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Authors

Georgiadis, Andrew P
Kishore, Archana
Zorrilla, Michelle
et al.

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High Quality RNA in Semen and Sperm: Isolation, Analysis and Potential Application in Clinical Testing

Andrew P. Georgiadis, **Archana Kishore**, **Michelle Zorrilla**, **Thomas M. Jaffe**, **Joseph S. Sanfilippo**, **Etta Volk**, **Aleksandar Rajkovic**, and **Alexander N. Yatsenko***

Departments of Obstetrics, Gynecology and Reproductive Sciences (APG, AK, MZ, JSS, AR, ANY), Urology (TMJ), Molecular and Cellular Biology (AR), Pathology (AR, ANY) and Human Genetics (AR), School of Medicine, University of Pittsburgh and Magee-Womens Hospital, University of Pittsburgh Medical Center (EV), Pittsburgh, Pennsylvania

Abstract

Purpose—Male infertility is a complex health condition. To our knowledge there are no molecular biomarkers of male infertility. Sperm RNA is a potential biomarker for detecting sperm abnormalities and viability at infertility clinics. However, RNA use is hindered by its inconsistent quantity, quality, multiple cell types in semen and condensed sperm structure.

Materials and Methods—We tested the usefulness of high quality RNA isolated from mature sperm and whole semen by our protocol, which reduces RNA degradation by maintaining semen and protocol components at 37C and decreasing processing time. We isolated RNA from 83 whole semen samples, 18 samples of motile sperm prepared by the swim-up protocol and 18 of sperm prepared by the standard Percoll gradient method.

Results—Electrophoretic and spectral analysis of RNA revealed high quality 18S and 28S rRNAs in 71 of 83 whole semen samples (86%) and 15 of 18 mature sperm swim-up samples (83%). However, high quality RNA was isolated from only 7 of 18 Percoll gradient sperm samples (39%). Interestingly quantitative reverse transcriptase-polymerase chain reaction analysis of 4 somatic and 10 germ cell markers showed that whole semen and swim-up samples had similar RNA profiles. RNA sequencing revealed that most encoded proteins were involved in mature sperm function, regulation of DNA replication, transcription, translation, cell cycle and embryo development.

Conclusions—We believe that semen and sperm specific RNAs are highly informative biomarkers for germ cell stages and somatic cell contribution. Therefore, these RNAs could be valuable diagnostic indicators of sperm survival, fertilization and early embryogenesis, and could serve as a predictor of the in vitro fertilization prognosis.

Keywords

fertilization; infertility; male; RNA; biological markers; spermatozoa

*Correspondence: Department of Obstetrics, Gynecology and Reproductive Sciences, Magee-Womens Research Institute, School of Medicine, University of Pittsburgh, 204 Craft Ave., Room A206, Pittsburgh, Pennsylvania 15213 (telephone: 412-641-6290; FAX: 412-641-8519; yatsenkoan@mwri.magee.edu).

Male infertility affects almost 7% of men worldwide. [1,2] It is diagnosed by routine semen analysis, ie cell count, morphology and motility.[3] Although sperm abnormalities are common in infertile men, almost 50% of patients are diagnosed with idiopathic infertility to which genetic factors are likely contributors.[4,5] More than 3,000 genes are expressed in the male reproductive system and more than 500 animal knockout models were reported with male infertility.[6–8] Progress in understanding human infertility is limited due to its clinical and genetic heterogeneity.[7,9]

Despite transcriptional silencing and cytoplasmic expulsion of RNA in mature sperm several types of RNA have been successfully identified.[10–13] Two models of the biological role of sperm RNA have been offered, including 1) existing RNAs are residual material of no functional significance and 2) sperm RNAs have a vital biological role in maturation, fertilization and/or post-fertilization events.[14,15] However, the lack of high quality RNA isolation techniques has hampered sperm RNA studies.[16–21]

Recently groups studying human sperm RNA attempted to develop a reliable RNA isolation protocol devoid of contaminating somatic cells, debris and DNA.[12,13] However, sperm RNA quality assessed by RIN was suboptimal, mainly due to high RNA fragmentation. To investigate the natural RNA profile in whole semen and mature sperm we extensively studied RNA integrity, quality and content in a large cohort of patients in diverse clinical semen categories. We report the usefulness of sperm and semen RNA for diagnosing male infertility and assessing sperm quality for in vitro fertilization.

