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Short communication

Total RNA quality in boar spermatozoa with different freezability

L. Fraser[1], P. Brym[2], M. Mogielnicka-Brzozowska[1], K. Wasilewska[1]

[1] Department of Animal Biochemistry and Biotechnology, Faculty of Animal Bioengineering, University of Warmia and Mazury in Olsztyn, 10-719 Olsztyn, Poland
[2] Department of Animal Genetics, Faculty of Animal Bioengineering, University of Warmia and Mazury in Olsztyn, 10-719 Olsztyn, Poland

Abstract

In this study the quality of total RNA, isolated from fresh spermatozoa, was compared between boars with good and poor semen freezability (GSF and PSF, respectively). Semen from 3 boars with GSF exhibited significantly higher total motility, mitochondrial function, plasma membrane integrity and reduced lipid peroxidation compared with 3 boars with PSF after cryopreservation. There were variations in the quality of RNA isolated from spermatozoa of boars with GSF and PSF. Boars with GSF exhibited mainly full-length, intact RNA, whereas substantial amounts of degraded RNA were detected in spermatozoa from boars with PSF. Further understanding of the biological relevance of RNAs in sperm function is critical to improve the freezability of boar semen.

Key words: boar, spermatozoa, semen freezability, RNA profiles

Introduction

Cryopreservation causes changes in the membrane structure of spermatozoa, resulting in reduced fertilization ability (Yeste 2016). Analysis of different sperm attributes has confirmed that cryo-induced damage to spermatozoa differs among individual boars, suggesting varying sperm response to the freezing-thawing procedure (Fraser et al. 2010). Moreover, it has been demonstrated that the susceptibility of boar spermatozoa to sustain injury after cryopreservation has a marked effect on the sperm cryo-survival, and that the identification of boars with high cryo-tolerance is the most practical way to improve the fertility potential of frozen-thawed (FT) semen (Fraser et al. 2014, Yeste 2016). It has been reported that the identification of sperm freezability markers might be useful in the selection of boars for cryopreservation (Yeste 2016).

Accumulating evidence has confirmed that spermatozoa carry a wide diversity of RNA that is implicated in capacitation, sperm-egg interactions and embryo development (Card et al. 2013, Schuster et al. 2016). Numerous messenger RNA (mRNA) transcripts have been reported to be markers for post-thaw sperm motility and viability (Card et al. 2013). More recently, it has been suggested that the quality of sperm-borne

