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Development and optimization of an efficient RNA isolation protocol from bovine (*Bos indicus*) spermatozoa

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ABSTRACT

Achieving the optimum extraction of RNA from spermatozoal cells is crucial for carrying out effective high-throughput analysis regarding its role in fertility and other reproduction processes in *Bos indicus*. Nevertheless, semen comprises spermatozoa and several other secretions from the male reproductive system, which as well consist of diverse somatic cell types. Therefore, the elimination of somatic cells guarantees the purity of the sperm RNA. In the present study, we tested five different RNA isolation protocols and evaluated them for their yield and purity using spectrophotometer and polymerase chain reaction. Among the five RNA isolation protocols, the Triazol + RNAeasy plus Kit + TCEP method revealed optimum performance. We successfully achieved isolation of spermatozoal RNA without any spermatozoal DNA contamination from *Bos indicus* spermatozoa that contains approx. 1000 to 10,000 times less RNA as compared to other mammalian somatic cells. RNA quality was assessed using primers *Protamine1* (spermatozoal RNA and spermatozoal DNA), *CDH1* (epithelial cell), *KIT* (germ cell) and *PTPRC* (leukocytes) designed using primer BLAST where there was no product amplified except *Prm1* whose product size was specific for spermatozoal RNA. The results of our investigation on RNA isolation procedures indicate that the inclusion of a disulphide reducing agent (TCEP) is crucial for the process of sperm cell lysis.

1. Introduction

A macromolecule that is indispensable for numerous biological activities in all species is ribonucleic acid (RNA). It manifests in many forms and lengths, and exhibits a multitude of functions. Eukaryotic messenger RNA (mRNA) is a type of coding RNA that is transcribed from a gene which codes for proteins. The primary role of sperm is to transport a haploid genome to the target oocyte. Although sperm cells lack the ability to conduct transcription, they do possess RNA [1]. The majority of these RNA molecules are actually segments of larger transcripts, including both rRNAs and mRNAs [1]. Conventional beliefs assert that the main function of spermatozoa is to transmit the paternal genetic material (DNA) into the ovum during the process of fertilisation. Numerous studies conducted in the past decade have shown that spermatozoa transport their DNA and a diverse range of large and small RNA molecules into the oocytes. This process is crucial for the early development of the embryo. Furthermore, these RNA molecules remain stable until the embryonic genome is activated [2,3]. Moreover, the potential of utilising sperm RNA as a diagnostic marker for male fertility and infertility has lately attracted new interest [4,5]. A spermatozoon has around 10–20 fg of RNA equivalent to 10–20 pg of RNA in a diploid somatic cell [6,7]. Recent investigations have indicated that some characteristics inherited from the parent generation are transmitted to the offspring via sperm [8,9]. Moreover, it has been proposed that sperm RNAs can transmit the message for an individual's life progression, therefore enabling the investigation of health at critical periods spanning from preconception to the conception of offspring [10]. Tissue and cellular RNA are often obtained using two primary techniques: acid guanidinium thiocyanate-phenol-chloroform (AGPC) and silica-based extraction columns [11]. AGPC extraction is a straightforward, cost-effective, and high-yielding technique for obtaining total RNA from cells and tissues [12]. Nevertheless, the purity and quality of RNA

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obtained using this approach are mostly determined by the proficiency of the experimenter and the meticulous management of the material. Silica-based columns facilitate the extraction of nucleic acids by their binding to silica crystals in the presence of chaotropic salts. Silica-based columns have a preference for capturing nucleic acids that are at least 200 nucleotides long. However, they offer limited recovery of short RNA due to its strong binding to the silica, which reduces their likelihood of elution [13]. The RNA obtained is of great purity, while the yield is often lower in comparison to AGPC. Since spermatozoa contain a very small quantity of RNA, developing a suitable protocol for extracting RNA with high quality and yield would serve as a dependable and precise indicator for male fertility and semen assessment. The objective of the present work was to experiment with several protocols for extracting optimum RNA from frozen cattle spermatozoa and then assess their quality and yield.

2. Methods

2.1. Sample preparation

Frozen semen samples from reputed bull station were procured for the experiment. The semen samples (n = 6 bulls for each method) were thawed in a laboratory thawing device at 37 °C for 1 min and then transferred to a 1.5 ml microcentrifuge tube (MCT). The samples were subsequently subjected to centrifugation at 600×*g* for 20 min at 4 °C to remove dilutors and induce precipitation of spermatozoa. Once the dilutors were removed, the sperm cell pellets were reconstituted in cold PBS (1X) and subjected to centrifugation at 700×*g* for 10-min. at 4 °C. This washing process was repeated 2–3 times. A complete RNA extraction from sperm cells was performed using 250 μl of an initial cell suspension comprising 4×10^6 cells.

