Short communication

Isolation of total ribonucleic acid from fresh and frozen-thawed boar semen and its relevance in transcriptome studies

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Abstract

The main objective of this study was to isolate high-quality total ribonucleic acid (RNA) from raw fresh semen and frozen-thawed boar semen, using a protocol comprising the conventional TRIzol assay and a membrane-based technique, the PureLink RNA mini kit. Bioanalyzer profile revealed that the sperm RNA size distributions comprised mainly intact RNA ranging from 1500 to 1800 bp, without any detectable residual genomic deoxyribonucleic acid (DNA) or 28S ribosomal RNA (rRNA). Spectrophotometric quantifications of the total RNA yielded 1.64 to 2.44 µg/10^6 spermatozoa, irrespective of the sperm source. The TRIzol/PureLink protocol allowed the isolation of high-quality intact RNA from boar spermatozoa, which is required for transcriptome analysis on high-throughput RNA-sequencing (RNA-Seq) data. Such an approach is relevant to identifying sperm messenger RNA (mRNA transcripts) that are associated with boar semen freezability.

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Frozen-thawed boar semen is not used on an industrial scale because of reduced sperm cryo-survival and subsequent compromised fertility (Fernández-Gago *et al*., 2013; Yeste, 2016). Selection of boars with good semen freezability is one of the main challenges in cryopreservation technology. Moreover, selection of boars with poor and good semen freezability ejaculates depends on conventional semen analyses, and there is compelling evidence that individual boar variability affects the success of the cryopreservation technology (Fraser *et al*., 2010; Yeste, 2016). Using amplified restriction fragment length polymorphism (AFLP) technology, it has been confirmed that there are molecular markers linked to genes that control the freezability of boar semen, suggesting that there is a genetic basis for the significant variations in post-thaw semen quality (Thurston *et al*., 2002, Fraser *et al*., 2008). Several mRNA transcripts have been shown to be useful markers for sperm phenotypes, such as motility, viability, capacitation and chromatin condensation. There is also evidence to suggest that sperm-derived RNA contributes to fertilization and embryo development (Cappallo-Obermann *et al*., 2011; Card *et al.*, 2013: Georgiadis *et al*., 2015).The main objective of this preliminary study was to isolate high-quality total RNA from raw fresh and frozen-thawed boar spermatozoa using a modified RNA extraction protocol.

Ejaculates were collected from six Polish Large White (PLW) boars (n = 6) and were frozen, using a standard cryopreservation protocol (Fraser *et al*., 2008; 2010). The frozen samples were stored in liquid nitrogen (-196 °C) for a week, prior to post-thaw semen analysis. Three boars each (n = 3) were characterized as poor and good freezability ejaculates. To enable this characterization, a plethora of sperm phenotype parameters were used, such as total and progressive motility (TMOT and PMOT, respectively), which were analysed by the computer–assisted semen analysis (CASA) system, mitochondrial function, plasma membrane integrity (PMI), normal apical ridges (NAR) acrosome integrity, and DNA fragmentation (Garner & Johnson, 1995; Thomas *et al*., 1998; Fraser *et al*., 2010). Repeated measures ANOVA, including a 6 × 2 factorial design (boar × freezability group) was used to analyse the interactions of the main effects on sperm quality characteristics after freezing-thawing. Significant main effects were compared using the

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Neuman–Keuls post hoc test (P <0.05). Raw fresh and frozen-thawed semen (100 × 10^6 spermatozoa/ml) from boars with different freezability were washed in phosphate buffer solution and the sperm pellets were re-suspended in the same buffer and stored at -80 °C, until required for RNA isolation.

