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COMPARISON OF THE SEMEN SWIM-UP AND SOMATIC CELL LYSIS PROCEDURES FOR RAM SPERM RNA EXTRACTION

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ABSTRACT

Male infertility is an important aspect of animal reproduction, which has a high economic impact on the livestock industry. From rapidly increasing list of different sperm fertility biomarkers, the sperm RNA could serve as a promising diagnostic tool to assess male fertility or could have prognostic value for fertilization and embryo development. The aim of this preliminary study was to compare swim-up and somatic cell lysis buffer (SCLB) procedures for extraction of high-quality ram sperm RNA suitable for downstream molecular biology applications.
A modified TRI REAGENT RT procedure with glycogen and lysis step at 65 °C was carried out in order to extract total RNA. Spectrophotometric measurement of quality and quantity of extracted RNA showed A260/280 ratio 1.8 – 1.9, indicating the absence of contaminants and the amount of RNA 24 ± 3.9 µg (unpurified sperm), 0.9 ± 0.11 µg (swim-up) and 1.5 ± 0.2 µg (SCLB). Sperm RNA quality was further validated by RT-qPCR using primers for WBP2NL and MKRN1 genes. The CD18 and CDH1 markers for leucocytes and endothelial cells, respectively, have been used to check a successfull removal of somatic cells from ram sperm by both procedures. Unlike comparable relative amount of WBP2NL and MKRN1 transcripts among unpurified and swim-up or SCLB purified sperm RNA samples, relative amounts of CD18 and CDH1 transcripts were significantly lower in purified sperm RNA samples ($P < 0.001$), confirming an effective removal of leucocytes and endothelial cells from sperm by both purification techniques. Further investigations could reveal a potential of sperm RNA as a novel biomarker and promising diagnostic tool to assess ram fertility.

Key words: ram semen; swim-up; somatic cell lysis buffer; total RNA extraction

INTRODUCTION

Male infertility is an important aspect of human and animal reproduction, characterized by a diminished or absent capacity to produce spermatozoa capable of fertilizing the oocyte and supporting embryonic and fetal development. Semen analysis is a routine diagnostic tool used to assess male fertility (Hwang *et al*., 2011). Conventional, light microscopy-based semen analysis provides a useful baseline of information on sperm count, motility and morphology of semen samples, however, a limited degree of correlation is usually observed between semen parameters and the actual fertility of an individual. Several advanced methods for evaluation of sperm quality have been developed, like computer-assisted semen analysis (CASA), flow cytometry (FC), sperm penetration assay, quantification of reactive oxygen species, sperm DNA damage assay or biomarker-based sperm quality testing (Sutovsky *et al*., 2015). Among rapidly increasing list of different sperm fertility

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biomarkers, the sperm RNA could serve as a promising diagnostic tool to assess male fertility (Lalancette *et al*., 2009) or could have prognostic value for fertilization and embryo development (Boerke *et al*., 2007). RNA in mature sperm cells was first assumed to be lost or degraded during spermiogenesis. However, a lot of studies have shown the presence of RNA in sperm using RT-PCR (Miller *et al*., 1999), differential display method (Geisinger *et al*., 1996), targeted microarrays (Liu *et al*., 2012) and subtractive hybridization experiments (Chen *et al*., 2014). Studies exploring the complexity of spermatozoal RNA population indicated the presence of rRNA (ribosomal ribonucleic acid), mRNA (messenger ribonucleic acid), sncRNAs (small non-coding RNAs) including microRNA and large non-coding RNAs (Kawano *et al*., 2012). A precise biological function remains to be assigned for a majority of the thousands of RNA transcripts carried by sperm. The utility of sperm RNA as markers for infertility has been explored (Miller, 2000), where differences in transcript levels in sperm of different motility (Lambard *et al*., 2004), as well as between normal and abnormal sperm samples have been reported (Platts *et al*., 2007). Moreover, sperm RNA may also play a role in early embryo development (Ostermeier *et al*., 2004). The study of sperm RNAs has been challenging because of the difficulty associated with sperm RNA isolation. Several sperm RNA isolation protocols have been developed showing highly variable sperm RNA yield due not only to the different approaches used but also to the heterogeneity of RNA within an individual sperm sample (Lalancette *et al*., 2009). In addition, isolating RNAs is variable among different species due to differences in sperm morphology and chromatin packaging (Schuster *et al*., 2016). Since semen contains somatic cells including leukocytes and epithelial cells along with spermatozoa, somatic cell removal is essential to avoid contamination of the sperm transcripts (Jodar *et al*., 2013), as the amount of RNA per spermatozoa is on the order of femtograms, rather than picograms as in somatic cells (Krawetz, 2005).