Materials and Methods

Men were recruited at the Center for Fertility and Reproductive Endocrinology at Magee-Womens Hospital, University of Pittsburgh Medical Center. Semen specimens (119) were de-identified and collected according to our institutional review board protocol. Samples were maintained at 37C (fig. 1). Specimens were classified into clinical semen categories, including oligozoospermia (24), severe teratozoospermia (7), asthenozoospermia (15) and normozoospermia (55) (supplementary table 1, http://jurology.com/). We applied our HQSR method to 119 samples, including 83 whole semen specimens, 18 sperm aliquots prepared by the swim-up method and 18 sperm samples sorted by the Percoll gradient protocol (supplementary table 1, http://jurology.com/). The Percoll gradient sperm separation procedure was used to compare our HQSR procedure to published isolation protocols.[22]

Whole semen was washed and pelleted with an equal volume of 37C Sperm Washing Medium (Irvine Scientific®). Samples were minimally mixed and spun to preserve RNA. Using the swim-up method we aliquoted semen under 37C Quinns Advantage® Fertilization HTF Universal Medium. Specimens were incubated at a 45-degree angle at 37C in 5% CO_2. The top layer containing mature motile sperm was washed with 37C Sperm Washing Medium. Using the Percoll gradient method we placed whole semen on 50% and 90% layered ISolate® Stock Solution at 37C. After spinning the pellets were washed twice in 37C Complete P-1® Medium with SSS™. For sperm suspensions 37C TRIzol® was used and RNA was isolated according to the manufacturer protocol. Total RNA was purified with the RNeasy® Mini Kit. RNA quality and concentration were assessed by a NanoDrop®

device (260/280 >1.8 and 260/230 >2) and a bioanalyzer (Agilent, Santa Clara, California) with RIN greater than 3 indicating the presence of 18S and 28S rRNA.[23]

cDNA was synthesized according to manufacturer instructions (Epicentre® Biotechnologies). We performed qRT-PCR with iQ™ SYBR® Green Supermix on 14 genes and a *GAPDH* control in 12 semen and 12 SU samples (supplementary table 2, http://jurology.com/). Relative transcript quantities were calculated by the primer efficiency corrected ΔCt method as $E = 10^{(-1/slope)}$ with quantities of control and experimental transcripts = E^{-Ct} and relative quantity = average tested cDNA quantity/ average reference cDNA (*GAPDH*) quantity. Relative transcript quantities of whole semen and post swim-up cDNAs were averaged for each marker and are shown individually with the SD. Embryogenesis markers were selected by mining the DAVID Bioinformatics Database (http://david.abcc.ncifcrf.gov/) for binding partners, ie KRAB domains, and annotated with the PANTHER Classification System (http://www.pantherdb.org/).[19,24,25]

Three fertile WS RNA samples were subjected to next generation RNA-seq. Two pooled RNA samples consisted of an equimolar amount of RNA from 5 individual patients with similar semen parameters, including RS4 (64 to 77 × 10^6/ml, normal morphology 12% to 15%) and RS6 (115 to 155 × 10^6/ml, normal morphology 14% to 21%). Ribosomal RNA reduction was performed with the RiboMinus™ Eukaryote Kit for RNA-Seq. Whole transcriptome library preparation was done with random primers using Total RNA-Seq Kit (Life Technologies, Grand Island, New York) and applied to a SOLiD® flow cell. Sequencing reads (75 nt) were collected using the SOLiD 5500×l Sequencer. One normozoospermic sample (RS7, 111 × 10^6/ml, normal morphology 13%) was sequenced without rRNA reduction using the described protocol. RNA-seq reads were mapped to hg19 using the TopHat 2.0.6 mapper (http://ccb.jhu.edu/software/tophat/index.shtml) with Bowtie aligner (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml).[26,27] Expression was measured as normalized supertranscript quantity with the combined coverage of each exon normalized against transcript length using RNASeqnator software (R. Sethi and J. Lyons-Weiler, personal communication).

A threshold value of significant expression was determined by plotting a density curve of normalized transcript quantity to find the first inflection point, where distribution switched concavity. Threshold expression values less than 1 were considered noise. Proteins encoded by selected mRNAs were classified into functional categories using IPA. IPA functional annotations were derived from the Ingenuity Knowledge Base, including the Gene Ontology (http://geneontology.org/), NCBI (National Center for Biotechnology Information) ENTREZ Gene, MGI (Mouse Genome Informatics http://www.informatics.jax.org/) and PubMed® databases. RNA-seq data is submitted in NCBI GEO (Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/).