Correspondence to: L. Fraser, e-mail: fraser@uwm.edu.pl, tel.: +48 89 523 36 26

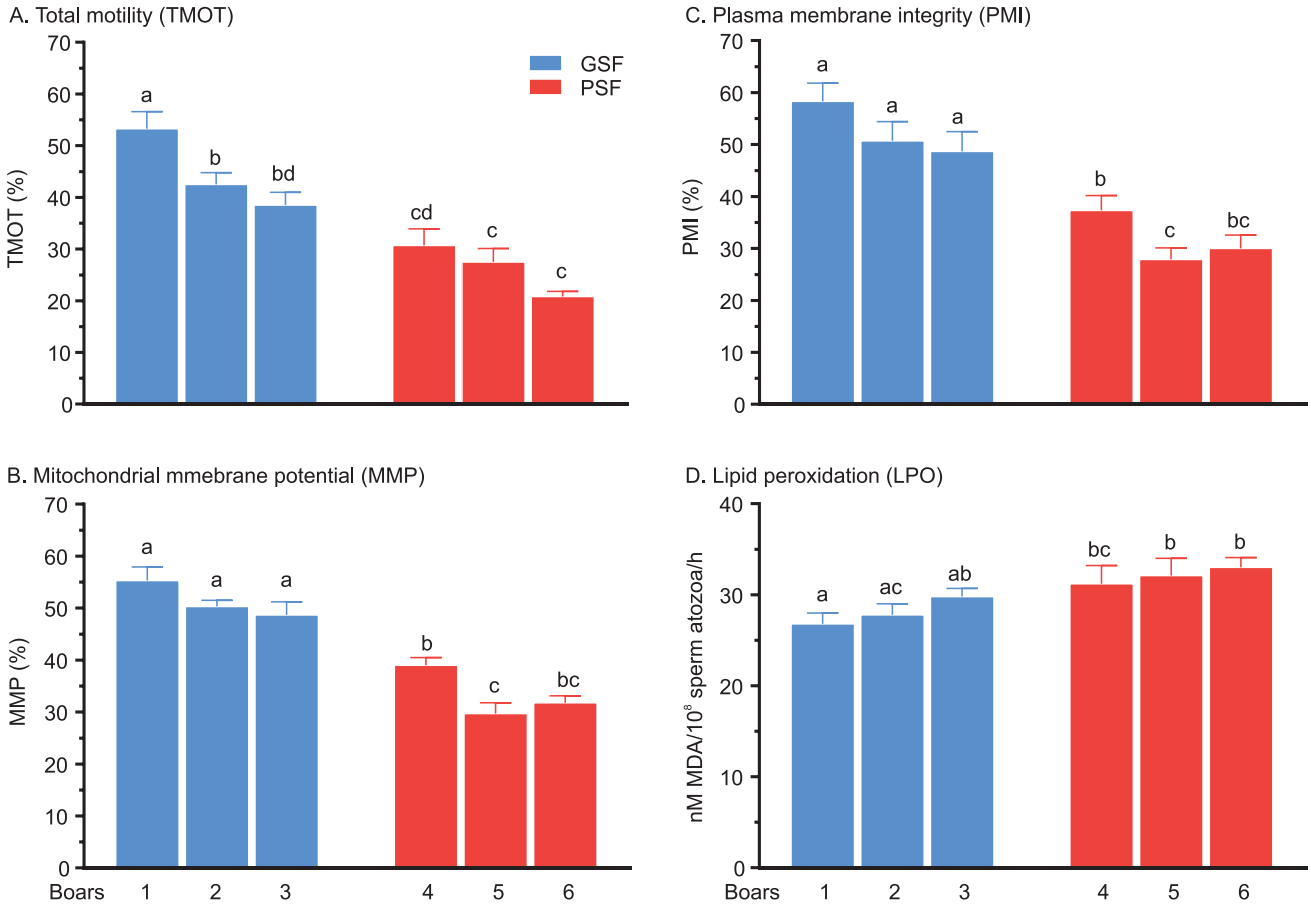

Fig. 1. Post-thaw quality characteristics of semen from boars with good and poor freezability (GSF and PSF, respectively). Values are expressed as the mean (± SEM) of 3 samples each from boars with GSF and PSF, respectively. Values with different letters (a, b, c, and d) are significant at $p<0.05$.

RNAs might be associated with the cryo-survival of spermatozoa (Card et al. 2013). In this study a modified RNA protocol was used to isolate total RNA from fresh spermatozoa, and the quality of RNA was compared between boars with good and poor semen freezability (GSF and PSF, respectively).

Materials and Methods

Sperm-rich fractions were collected from six Polish large white (PLW) boars. Animal experiments were carried out in accordance with the guidelines set out by the Local Ethics Committee. Semen samples were frozen, according to a cryopreservation procedure (Fraser et al. 2010). The sperm quality parameters analyzed in the fresh pre-freeze and FT semen included total motility (TMOT), mitochondrial membrane potential (MMP), plasma membrane integrity (PMI) and lipid peroxidation (LPO) (Garner and Johnson 1995, Fraser et al. 2010, Fraser et al. 2014).

In this study a column-based RNA purification protocol was used to isolate total RNA from the fresh spermatozoa of the PLW boars, according to a previously described study (Fraser et al. 2017), with some modifications. The modified protocol was based on the application of the TRIzol/Pure Link RNA Mini kit (Invitrogen, Thermo Fisher Scientific Inc.) followed by a 2× DNase digestion procedure to ensure effective removal of genomic (g) DNA (Turbo DNase, Ambion, USA). Following treatment of fresh sperm samples (150×10^6 spermatozoa/ml) with a Lysis Buffer and TRIzol (Invitrogen), the PureLink RNA Mini and PureLink DNase kits (Invitrogen) were used to purify total RNA. Prior to measurements, the RNA isolate was treated with Turbo DNase (Ambion), according to the manufacturer's instructions. Isolated RNA samples were quantified spectrophotometrically (ND-1000, NanoDrop) and by capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc.). RNA purity and integrity were examined by reverse transcription PCR (RT-PCR) analysis using intron-spanning primers for the type C tyrosine phosphatase receptor (*PTPRC* also known as the somatic specific CD45 marker) and sperm- and testes-specific *PRM1* (protamine 1). In three randomly selected RNA isolates, analysis was performed using the primers for