Effective methods for isolation of high-quality human sperm RNA (Goodrich *et al*., 2013) as well as from different animal species, like mouse, rat, cattle, horse, rabbit, chicken and others, have been published (Schuster *et al*., 2016). Most of them utilized swim-up procedure, gradient centrifugation (using Percoll, PureSperm, etc.) or somatic cell lysis buffer (SCLB) technique based on a detergent mixture to purify spermatozoa from contaminating leukocytes and epithelial cells.

The aim of this preliminary study was to compare swim-up and SCLB techniques for extraction of high-quality ram sperm RNA suitable for downstream applications, e.g. gene expression profiling, as a novel tool to evaluate male fertility in endangered national Wallachian sheep breed in Slovakia.

MATERIAL AND METHODS

Animals and semen collection

Two sexually mature and clinically healthy Wallachian rams aged 3–4 years were used in this study. Semen samples were collected by an electro-ejaculation, as described previously (Baláži *et al*., 2020) and immediately transferred to the laboratory for the subsequent processing.

Semen purification

The concentration of spermatozoa was measured by an Automatic Cell Counter (NanoEnTek, Korea) according to Baláži *et al*. (2020) and semen samples were diluted in a sterile phosphate buffered saline (PBS) to final concentration of 10^8/mL. One mililiter semen aliquots were used for purification by swim-up procedure and SCLB method or used directly for RNA extraction. For purification by a swim-up procedure, 1 mL of diluted semen was divided by 100 µL underneath of 1 mL prewarmed SpermTALP medium (37 °C) in 5 ml flat bottom tubes. Following incubation for 1 hour at 37 °C, approximately 800 µL of the solution were carefully aspirated from the top of each tube and blended together. Spermatozoa were sedimented by centrifugation at 200 x g for 15 min at 4 °C, gently resuspended in 1 mL of ice-cold sterile PBS to remove residual medium and then centrifuged as in the previous step.

To eliminate contaminating somatic cells, 1 mL of diluted semen was incubated with SCLB (0.1 % SDS, 0.5 % Triton X-100 in nuclease-free water). Briefly, following sperm centrifugation at 200 x g for 15 min at 4 °C sperm pellet was resuspended in 4 mL of SCLB and incubated on ice for 30 min with intermittent mixing. Further, the incubation was extended until microscopic inspection confirmed the absence of somatic cells in small volume

sampled from sperm suspension. Finally, the sperm suspension was centrifuged at 400 x g for 15 min at 4 °C and the pelleted spermatozoa were washed twice with 1 ml of ice-cold sterile PBS and finally centrifuged as in the previous step.

Sperm RNA extraction

Total RNA was extracted from sperm using modified TRI REAGENT RT (MRC, USA) method. The sperm pellet (unpurified and purified by swim-up or SCLB) was suspended in 1 mL of TRI REAGENT RT, containing 1 µL of glycogen (20 µg/µL, Invitrogen, USA), incubated on a dry bath at 65 °C for 10 min and passed through 26-gauge needle using 2 mL syringe until a smooth flow of the suspension was observed. To this suspension 50 µL of bromoanisole was added. Microtubes containing the suspension were vortexed for 20 seconds, incubated at room temperature for 2 min and then centrifuged at 12000 x g for 15 min at 4 °C. Following centrifugation, the upper aqueous phase containing RNA was transferred to a fresh tube and RNA was precipitated by adding 0.5 mL of chilled isopropanol. Further, the pellet was washed twice with 1 mL of 75 % ethanol, the RNA pellet was air-dried for 5 – 10 min and dissolved in RNase-free water. RNA quantification was done spectrophotometrically using BioSpec-nano (Shimadzu, Japan) at 260 nm, A260/280 ratio (approx. 1.8 – 1.9) for RNA samples calculated by BioSpec-nano was also considered to assess quality of the RNA preparation. RNA samples were processed immediately for cDNA synthesis and stored at -80 °C.