2.2. Isolation of spermatozoal RNA

Sperm RNA was extracted using five different protocols: Triazol method, Modified Triazol method, PureLink RNA Mini Kit (Invitrogen), Quick-DNA/RNA MagBead kit (Zymo Research), and Triazol + RNAeasy plus Kit + TCEP. The methods adopted for extracting RNA from sperm cells differed in terms of the lysis condition and the number of stages required.

2.2.1. Triazol method

The sperm pellet after washing steps was suspended in 1 ml of Qiazol (Qiagen). The suspension was passed through a 5-ml syringe fitted with a 24-gauge needle 20-times in order to lyse the sperm cells properly. The suspension was then vortexed for 5 min and incubated for 10-min. To this lysate, we added 200 μl chloroform and mixed vigorously for 15-sec. until the colour of the lysate turned light pink. After this, we allowed the MCT to stand for 3 min at room temperature. Then the tubes were centrifuged at 15,000×*g* for 25-min. at 4 °C. The upper aqueous layer containing the RNA was transferred to 1.5-ml tube. An equal volume of isopropanol (HiMedia) was added and mixed gently by inverting the tubes, and incubation for 10-min. at room temperature was performed. Then the solution was centrifuged at 12,000×*g* for 15-min. at 4 °C. The supernatant was discarded, and the RNA pellet was washed twice with ethanol (HiMedia). RNA pellets were air dried to remove traces of ethanol. After that, RNA pellets were dissolved in 25 μl nuclease free water and stored at −80 °C for further studies.

2.2.2. Modified Triazol method

The sperm pellet after washing steps was suspended in 100 μl of RLT buffer (Qiagen) supplemented with 1 μl of beta-mercaptoethanol (Qiagen) and incubated for 15-min. at room temperature. To this, one ml of Qiazol was added, and samples were incubated at room temperature for 5-min. followed by the addition of 100 μl of chloroform and vigorous shaking for 15-sec. Samples were then incubated at room temperature for 2-min., followed by centrifugation at 12,000×*g* for 15-min. at 4 °C. The upper aqueous layer was removed and transferred to a new 1.5-ml tube, and again 100 μl of chloroform was added, followed by centrifugation at 12,000×*g* for 15-min. at 4 °C. Again, the aqueous phase was removed and added with an equal volume of 100 % ethanol and kept at −20 °C supplemented with 25 μl glycogen. Then the samples were centrifuged at 12,000×*g* for 15-min. at 4 °C, and pellets obtained were washed with 70 % ethanol and dried under vacuum (Eppendorf). The dried pellets were resuspended in 25 μl of RNase free water and incubated for 1 h at 4 °C. It was thereafter stored at −80 °C for further use.

2.2.3. RNA isolation using PureLink RNA mini kit

The sperm pellet after washing steps was suspended in 0.6-ml lysis buffer with 2-mercaptoethanol and vortexed until the cell pellet was dispersed. The lysate was then passed 5–10 times through a 21-gauge syringe needle. The lysate was transferred into a new tube and centrifuged at 20,000×*g* for 5-min. Then the supernatant was transferred to a new RNase-free tube, and one volume of 70 % ethanol was added to each volume of cell homogenate. The homogenate was vortexed to mix thoroughly and to disperse any visible precipitate that was formed after the addition of ethanol. From this mixture, 700 μl of the sample (including any remaining precipitate) was transferred to the spin cartridge (with the collection tube). This tube was centrifuged at 12,000×*g* for 15-sec. at RT. The flowthrough was discarded, and the spin cartridge was reinserted into the same collection tube. The above steps were repeated twice. This was followed by washing of the spin cartridge with Wash Buffer I and Wash Buffer II, respectively. Then the spin cartridge was centrifuged at 12,000×*g* for 2-min. to dry the membrane with bound RNA. The collection tube was discarded, and the spin cartridge was reinserted into a recovery tube. To the center of the spin cartridge, 25 μl of RNase free water was added and incubated for 1-min. The spin cartridge was centrifuged for 2-min. at 12,000×*g* at RT to elute the RNA from the membrane into the recovery tube. The purified RNA was stored at −80 °C.