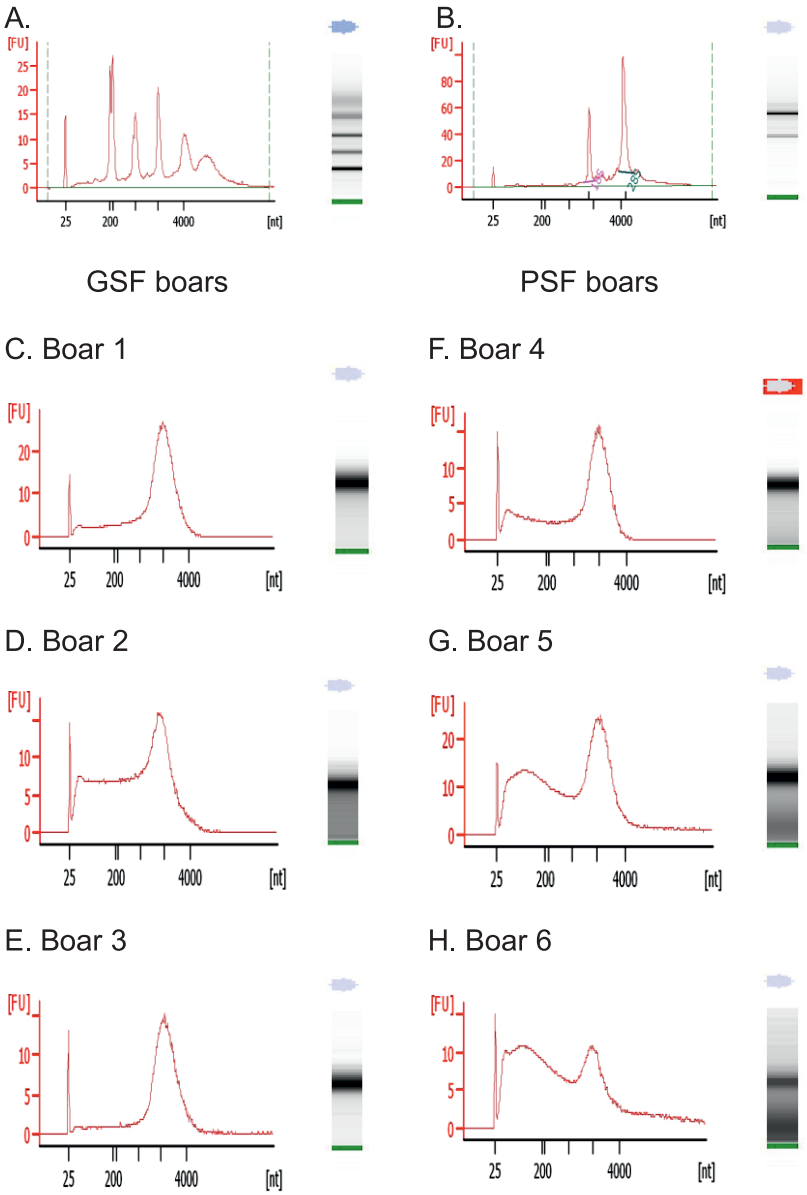

Fig. 2. Spectral and electrophoregram profiles of RNA size ladder from 6000 Nano kit (A), RNA isolated from peripheral blood mononuclear cells (PBMCs) showing the 18S and 28S rRNA reference (B) and total RNA isolated from spermatozoa of boars with good semen freezability, GSF (C-E) and boars with poor semen freezability, PSF (F-H). The values on the x-axis represent the nucleotide (nt) length, whereas the values on the y-axis represent the relative fluorescence (FU).