Results

We studied the RNA of 119 diverse semen samples representing oligozoospermia, severe teratozoospermia, asthenozoospermia and normozoospermia. Using our HQSR protocol we isolated total RNA and performed molecular analysis of 18 WS samples and 18 swim-up

separated motile sperm samples prepared from the same ejaculates as well as 18 total sperm separated by the standard Percoll gradient protocol. We expanded the RNA study by an additional 65 WS samples for a total of 83 (fig. 1). Most semen specimens contained 0.1% to 2% round cells except 4 samples with 11% to 17% round cells (supplementary table 1, http://jurology.com/).

RNA isolation with warmed TRIzol (37C) produced better quality RNA than the standard room temperature TRIzol protocol. Whole semen RNA showed uniform electrophoretic and spectral profiles, including visible fractions of about 100, about 500, about 1,500, about 2,000 and about 4,000 nt with the latter 2 values equivalent to intact 18S and 28S rRNAs, respectively (fig. 2, A). In 17 of 18 and 14 of 18 WS and SU specimens RNA showed 18S and 28S rRNA peaks, respectively (fig. 2, B). Sperm RNAs prepared by the Percoll gradient protocol were of lower quality. While 12 of 18 samples had visible 18S rRNA, only 1 of 18 showed intact 28S rRNA. In addition, 6 of 18 PG samples were substantially degraded (fig. 2, C).

Most RNAs from WS and SU samples demonstrated a RIN value of 5-6 while most PG RNAs showed a RIN of only 1 (fig. 3, A). Overall RNA of moderate to high quality (RIN 3-8) was obtained in 71 of 83 WS samples (about 86%) and in 15 of 18 SU samples (about 83%) (fig. 3, B). Independent assessment of ribosomal RNA quality according to the 28S/18S rRNA ratio (greater than 0) showed that rRNAs were present in 56 of 83 WS (about 69%) and 12 of 18 SU samples (about 67%) (fig. 3, B). RNA quality of sperm and whole semen is similar, suggesting that the contribution of round cells to whole semen RNA is measureable and minimal. This contrasted with PG samples of which only 7 of 18 (39%) showed high quality RNA (RIN greater than 3 with 18S and 28S rRNA present) and 2 of 18 samples (11%) had a 28S/18S rRNA ratio of greater than 0 (fig. 3, B). We plotted a graph with RIN values for WS, SU and PG samples, and found that they were randomly distributed ($R^2 = 0.05$, $p = 0.007$, no correlation) and did not correlate with the sperm amount (fig. 4, A).

To estimate rRNA in semen we removed rRNA using RiboMinus™ in 6 whole semen RNA samples and compared bioanalyzer readings before and after the procedure (supplementary fig. 1, http://jurology.com/). After rRNA removal we did not detect the 2 major 2,000 and 4,000 nt rRNA peaks (supplementary fig. 1, http://jurology.com/). The average amount of total RNA before rRNA removal was about 4.02 μg per sample while the post-procedure RNA amount was about 1.25 μg per sample, suggesting that approximately 69% of total RNA (rRNA) was removed (supplementary fig. 1, http://jurology.com/). This was independently corroborated by RNA sequencing of total RNA without rRNA removal in a fertile control sample. Ribosomal RNAs accounted for about 9,803,574 of the total of 13,456,512 aligned reads (73%).

We analyzed the relationship between sperm count and RNA amount in 3 groups of samples (fig. 4, B). RNA yield strongly correlated with the amount of sperm in WS ($m = 0.052$, $R^2 = 0.80$) and SU samples ($m = 0.046$, $R^2 = 0.76$) while PG samples showed a weaker correlation, perhaps due to stringent germ cell removal ($m = 0.005$, $R^2 = 0.37$, fig. 4, B). Percoll gradient cell selection retained only mature motile sperm, removing about 60% of

total sperm in a given sample (supplementary fig. 2, http://jurology.com/). The round cell count did not correlate with the amount of RNA (fig. 4, B, inset), suggesting that somatic RNA is a nonsignificant contributor to whole semen RNA quantity.