2.2.4. Isolation of RNA using Quick-DNA/RNA MagBead kit

The sperm pellet after washing steps was suspended in 500 μl DNA/RNA Lysis Buffer supplemented with 10 μl Proteinase K. This was followed by the addition of 30 μl of MagBinding Beads and mixed well for 20-min. As the MagBinding Beads settle quickly, we ensured that the beads were kept in suspension while dispensing. The tubes were moved to the magnetic stand where the beads (bound with DNA) were pelleted, followed by the transfer of the cleared supernatant (RNA) into a new tube. After this, we proceeded to isolate both RNA and DNA from the same sample. For RNA, we added 700 μl ethanol (100 %) to the supernatant and mixed well. Then steps given in the kit manual were followed. The RNA was eluted in 25 μl of nuclease free water, and it was subsequently stored at −80 °C.

2.2.5. Triazol + RNAeasy Plus Kit + TCEP method

Following washing, the sperm pellet was reconstituted in 100 μl of RNeasy Buffer RLT+ and homogenised completely using a syringe three to four times. Subsequently, 900 μl of Qiazol and 100 μl of TCEP (50 mM) (Sigma) were added and homogenised thoroughly by passing through a 24-gauge needle connected to a 2-ml syringe 20-25-times until no precipitate developed. Following homogenisation, the lysate was allowed to rest at RT for 5-min., and then vortexed for 1 min. The RNA isolation was performed using this lysate. To each ml of lysate, 200 μl of chloroform (HiMedia) was added and aggressively agitated for 20-sec. Observation was made of the transition in colour from dark pink to bright pink at this period. Following a 5 min incubation at RT, the lysate was centrifuged at 12,000×*g* for 15-min. at 4 °C. Thorough care was taken to remove the MCT from the centrifuge to avoid any intermingling of the aqueous phase and the interphase layer. The aqueous phase was separately aspirated into the gDNA wipeout spin column of the Qiagen RNeasy Plus mini kit. The column was then placed in a 2-ml collection

Table 1
Primers used for assessing spermatozoal gDNA and other cell (epithelial cell, germ cell, leukocyte) contamination, confirming transcripts presence in spermatozoa.

Gene	GenBank Acc. No	Primer Sequence (5′-3′)	Amplicon Size (base pairs)	Intron Span (YES/NO)	Purpose
CDH1	NM_001002763.1	F- CTGCATTCCTGGCTTTGGTG R- GTAAGCACGCCATCTGTGTG	171	Yes	Epithelial cell
KIT	AF263827.1	F- GAATAGCTGGCATCAGGGTG R- CCAGATCCACATTCTCTCCATC	224	Yes	Germ cell
PTPRC	NM_001206523.1	F- ACCCAACCTTCTACTCAAGATG R- CGTATTTGTTCTCACATGGTGG	124	Yes	Leukocytes
PRM1	NM_174156.2	F- AAGATGTCGCAGACGAAGGAG R- GTGGCATTGTTCGTTAGCAGG	248	Yes	Sperm RNA and gDNA

tube and subjected to centrifugation at 8000×*g* for 30-sec. An equivalent volume of 70 % ethanol was added to the flow-through and mixed well by pipetting 2–3 times. After allowing the tubes to stand at RT for 5-min., 700 μl of the sample was moved to the RNeasy spin column in a 2-ml collection tube and subjected to centrifugation at 8,000×*g* for 15-sec. The flow-through was eliminated, and the procedure was repeated until the entire volume had been filtered through the column. Next, 700 μl of Buffer RW1 was introduced into the spin column and subjected to centrifugation for 15-sec at 8,000×*g*. Once again, the flow-through was discarded and 500 μl of Buffer RPE was added to the spin column. The column was again centrifuged at 8,000×*g* for 15-sec. Following the removal of the flow-through, 500 μl of Buffer RPE was reintroduced into the column and subjected to centrifugation at 8,000×*g* for 2-min. The flow-through was disposed of together with the collection tube. The spin column was inserted into a fresh 2-ml collection tube and subjected to centrifugation at 20,000×*g* for 1 min to ensure thorough drying of the membrane. To elute the RNA, the spin column membrane was inserted into a fresh 1.5-ml collection tube, and 25 μl of RNase-free water was carefully placed inside the center of the tube, followed by centrifugation at 8,000×*g* for 1-min. The RNA was stored at −80 °C.

2.3. Evaluation of total RNA yield and quality assessment

Quantification of RNA and DNA was performed using an UV–Vis spectrophotometer (Nanodrop, Thermo Scientific), and initial quality estimation was done by taking the 260/280 absorbance ratio.