Sperm cells were treated according to the method described by Rauber (2008). The authors made a slight modification in the RNA isolation procedure comprising the conventional TRIzol protocol and Lysis Buffer (PureLink RNA mini kit, Ambion, USA), which was used to isolate total RNA from fresh raw bull semen (Parthipan *et al*., 2015). Briefly, besides 2-Mercaptoethanol (as a reducing agent), proteinase K was added to the Lysis Buffer. Following homogenization (QIAshredder homogenizer, Qiagen Inc.), TRIzol and 200 µl chloroform were added to the mixture. The mixture was centrifuged (12000 × g for 15 min at 4 °C) and subjected to phase separation, using absolute ethanol and subsequently the spin column (PureLink RNA mini kit). All subsequent steps of RNA isolation (initial washing, on-column DNase treatment and purification, subsequent washing and elution) were performed according to the manufacturer's protocol (PureLink RNA mini kit). The eluted RNA was quantitated using fluorometric (RiboGreen RNA assay on Ascent FL, Thermo Scientific) and UV spectrophotometric (ND-1000, NanoDrop) methods. The quality of the isolated RNA was analysed with Bioanalyzer 2100 and RNA 6000 Nano Kit (Agilent Biotechnologies Inc. Waldbronn, Germany). All animal experiments were carried out in accordance with the approved guidelines set out by the local ethics committee.

In raw fresh semen, the phenotype sperm parameter for TMOT was 88.7 ± 4.4% (mean ± SD); PMOT was 66.7 ± 6.5%; mitochondrial function was 86.5 ± 2.9%; PMI was 87.3 ± 2.8%; NAR acrosome integrity was 92.3 ± 2.3%; and DNA fragmentation was 2.4 ± 1.4%. Boar variability and differences in the freezability group (poor and good post-thaw semen quality) were significant (P <0.05) sources of variations in the quality characteristics of frozen-thawed spermatozoa. Boars with poor semen freezability were characterized by reduced (P <0.05) sperm cryo-survival, which was manifested mainly in lower motility and mitochondrial function, and increased deterioration in the plasma membrane, acrosome and DNA integrity compared with boars with good semen freezability (Table 1). The authors' previous studies (Fraser *et al*., 2008; Fraser *et al*., 2010) and those of others (Thurston *et al*., 1998, Yeste, 2016) confirmed that these sperm phenotype parameters are reliable predictors of the freezability of boar semen.

Table 1 Quality characteristics of boar spermatozoa following cryopreservation

Sperm phenotype parameters (%)	Poor post-thaw semen quality			Good post-thaw semen quality		
	Boars 1 (n = 7)	2 (n = 10)	3 (n = 7)	4 (n = 10)	5 (n = 10)	6 (n = 7)
Total motility (TMOT)	27.6^{a} ± 2.2	20.5^{a} ± 2.1	25.5^{a} ± 2.1	43.8^{b} ± 2.9	42.5^{b} ± 1.2	48.5^{b} ± 4.2
Progressive motility (PMOT)	17.8^{a} ± 4.3	15.5^{a} ± 4.4	22.4^{ac} ± 3.8	30.9^{bc} ± 2.3	32.5^{b} ± 3.9	36.8^{b} ± 2.8
Mitochondrial function	32.5^{a} ± 3.9	39.9^{a} ± 2.6	32.5^{a} ± 4.2	58.5^{b} ± 2.4	56.1^{b} ± 2.3	52.4^{b} ± 2.3
PMI	39.5^{a} ± 1.6	36.7^{a} ± 2.2	40.9^{ac} ± 3.3	54.8^{b} ± 1.7	56.4^{b} ± 1.5	50.5^{bc} ± 3.5
NAR acrosome integrity	40.5^{a} ± 3.2	47.5^{a} ± 1.3	43.4^{a} ± 1.4	57.8^{b} ± 2.6	56.8^{b} ± 2.8	55.8^{b} ± 3.2
DNA fragmentation	7.3^{ab} ± 1.4	10.8^{ab} ± 4.5	14.5^{b} ± 4.2	5.6^{a} ± 1.5	4.6^{a} ± 2.8	5.2^{a} ± 3.2

^{a,b,c}Row means with different superscripts differ significantly at P <0.05 (Neuman-Keuls post hoc test)
PMI: plasma membrane; NAR: normal apical ridge

