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Spermatozoa input concentrations and RNA isolation methods on RNA yield and quality in bull (*Bos taurus*)

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ABSTRACT

Sperm RNA can be used to understand the past spermatogenic process, future successful fertilization, and embryo development. To study the sperm RNA composition and function, isolation of good quality RNA with sufficient quantity is essential. The objective of this study was to assess the influence of sperm input concentrations and RNA isolation methods on RNA yield and quality in bull sperm. The fresh semen samples from bulls ($n = 6$) were snap-frozen in liquid nitrogen and stored at −80 °C. The sperm RNA was isolated using membrane-based methods combined with TRIzol (RNeasy + TRIzol and PureLink + TRIzol) and conventional methods (TRIzol, Double TRIzol, and RNAzol RT). Based on fluorometric quantification, combined methods resulted in significantly ($P < 0.05$) higher total RNA yields (800–900 ng/30–40 × 10^6) as compared with other methods and yielded 20 to 30 fg of RNA/spermatozoon. The quality of RNA isolated by membrane-based methods was superior to that isolated by conventional methods. The sperm RNA was observed to be intact as well as fragmented (50–2000 bp). The study revealed that the membrane-based methods with a cocktail of lysis solution and an optimal input concentration of 30 to 40 million sperm were optimal for maximum recovery of RNA from bull spermatozoa.

The presence of RNA in spermatozoa has been confirmed in mammals [1–4] and poultry [5]. The expression levels of some of the sperm transcripts were correlated with sperm functional parameters [6–8], early embryonic development [9], and fertility [10,11]. Because the spermatozoa transcripts reflect the spermatogenic process and have a prognostic value in fertilization, the absolute expression level of sperm transcripts may be a valuable marker to identify high-fertile males [4,7,12]. To find accurate expression levels, isolation of good quantity and good quality RNA from sperm becomes essential.

The sperm contains reduced cytoplasm, low amounts of full-length RNAs along with biologically degraded RNAs, and highly condensed DNA by protamine [13]. Due to this complex nature, a unique RNA isolation strategy is needed for sperm as compared with other cells. To isolate total RNA, the sperm membrane and nucleoprotamine complex should be dissolved completely. Protocols used by various studies to isolate sperm RNA from different species—human [7,14–16], bovine [3,4,10,17,18], porcine [9,19], stallion [12], and chicken [5]—have been described. Because the sperm structure and composition vary between species, development of a sperm RNA isolation protocol for bulls becomes necessary. Although the earlier studies [4,17,18] provided valuable methodological guidance, the input spermatozoa concentration, a suitable isolation method, and a reliable method for RNA quantification and quality check were not described in detail. This information is highly essential for establishing a sperm RNA isolation procedure, using high-quality RNA for downstream analysis, and drawing an effective conclusion. Hence, the objectives of the current study were (i) to find the optimal sperm concentration and suggest a suitable protocol for obtaining high-quantity and high-quality RNA and (ii) to study the nature of bull sperm transcript quality.

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Materials and methods

Semen collection and storage

Fresh semen samples were collected from Holstein Friesian (HF)[2] bulls ($n = 6$) maintained at Nandini Sperm Station, Hessarghatta, Bengaluru, India. From each bull, four ejaculates were collected based on the initial motility (>70%), and 1 ml of whole semen from each ejaculate was aliquoted in a 1.5-ml tube, snap-frozen in liquid nitrogen, and stored at −80 °C until RNA isolation. Another 1-ml aliquot of semen was centrifuged at 5000*g* for 5 min at 4 °C, and after removing the seminal plasma, the sperm pellet was snap-frozen in liquid nitrogen and stored at −80 °C until RNA isolation.

Effect of storage on recovery rate of spermatozoa

The stored whole semen and sperm pellet were purified using 50% BoviPure solution (Nidacon, Sweden) in phosphate-buffered saline (PBS, pH 7.2, Amresco, USA). Sperm cells were counted before and after BoviPure purification using a hemocytometer. The recovery percentage of the sperm was compared between storage types.

Sample preparation for RNA isolation

The whole semen aliquot were centrifuged at 700*g* for 5 min at room temperature (RT, 28 °C), and the supernatant was removed without disturbing the sperm pellet. The pellet was resuspended in 1 ml of PBS, and the sperm sample in PBS was slowly layered onto 4 ml of 50% BoviPure solution and centrifuged at 200*g* for 20 min at RT to purify the spermatozoa from contaminating somatic cells, germ cells, and leukocytes. After centrifugation, the top layer was carefully removed and the sperm pellet was washed twice with 10 ml of PBS at 700*g* for 5 min at 4 °C. Finally, the sperm pellet was resuspended in 1 ml of PBS and the concentration was estimated using a hemocytometer to calculate the sperm input concentration for RNA isolation. The purified sperm samples were used for RNA isolation. The plasticware and chemicals used for RNA isolations were nuclease free (RNase and DNase free).