RT-qPCR

All RNA samples were treated with the dsDNase (Thermo Fisher Scientific, Waltham, USA) before reverse transcription. The first strand cDNA synthesis and quantitative real-time PCR (qPCR) were performed as described previously (Kulikova *et al*., 2019) with minor modifications. Briefly, 50 ng of total RNA from each sample were used for the first strand cDNA synthesis and qPCR contained 5 pmol of each corresponding primers for tested and GAPDHS reference genes (Table 1). The amplification protocol was as follows: an initial denaturation and activation of Taq DNA polymerase at 95 °C for 7 min followed by 45 cycles of denaturation at 95 °C for 10 s, annealing – at 60 °C for 10 s and 72 °C for 10 s. A relative quantification of WBP2 N-terminal like (WBP2NL), makorin ring finger protein 1 (MKRN1), E-cadherin (CDH1) and CD18 gene transcripts to reference spermatogenic glyceraldehyde-3-phosphate dehydrogenase (GAPDHS) was calculated according to Pfaffl (2001).

Statistical analysis

Data obtained from analyses were evaluated using the GraphPad Prism version 9.1.0 for Windows (GraphPad Software, USA) with two-way ANOVA (Tukey's test). Results are expressed as the means ± standard deviation (SD). *P*-values at $P < 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

Gene expression profiling of mammalian sperm has been proposed as a novel non-invasive tool to evaluate male fertility (Krawetz, 2005). Since the accuracy of functional genomics studies strongly depends on RNA quality and there are inter-species sperm differences, sperm RNA isolation protocols must be adjusted to individual species. Semen contains somatic cells, including leukocytes and epithelial cells, along with spermatozoa, therefore,

Table 1. Genes, primer sequences and size of PCR products

Gene	Forward primer [5´- 3´]	Reverse primer [5´- 3´]	PCR product size [bp]
WBP2NL	ATGGCACAAAGAAAGGAACG	TGGTTGTTCAATGGTGCAGT	134
MKRN1	AATGCCATCGAGTTTGTTCC	TTGCTCCTTCTCCGTGTCTT	111
CD18	CAGACGATGGGTTCCACTTT	TTGCTTTCTGCCAGTTTGTG	157
CDH1	CCGTGAGAGTTTTCCCACAT	CATTGGTGTCCAGGACTGTG	107
GAPDHS	TAAGAAGGTTCGGGAGCTGA	ATGGGTCGTTCACTGCTACC	113

somatic cell removal is essential to avoid contamination of the sperm transcripts (Jodar *et al*., 2013).

The aim of this study was to compare two commonly used methods eliminating somatic cells from ram semen samples, sperm swim-up and lysis of somatic cells by a mixture of detergents (SCLB). Both methods have been widely used for sperm cell purification from many animal species, as well as from human (Sieme and Oldenhof, 2015; Ostermeier *et al*., 2005). In our experiment, microscopic inspection confirmed the absence of somatic cells in small sperm samples subjected to either swim-up or SCLB purification. Thereafter, a modified TRI REAGENT RT procedure, including the addition of glycogen, homogenization through 26 gauge needle and lysis step at 65 °C, was carried out in order to extract total RNA suitable for downstream applications, such as RT-qPCR. The glycogen molecule binds to RNA as soon as they are available in solution and, therefore, enhances the yield of RNA recovery. According to spectrophotometric measurement of quantity (260 nm) and purity (A260/280 ratio) of extracted RNA, the amount of RNA was 24 ± 3.9 µg for unpurified sperm, 0.9 ± 0.11 µg for swim-up and 1.5 ± 0.2 µg for SCLB. All RNA samples showed A260/280 ratio 1.8 – 1.9, indicating absence of phenols, proteins or other contaminants that absorb at or near 280 nm.