These preliminary studies showed that the modified RNA extraction protocol, comprising the TRIzol protocol and PureLink RNA mini kit, allowed the removal of highly abundant rRNA in spermatozoa, resulting in the extraction of high-quality total RNA from the raw fresh semen and the frozen-thawed boar semen. The RNA ladder from the 6000 Nano Kit (Agilent Biotechnologies Inc. Waldbronn, Germany) is shown in Figure 1A. Figure 1B illustrates two well-defined peaks, corresponding to 28S rRNA (~5kb) and 18S rRNAs (~1.8kb) on the electrophoregrams for the reference RNA isolated from peripheral blood mononuclear cells (PBMCs). Using capillary electrophoresis (Agilent 2100 Bioanalyzer), it was found that the RNA samples, isolated with the TRIzol/PureLink protocol, had spectrophotometry values of 1.85-1.95 for absorbance ratios A_{260}/A_{280} and greater than 1.87 for ratios A_{260}/A_{230}, indicating that the purified RNA was free from proteins and organic substances. The Bioanalyzer profile revealed that the sperm RNA size distributions comprised mainly full-length intact RNA isolate, ranging from 1500 to 1800 bp (with no detectable residual genomic DNA or 28S rRNA), from boar spermatozoa originating from the raw fresh semen (Figure 1C) and frozen-thawed semen (Figure 1D), suggesting the efficiency of the extraction procedure.

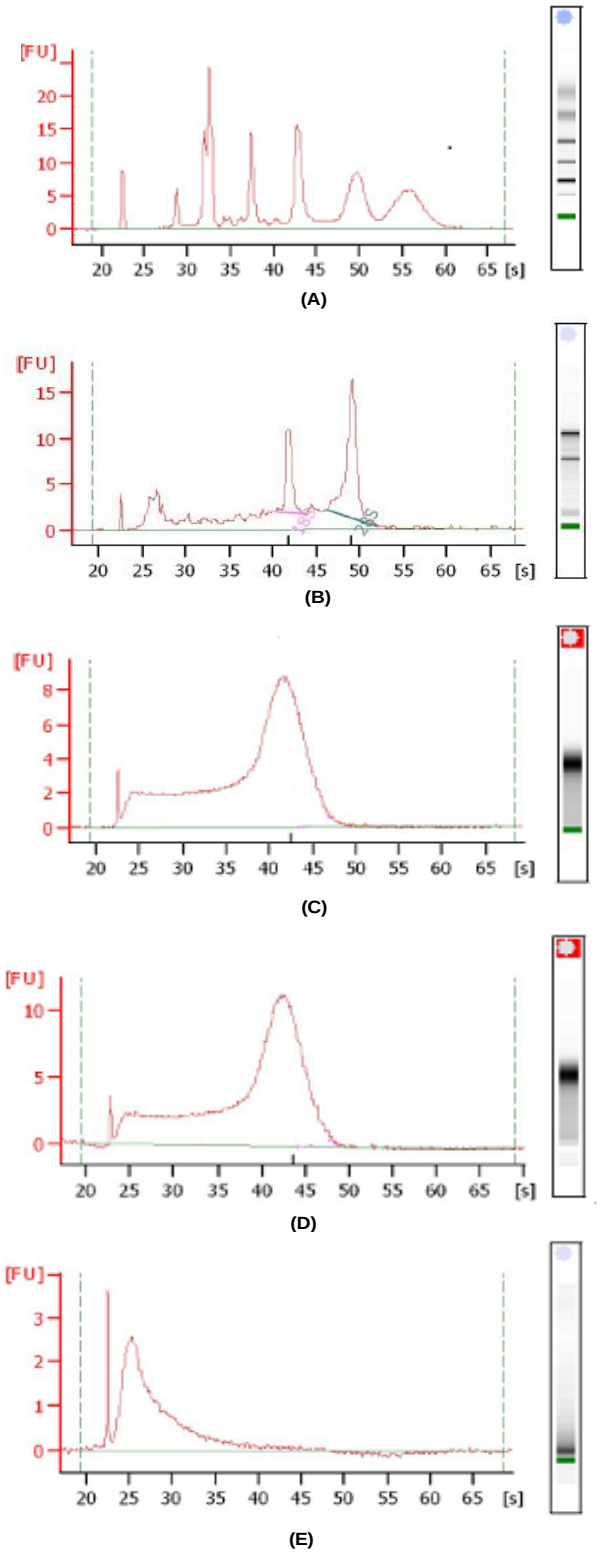

Figure 1 Characteristics of capillary electrophoresis patterns for ribonucleic acid isolated from peripheral blood mononuclear cells and boar spermatozoa. RNA size ladder from 6000 Nano Kit (A). Reference RNA isolated from PBMC cells (B). Total RNA isolated from (C) raw fresh semen and (D) frozen-thawed semen with the TRIzol/PureLink protocol. Total RNA isolated from raw fresh semen with the TRIzol protocol (E)). The value on the x-axis represents seconds (s), whereas the value on the y-axis represents the relative fluorescence units (FU)