2.4. cDNA synthesis

The cDNA was synthesized using the SuperScript II Reverse Transcriptase kit (Invitrogen) from the RNA samples. The reaction mix (10 μl) was comprised of 250 ng/μl Oligo dT, 150 ng random primers, 10 mM dNTP mixture, and 100–150 ng RNA as template. The reaction mix was properly mixed and incubated at 65 °C for 5-min., followed by immediate chilling on ice. To the template RNA primer mixture, Prime Script Buffer (1X) and DTT (0.1 M) were added and mixed gently, followed by incubation at 42 °C for 2-min. and 25 °C for 2-min. After incubation, 200U SuperScript II Reverse Transcriptase was added and mixed gently by pipetting. The synthesis was performed at 25 °C for 10-min., 42 °C for 50-min., and 70 °C for 15-min., respectively.

2.5. Estimation of spermatozoal gDNA contamination and RNA contamination from other cells

Primers used for determination of contamination from spermatozoal gDNA and other cell's RNA were designed using NCBI Primer-BLAST tool. The information regarding the primers are provided in Table 1. The gDNA contamination was detected using intron-spanning primers, Protamine1 (*PRM1*). To confirm that the RNA was free from other cell contamination, cell-specific intron-spanning primers (Table 1) Cadherin1 (*CDH1* for epithelial cells), Protein Tyrosine Phosphatase Receptor Type C (*PTPRC* for leukocytes) and KIT oncogene (*KIT* for germ cells) were used. Quality determination was done using Taq PCR master mix (Qiagen) carried out in a thermal cycler (ProFlex, Applied Biosystems). Each reaction consisted of final concentration of PCR buffer 1x, dNTP 200uM, forward and reverse primer 10 pmol each, Taq Polymerase 1.25 units added to 2 μl of cDNA, and nuclease free water was added to make the final volume to 25 μl. The conditions adjusted in a thermal cycler for the amplification of product were initial denaturation at 94 °C for 4-min., followed by 30 cycles of denaturation at 94 °C for 30-sec., annealing temperature of 59 °C for 45-sec., and extension at 72 °C for 1-min. This was followed by the final extension step at 72 °C for 8-min.

Table 2
Total spermatozoal RNA yield (ng) and quality of five different isolation methods.

Isolation Method	Total RNA yield (ng)	260/280 ratio
Triazol method	3989.5 ± 213.04^{a}	1.27 ± 0.05^{a}
Modified Triazol method	690.33 ± 18.33^{b}	1.57 ± 0.04^{b}
PureLink RNA Mini Kit	483.83 ± 58.06^{c}	1.81 ± 0.01^{c}
Quick-DNA/RNA MagBead Kit	69.17 ± 10.25^{d}	1.25 ± 0.06^{d}
Triazol + RNAeasy Plus Kit + TCEP method	536.83 ± 21.64^{e}	1.68 ± 0.01^{e}

Note: Means with different superscripts differ significantly ($p < 0.01$).

3. Results and discussion

3.1. Comparative spermatozoal RNA yield and quality obtained by different methods

The average yield of RNA using 250 μl of an initial cell suspension comprising 4×10^6 cells and possible protein contamination measured using a 260/280 ratio for five different protocols are presented in Table 2 (Supplementary Fig. 1). The yield of 3989.5 ± 213.04 ng of RNA was found to be highest with the Triazol method. In this method, we performed the homogenisation using a 24-gauge needle connected to a 5-ml syringe. However, 260/280 ratio was found to be 1.27 ± 0.05 which suggested that the RNA samples were contaminated with proteins. In the modified Triazol method, an average RNA yield of 690.33 ± 18.33 with the 260/280 ratio of 1.57 ± 0.04 was observed. Although the yield of RNA was reduced, the protein contamination was also reduced in this method. This may be attributed to the use of beta-mercaptoethanol, which helps to break the protein-protein bonds. Also, we used chloroform phase separation twice, which helped in further purification of the RNA samples from the protein contamination. Earlier, Parthipan et al. [16] had used the double Triazol method which increased the concentration and improved the 260/280 ratio. The RNA yield of 483.83 ± 58.06 ng was obtained from the PureLink RNA Mini Kit isolation protocol. The 260/280 ratio also improved to 1.81 ± 0.01, which shows that silica column-based isolation helps in removing the protein contamination and improves the RNA quality at the cost of the RNA yield, which was found to be less than the traditional Triazol based methods of RNA isolation. We observed that the RNA yield from sperm was lowest in the case of the Quick-DNA/RNA MagBead Kit of all the five