Sperm concentration for total RNA isolation

To select a concentration of sperm for subsequent experiments, a preliminary study was conducted to isolate total RNA from bull spermatozoa with different concentrations ranging from 10 to 20, 30 to 40, 50 to 60, and 70 to 80 $\times$ 10^6 sperm cells using two different methods: conventional (TRIzol) and membrane based (RNeasy Mini Kit + TRIzol). After analyzing total RNA yield and RNA yield/spermatozoon (as described later), the two different input spermatozoa concentrations of 30 to 40 and 70 to 80 $\times$ 10^6 were chosen for subsequent experiments for optimizing the total RNA isolation protocol.

Sperm total RNA isolation

Conventional RNA isolation methods

TRIzol method. The total RNA was isolated from sperm using TRIzol (Invitrogen, USA) as per the manufacturer's protocol with modifications. In brief, 1 ml of TRIzol was added to the sperm pellet and homogenized by passing through a 20-G needle attached to a 5-ml syringe 8 to 10 times. Then the samples were vortexed for 5 min and incubated for 5 min at RT for complete dissociation of the sperm membrane. To the lysate, 200 µl of chloroform was added and mixed vigorously by hand for 20 s, and tubes were allowed to stand at RT for 5 min. The mixture was centrifuged at 12,000*g* for 20 min at 4 °C without applying a break to separate the phases. After centrifugation, the upper aqueous layer containing RNA was transferred to a new 1.5-ml conical bottom tube. An equal volume of isopropanol was added to the aqueous solution and mixed gently by inverting the tubes. The mixture was kept at RT for 10 min and centrifuged at 12,000*g* for 15 min at 4 °C. The supernatant was discarded, and 1 ml of 96 to 100% ethanol was added to the RNA pellet and centrifuged at 12,000*g* for 10 min. Ethanol was removed, and the RNA pellet was air-dried to remove traces of ethanol. The pellet was dissolved in 40 µl of nuclease-free water, and 2 µl of RNase inhibitor (20 U/µl, Invitrogen) and dithiothreitol (DTT) to a final concentration of 10 mM were added.

Double TRIzol method. The total RNA was isolated from spermatozoa using TRIzol. This method, the samples were lysed twice with TRIzol for complete dissociation of sperm membrane and nuclear components. In brief, 1 ml of TRIzol was added to the sperm pellet and homogenized by passing through a 20-G needle attached to a 5-ml syringe 8 to 10 times. Then the samples were vortexed for 5 min and incubated for 5 min at RT, the samples were centrifuged at 16,000*g* for 30 s, 1 ml of fresh TRIzol was added to the supernatant and vortexed for 2 min, and the remaining protocol was followed as per the TRIzol method given above.

RNAzol RT method. Total RNA was isolated using RNAzol RT reagent (Sigma, USA) as per the manufacturer's protocol with slight modifications. In brief, 1 ml of RNAzol RT was added and homogenized using a syringe as described in previous methods (TRIzol method), the samples were incubated in RNAzol RT for 15 min at RT, and 0.4 ml of nuclease-free water was added and centrifuged at 16,000*g* for 15 min at RT, followed by precipitation of total RNA with isopropanol. The RNA pellet was dissolved in 40 µl of warm (60 °C) nuclease-free water, and 2 µl of RNase inhibitor (20 U/µl) and DTT to a final concentration of 10 mM were added.

Membrane-based RNA isolation methods

RNeasy Mini Kit. Total RNA was isolated using an RNeasy Mini Kit (Qiagen, USA) as per the manufacturer's instructions with slight modifications by employing syringe homogenization and using 96 to 100% ethanol, instead of 70% ethanol, to facilitate the binding of RNA with the membrane. Elution was done three times with volumes of 40, 25, and 25 µl of warm (60 °C) nuclease-free water. After isolation, 2 µl of RNase inhibitor (20 U/µl) and DTT to a final concentration of 10 mM were added.

RNeasy + TRIzol method. Total RNA was isolated by combining the conventional and kit methods described by Goodrich and coworkers [15] with minor modifications (Fig. 1). In brief, to the sperm pellet, 0.5 ml of RLT lysis buffer containing β-mercaptoethanol (10 µl/ml) was added and homogenized by passing through a 20-G needle attached to a 5-ml syringe 8 to 10 times. The homogenized mixture was vortexed for 2 min, and 0.5 ml of TRIzol was added and vortexed again for 2 min. Then the samples were allowed to stand at RT for 5 min, and 200 µl of chloroform was added. The contents were mixed vigorously by hand for 20 s and kept at RT for 3 to 5 min, followed by centrifugation at 12,000*g* for 20 min at 4 °C. The upper aqueous layer was transferred to a new 2.0-ml tube, and an equal volume of 100% ethanol was added and mixed by pipetting. The mixture was transferred to an RNeasy

[2] *Abbreviations used:* HF, Holstein Friesian; PBS, phosphate-buffered saline; RT, room temperature; DTT, dithiothreitol; gDNA, genomic DNA; cDNA, complementary DNA; Prm1, protamine 1; PCR, polymerase chain reaction; Eif1, eukaryotic translation initiation factor 1; Ptprc, protein tyrosine phosphatase receptor type C; Cdh1, cadherin 1; Kit, kit oncogene.