The conventional TRIzol protocol yielded high amounts of degraded RNA, ranging from 150 to 250 bp (Figure 1E). Similar findings were found when the TRIzol protocol was used to extract RNA from fresh bull semen (Parthipan *et al*., 2015). It should be emphasized that the biological significance of the fragmented RNA in boar spermatozoa is unclear.

The current study showed that differences in semen quality did not appear to have marked effects on the extraction yield of total RNA. According to Das *et al*. (2010), lower amounts of RNA were isolated from stallion semen with marked reduction in post-thaw sperm motility. In the current study, there were no marked differences in the total RNA yield between the raw fresh and frozen-thawed boar spermatozoa (poor and good freezability ejaculates) (Table 1). The amounts of total RNA extracted from the fresh raw semen or either freezability group ejaculate ranged from 1.64 to 2.44 µg/10^6 spermatozoa. In this study the amounts of total RNA isolated from boar spermatozoa differ from other animal species, mainly because of differences in the RNA extraction procedures, quantification methods, and other factors (Das *et al*., 2010; Cappallo-Obermann *et al*., 2011; Parthipan *et al*., 2015). According to Cappallo-Obermann *et al*. (2011), highly purified RNA comprises 18S rRNA, with the 28S/18S rRNA ratio approximately 0.1, suggesting the inherent characteristic of human spermatozoa. In the current study, the full-length 28S/18S rRNA ratio was 0, which has been suggested as a strong indicator of RNA integrity because of the possibility of rapid deterioration of rRNA (Georgiadis *et al*., 2015). These are promising results, and in future studies the authors will use the TRIzol/PureLink protocol on a larger cohort of semen samples to isolate highly purified intact RNA for transcriptome analysis on high-throughput RNA-Seq data. Also, in future studies the isolated RNA samples would be subjected to reverse transcription polymerase chain reaction analysis of somatic markers (E-cadherin and CD45 transcripts) to remove the possibility of contamination from testicular germ cells and immunologic cells.

The authors suggest that the application of a high-quality RNA extraction protocol is a requisite for successful transcriptome analysis on high-throughput RNA-Seq data on spermatozoa of the domestic swine *(Sus scrofa).* The integration of RNA-Seq data analysis with functional genomics is required to address the molecular functions of the mRNA transcripts (Pareek *et al*., 2011; Card *et al*., 2013; Fraser, 2016). Transcriptome profiling of *Sus scrofa* spermatozoa would enable the development of a catalogue of mRNA transcripts, determining the transcriptional structure of genes, and quantifying the varying expression levels of each transcript. Furthermore, a wide range of bioinformatics analytical tools, including de novo assembly**,** transcriptome assembling, differentially expressed (DE) gene analysis, and detection of single nucleotide polymorphisms would be utilized to thoroughly assess sperm transcript sequencing in boars with poor and good post-thaw semen quality. In future studies, the authors would use bioinformatics screening to select candidate DE genes and putative SNP markers potentially associated with sperm freezability.

The results of this study show that a modification of the dual protocol, comprising the TRIzol assay and PureLink RNA mini kit, ensures the extraction of high-quality total RNA from boar spermatozoa originating from raw fresh semen and frozen-thawed semen. This on-going research study, including quality control of the RNA isolate and transcriptome study on RNA-Seq data, would establish a foundation for the discovery of novel sperm freezability markers, which would have tremendous potential to improve the cryopreservation technology of boar semen

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Authors' Contributions

LF conducted the semen quality research and was responsible for drafting and submitting the manuscript. PB contributed to the analysis of the RNA samples and CSP assisted in the revision of the manuscript.

Conflict of Interest Declaration

None of the authors have any conflict of interest to declare.

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