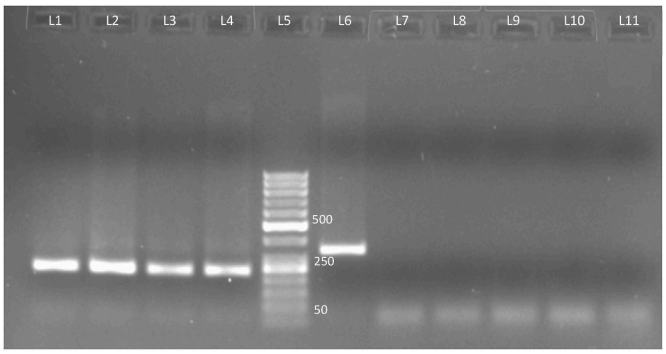

Fig. 1. L1-L4: cDNA synthesized from bovine spermatozoal RNA isolation by Triazol + RNAeasy Plus Kit + TCEP method using a set of intron-spanning primers for Protamine1 (*PRM1*) amplified a product of 248bp; L5: Marker (50bp-1Kb); L6: gDNA from sperm amplified a product size of 315bp; L7, L8, and L9: no amplified products for *PTPRC*, *CDH1,* and *KIT*; L10-11: NTC (no template control).

methods followed. This method used Proteinase K which we believe did not help in the proper lysis of the cells and breakage of the protein bonds between them. In the magnetic bead method, the supernatant is used for the RNA isolation, and the DNA is bound to the MagBead, which is separated using the magnetic stand. We believe that the reduced yield of RNA was due to binding a part of the RNA pool to the MagBead, which got separated along with the DNA during the magnetic separation. In the combined Triazol + RNAeasy Plus Kit + TCEP method, we obtained a RNA yield of 536.83 ± 21.64 with a 260/280 ratio of 1.68 ± 0.01. In this method, we used a combination of Triazol and RNAeasy Plus Kit and we used TCEP instead of beta-mercaptoethanol. According to Roszkowski & Mansuy [14] who used a similar recipe in mice sperm, Triazol supplemented with TCEP allows the complete and rapid lysis of sperm cells, increasing RNA yield. The routine chaotropic agents such as beta-mercaptoethanol do not work efficiently with bovine sperm due to presence of disulfide bonds that are responsible for the chemical resistance of sperm cells to RNA extraction reagents.

3.2.2. RNA quality assessment by PCR

The spermatozoal gDNA and other cell RNA (epithelial cells, germ cells, and leukocytes) contamination was assessed by performing PCR with the specially designed primers for quality check that included *Prm1, PTPRC, CDH1,* and *KIT*. The PCR products were run on 2 % agarose gel. Gel was photographed and visualized in a Gel Documentation System (Fig. 1). Had the samples been contaminated with the spermatozoal gDNA, these would have amplified products with amplicon size 248 bp (spermatozoal RNA) along with 314 bp (spermatozoal gDNA) in *Prm1*. We observed that the Triazol + RNAeasy Plus Kit + TCEP method was the only method in which a 248-bp product specific to spermatozoal RNA was amplified, which confirmed that the isolated RNA sample was devoid of any spermatozoal gDNA contamination. No amplified product was obtained using other primers (*PTPRC, CDH1,* and *KIT*). Therefore, it was obvious that there was no RNA other than of spermatozoal origin in the isolated samples. The reason for no DNA contamination in this method was due to the passing of the lysate through the gDNA wipe-out column, which contained the DNase enzyme. However, for other methods of RNA isolation, we observed the spermatozoal DNA contamination. In case of Triazol and modified Triazol method, DNA contamination was more prominent than PureLink RNA Mini Kit and Quick-DNA/RNA MagBead Kit method, as can be visualized in Fig. 2a and b. However, we did not find any contamination of RNA from other cells in these two methods.

Spermatozoa exhibit minimal transcriptional activity, resulting in an anticipated low RNA yield. Additionally, low RNA yield can complicate subsequent analyses, as RNA species like miRNAs may be selectively lost during AGPC RNA extraction when working with a limited number of cells [15]. Therefore, a robust method for RNA extraction is crucial for downstream RNA-seq analysis for better understanding of reproduction, male fertility and development. Our RNA isolation method from bovine spermatozoa using TCEP as a reducing agent demonstrated to harvest RNA of optimum quality and quantity.

