordingly, no *18S RNA* was detected by RT-PCR in sperm swim-up samples, which is consistent with previous reports showing that rRNAs are not present in sperm.

Little is known about the fate of rRNAs during and after spermiogenesis. These transcripts are the most abundant in all cell types, yet until now, were assumed to be absent from mature male gametes. Instead of exhibiting defined 18S and 28S rRNA peaks, electrophoretic analysis of sperm RNAs showed abundant short-length transcripts (Jodar et al., 2013; Sendler et al., 2013). Although intact mRNAs are detected in this pool of transcripts, full-length 28S and 18S rRNAs are not (Ostermeier et al., 2005). The large reduction in cytoplasmic volume that accompanies morphogenesis was thought to explain why rRNAs could not be found in sperm; indeed, expulsion of the translational machinery during sperm maturation is supported by detection of RNA and ribosomal proteins in the residual body/cytoplasmic droplet (Miller and Ostermeier, 2006). Taken together, these findings led to the assumption that spermatozoa lack rRNAs. In the present study, amplification of *18S RNA* was carried out using sperm non-swim-up and swim-up cDNA samples as negative and positive controls, respectively, and band specificity was confirmed by sequencing. Although the sperm used for RNA extraction were motile and collected after the swim-up procedure to avoid somatic cell contamination, the presence of leukocyte RNA in sperm RNA samples was also ruled out by the absence of *18S RNA*, indicating that the transcripts detected in sperm RNA by RT-PCR did not result from somatic cell contamination. Therefore, swim-up is an effective way of removing cellular debris from sperm samples.

From spermiogenesis to round spermatid development, most of the cytoplasm, including the RNA component, is depleted as a cytoplasmic droplet or residual body that is phagocytosed by Sertoli cells (Johnson et al., 2011). This results in sperm cells that lack intact rRNAs but contain multiple short-length transcripts (Miller et al., 2005; Ostermeier et al., 2002). Although intact mRNAs were detected in this pool of transcripts, full-length 28S and 18S rRNAs were not present (Ostermeier et al., 2005). Those authors prepared human spermatic RNA samples by heating the lysis buffer provided by Qiagen; this might explain the

low RNA yields observed (Johnson et al., 2011).

A few limitations of the present study should be noted. First, the sample size was relatively limited. In addition, most data available in the literature have been generated using human samples, while bull specimens were used here. Therefore, comparisons might not be completely accurate, and equivalent studies with human samples should be carried out, providing that ethics requirements are met, to confirm our findings.

In conclusion, total RNA of high quality and relatively high yield, with little contamination by somatic cells, protein, phenol, or guanidine salt, was obtained from frozen straw semen samples using the swim-up technique, illustrating the efficiency of this method.

Conflicts of interest

The authors declare that they have no conflict of interest.

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