Sperm RNA quality was further validated by RT-qPCR using primers for WBP2NL gene encoding testis-specific protein also known as post-acrosomal sheath WW domain-binding protein (PAWP), involved in fertilization in humans, mice and bulls (Kennedy *et al*., 2014) and MKRN1 that has been identified as a gene for E3 ubiquitin ligase in mammals (Kim *et al*., 2005) involved in post-transcriptional control of gene expression during gametogenesis and early development. Recently MKRN2, a member of makorins, was found to be essential for male fertility in mice (Qian *et al*., 2016). The CD18 markers for leucocytes (Vašíček at al., 2019) and CDH1 for endothelial cells (Bianchi *et al*., 2018) have been used to check a successful removal of contaminating somatic cells from ram sperm by swim-up or SCLB procedure. The data obtained from RT-qPCR analysis are shown on Figure 1.

A transcripts of all genes were detected in unpurified, as well as in purified ram sperm. Unlike more or less comparable relative amount of WBP2NL and MKRN1 transcripts among unpurified and swim-up or SCLB purified sperm RNA samples with exception of MKRN1 in SCLB vs. unpurified sperm showing statistically significant difference

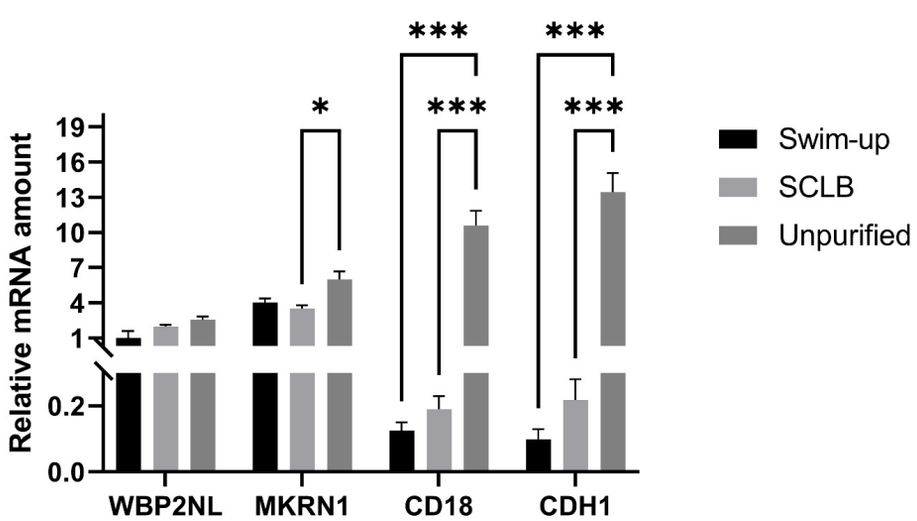

Figure 1. Relative mRNA amount (fold change) of WBP2NL, MKRN1, CD18 and CDH1 genes in purified/unpurified ram sperm samples normalized to GAPDHS reference. * – difference is statistically significant at $P < 0.05$; * – difference is statistically significant at $P < 0.001$.**

($P < 0.05$), relative amounts of CD18 and CDH1 transcripts were significantly decreased in purified sperm RNA samples ($P < 0.001$) confirming an effective removal of leucocytes and endothelial cells from sperm by both purification methods. SCLB is less time-consuming and cheaper method than swim-up, therefore, we will adopt this method in our further experiments.

In conclusion, this methodology results in reliable and consistent isolation of high-quality RNA from ram spermatozoa suitable for gene expression profiling using RT-qPCR, Custom RT^2 Profiler PCR Arrays or cDNA chips. Further investigations could reveal a potential of sperm RNA as a novel biomarker and promising diagnostic tool to assess ram fertility.

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