4. Conclusion

Our study on RNA isolation protocols demonstrated that the presence of a disulphide reducing agent (TCEP) is essential for the lysis of bovine sperm cells. The quantity, purity, and outcomes of downstream

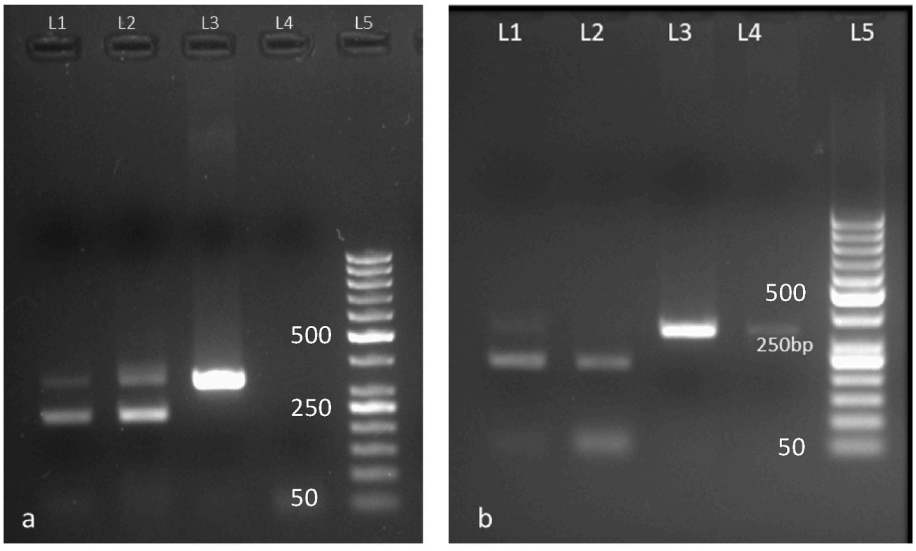

Fig. 2. (a) L1-L2: cDNA synthesized from bovine spermatozoal RNA isolated by the Trizol method and modified Trizol method, respectively, amplified both 248bp (RNA-specific) and 315bp (gDNA specific) products; L3: control gDNA (315bp); L4: NTC; L5: Marker (50bp-1Kb). **(b).** L1-L2: cDNA synthesized from bovine spermatozoal RNA isolated by the PureLink RNA Mini Kit and Quick-DNA/RNA MagBead Kit methods, respectively, amplified both 248bp (RNA-specific) and 315bp (gDNA-specific) products; L3-L4: control gDNA (315bp); L5: Marker (50bp-1Kb).

investigations are adversely affected by the contamination of RNA with phenol-based reagents. The modified combination protocol resulted in a substantial enhancement of both RNA quality and purity. Moreover, the RNA yield obtained by column-based extraction approach is generally lower than that of non-column-based purifying approaches. The flexible improved RNA extraction method we have developed efficiently eliminates remaining quantities of phenol-based impurities without imposing excessive requirements on time and without reducing the total RNA production. The proposed modified protocol for isolating RNA from bovine spermatozoa aims to enhance the quantity and quality of spermatozoal RNA isolation while minimising the presence of spermatozoal DNA or other cell RNA contaminants.

CRediT authorship contribution statement

Sofi Imran Ul Umar: Writing – original draft, Visualization, Validation, Software, Investigation, Formal analysis, Data curation. **Sushil Prasad:** Supervision, Resources. **Soumen Naskar:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Pooja Chowdhury:** Investigation. **Anju Rana:** Investigation. **Pranab Jyoti Das:** Writing – review & editing, Methodology.

Ethics approval

The experiment was conducted in accordance with the applicable rules and regulations of biosafety and ethics.

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Declaration of competing interest

The authors declare that they have no known competing interests.

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Abbreviations

AGPC	Acid guanidinium thiocyanate-phenol-chloroform
lncRNA	Non-coding RNA
cDNA	Complementary DNA
PBS	Phosphate buffer saline
TCEP	Tris(2-carboxyethyl) phosphine
fg	Femtogram
ng	Nanogram
SE	Standard error
CDH1	Cadherin 1
PRM1	Protamine 1
gDNA	Genomic DNA
PTPRC	Protein tyrosine phosphatase receptor type C
KIT	Tyrosine-protein kinase
ICG	Internal Control Gene
PCR	Polymerase chain reaction

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101862.

Data availability

Data will be made available on request.

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