PTPRC (forward: AGAATACTGGCCGTCGATGG, reverse: GCTGAACGCATTCACTCTCCT) with the amplicon lengths of 238 bp cDNA and 2285 bp gDNA (representing the template for gDNA), and the primers for *PRM1* (forward: TCACCATGGCCAGATACAGAT, reverse: TCTTCTCCGGCATTGAACCTAA) with the amplicon lengths of 184 bp cDNA and 290 bp gDNA (representing the template for gDNA), according to a previously described study (Yang et al. 2009). The cDNA was synthesized with Oligo(dT)$_{18}$ primers, in combination with the Transcriptor High Fidelty kit (Roche Life Science Inc.), according to the manufacturer's instructions. Positive control containing somatic cDNA (pig white blood cells) was run in parallel with the RT-PCR analysis. The quality of the pre-freeze and FT semen is presented as mean ± SEM (StatSoft Incorporation, Tulsa OK., USA). Significant main effects were compared using Neuman-Keuls *post hoc* test ($p<0.05$).

Results and Discussion

There were no marked differences ($p>0.05$) among the boars in the analyzed sperm parameters of the pre-freeze semen. Post-thaw semen analysis showed that the ejaculates of three boars (n=3) were classified as GSF, Boars 1 to 3, and the other three boars (n=3) as PSF, Boars 4 to 6 (Fig. 1). Boars with GSF were

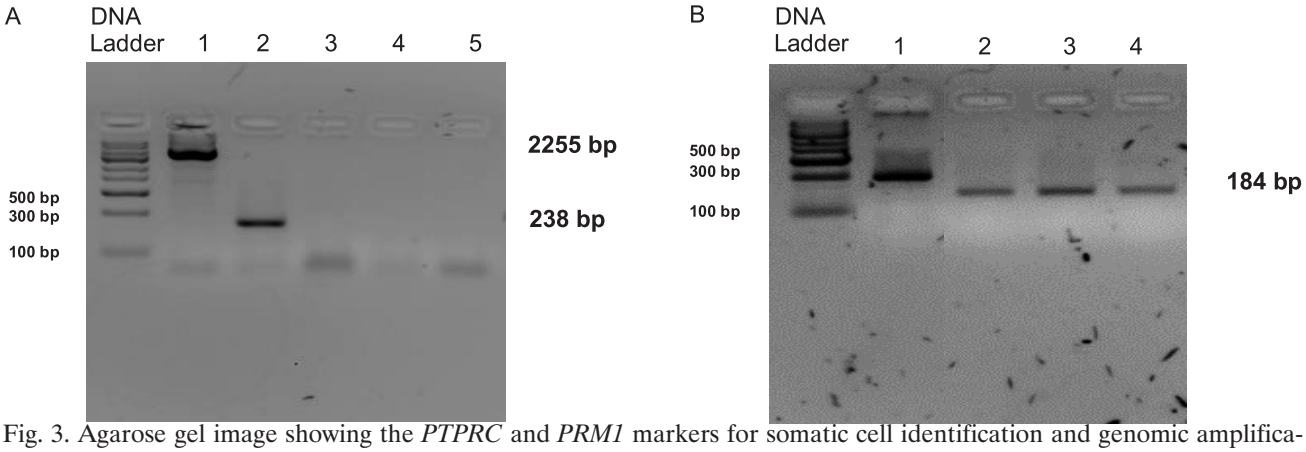

Fig. 3. Agarose gel image showing the *PTPRC* and *PRM1* markers for somatic cell identification and genomic amplification by reverse transcription PCR (RT-PCR) analysis. (A) Agarose gel image showing the *PTPRC* marker. Lane 1 shows the 2285 bp (genomic DNA) intron-spanning amplicon for *PTPRC*, lane 2 shows the 238 bp cDNA amplicon for the positive control with somatic cDNA (pig white blood cells), and lanes 3-5 show the absence of the amplification of the 238 bp cDNA amplicon. (B) Gel image of *PRM1*. Lane 1 shows the 290 bp (genomic DNA) intron-spanning amplicon for *PRM1*, whereas lanes 2-4 show the 184 bp PCR amplicon of cDNA. GeneRuler Express DNA Ladder 100-5000 (Fermentas).

characterized by significantly higher ($p<0.05$) TMOT (Fig. 1A) and membrane integrity, represented by MMP (Fig. 1B), and PMI (Fig. 1C). Wide variations were observed in post-thaw LPO, being significantly lower ($p<0.05$) in FT spermatozoa of Boar 1 (GSF) compared with the PSF boars (Fig. 1D).

Using the TRIzol/PureLink/Turbo DNase protocol, the RNA isolate displayed optical density (OD) ratio A_{260}/A_{280} ratio of 1.83, indicating samples with protein and organic-free substances. The total RNA yield did not differ significantly among the boars and averaged 2.28 ± 0.55 micrograms/150×10^6 spermatozoa, which is somewhat different from that reported for bull spermatozoa (Parthipan et al. 2015). The RNA size ladder from the 6000 Nano kit (Agilent) showed varying number of peaks in the spectral profile and electrophoregram bands (Fig. 2A). Furthermore, the RNA size ladder of the peripheral blood mononuclear cells (PBMCs) showed the 18S ribosomal RNA (rRNA), which comprises 1874 nucleotides (nt) and 28S rRNA peaks (4718 nt) in the spectral profile and two corresponding bands in the electrophoregram profile (Fig. 2B). Similarly to our recent study (Fraser et al. 2017), the absence of 18S RNA or 28S RNA or both RNA subunits in the isolated RNA samples (Fig. 2C-H) reaffirmed that the RNA was derived only from spermatozoa. The Bioanalyzer profiles showed that the sperm RNA profiles of two boars with GSF, Boar 1 (Fig. 2C) and Boar 3 (Fig. 2E) exhibited high proportions of full-length, intact RNA. Overall, the spectral RNA profiles of spermatozoa from boars with GSF comprised mainly full-length RNA, approximately 1000 to 3000 nt (Fig. 2C and 2E). In contrast, spermatozoa from most of the boars with PSF displayed large amounts of degraded RNA, comprising mainly fragments from 25 to 200 nt (Fig. 2G and 2H). These findings were confirmed by the differences in the sperm RNA electrophoregram profiles among the boars, which were reflected in the smeared appearance of partially degraded RNA, particularly for spermatozoa from Boar 5 (Fig. 2G) and Boar 6 (Fig. 2H). It is noteworthy that spermatozoa contain a heterogeneous population of degraded RNAs and some full-length intact RNAs that range about from 25 to 4000 nt (Zhang et al. 2015). Even though the RNA isolation procedure was performed twice in the fresh pre-freeze semen from each animal, our preliminary findings showed that spermatozoa from boars with PSF appeared to have high amounts of fragmented RNA compared to boars with GSF. It is unclear why the RNA profiles of spermatozoa from Boar 2 (Fig. 2D) and Boar 4 (Fig 2F) were different from those of the GSF and PSF groups, respectively. Presently, the basis of the differences in the sperm RNA quality, observed among the boars of the two freezability groups, is still not understood. In our laboratory we have considered to use different RNA isolation protocols on a large animal population to verify the differences in RNA profiles between the freezability groups. In addition, RNA-Seq analysis will be used to explain the finding of the current study.

Using the 2285 bp intron-spanning amplicon (lane 1), the *PTPRC* marker (lane 2, 238 bp RT-PCR amplicon) was observed in the positive control, whereas there was no amplification of the PCR product in the analyzed sperm RNA samples, as indicated in lanes 3 to 5 (Fig. 3A). The absence of the PCR amplification product provided strong evidence that there was no contamination of the isolated sperm RNA

samples by somatic cellular RNAs, indicating the effectiveness of the modified purification protocol. Likewise, the incorporation of the Turbo DNase digestion procedure in the extraction protocol allowed to obtain genomic free DNA samples, as indicated by the presence of only a 184-bp *PRM1* RT-PCR amplicon in the isolated sperm RNA samples (Fig. 3B). In contrast to other cell types, spermatozoa are depleted of 18S and 28S rRNA, which are often used to calculate the RNA integrity number (RIN), a measure of RNA quality (Schuster et al. 2016). In the current study the RIN values ranged from 2.4 to 3.0 in spermatozoa from either freezability group. Hitherto, it is still not clear whether the RIN values are useful in the assessment of RNA quality in spermatozoa.

In this study we reaffirm the findings of previous studies indicating that sperm RNA comprises a population of full-length, intact and degraded RNAs (Card et al. 2013, Zhang et al. 2017). Even though the biological relevance of full length intact and degraded RNAs in sperm function has not been elucidated as yet, it seems that variations in the quality of sperm RNA profiles among boars with different semen freezability might have significant relevance in transcriptome studies on RNA-Seq data. However, further studies are required on a larger animal population to fully evaluate whether the marked differences in the quality of sperm RNA profiles are associated with variations in the freezability of boar semen.

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