To independently measure somatic cell RNA in WS and swim-up separated mature sperm we performed qRT-PCR for the somatic mRNA markers in 12 WS and 12 SU RNA samples, including *PTPRC* (*CD45*) and *CD34* (leukocyte and lymphocyte), *MSMB* (epithelium) and *PIP* (prostate) markers (supplementary table 3 and fig. 3, http://jurology.com/, and fig. 5). We calculated transcript quantity in each sample and then calculated the average transcript quantity to compare WS and SU (mature sperm) mRNAs (supplementary fig. 3, http://jurology.com/ and fig. 5). There was no detectable quantity of round cell markers in SU samples. We detected a minimal number of somatic RNA markers (about 0.05% to 0.8% of the mature sperm marker *GAPDHS*) in whole semen RNA with a variable number of round cells (supplementary fig. 3, http://jurology.com/ and fig. 5, inset).

We performed a qRT-PCR series to measure 10 germ cell specific mRNAs in WS and SU RNA (supplementary table 3 and fig. 3, http://jurology.com/, and fig. 5). These well established germ cell specific transcripts were previously documented in mouse models, and functional and expression studies. They include the spermatogonia markers *KIT* and *PLZF*, the premeiotic spermatocyte markers *TEX11* and *KLHL10*, the mature sperm markers *ZPBP* and *GAPDHS*, and the post-fertilization and early embryogenesis markers *SLFNL1*, *OXCT2*, *FAM187B* and *POU5F1*. Mature sperm do not have a measurable quantity of the spermatogonial transcripts *KIT* and *PLZF* but they have detectable amounts of the spermatocyte transcripts *TEX11* and *KLHL10* (about 0.1% of the *GAPDHS* amount) (supplementary fig. 3, http://jurology.com/ and fig. 5). Whole semen has a low quantity of spermatogonial transcripts (about 0.2% that of *GAPDHS*) and a significant number of spermatocyte transcripts (about 3.5% that of *GAPDHS*). WS and SU RNAs showed almost identically high quantities of mature sperm markers (*ZPBP* and *GAPDHS*). Interestingly each group showed relatively high and uniform amounts of 4 embryogenesis markers (*SLFNL1*, *OXCT2*, *FAM187B* and *POU5F1*) (supplementary fig. 3, http://jurology.com/ and fig. 5). OXCT2 is a 3-oxoacid coenzyme A-transferase 2, which along with SLFNL1 and FAM187B interacts with KRAB domain transcription factors according to the DAVID database. These RNAs encode proteins that probably function as gene silencers via DNA methylation of certain gene promoters in post-fertilization zygote divisions.[28]

To characterize the sperm specific transcriptome we performed RNA sequencing on 2 pooled rRNA depleted, whole semen RNAs from normozoospermic fertile males as isolated by our modified protocol (RS4 and RS6, 64 to 77 × 10^6 and 115 to 155 × 10^6 sperm per ml, respectively, NCBI GEO Accession No. GSE52665). Whole semen RNA-seq produced an average of 52 million reads per sample with an average of about 50-fold coverage across the transcriptome, producing 4,614 (RS6) and 10,141 (RS4) significant transcripts. We performed IPA functional analysis of the significantly expressed genes (fig. 6, A). The analysis annotated 3,149 and 5,009 known genes primarily related to mature sperm function, DNA replication, transcriptional and translational regulation, cell cycle, cell signaling and embryogenesis. Analysis also identified 1,465 and 5,132 novel genes without functional annotation. The 2 transcriptomes showed similar profiles except for a decrease in

spermatogenesis associated transcripts and an increase in apoptosis related mRNAs in the RS4 reduced sperm count sample.

We analyzed the NQ of the same 4 somatic and 10 germ cell transcripts previously tested by qRT-PCR (supplementary table 4, http://jurology.com/ and fig. 6, B). The white blood cell markers *CD45* and CD34 showed consistently minimal NQ values. These results were consistent with qRT-PCR data. The epithelial marker *PIP* had a low NQ value of about 0.5 to 0.8, approximately 12% that of *GAPDHS*. *MSMB* encoding prostate secreted seminal plasma protein showed a variable NQ between the 2 samples of about 0.06 to 0.5, approximately 1.2% to 11% that of *GAPDHS*. The spermatogonial markers *KIT* and *ZBTB16* had similarly low NQs of 0.4 to 0.6 for an average of about 10.9% that of *GAPDHS*. The spermatocyte markers *TEX11* and *KLHL10* had moderate NQs of 0.5 to 0.7, about 19.5% that of *GAPDHS*. The post-meiotic markers *ZPBP* and *GAPDHS* had high overall NQs of about 2.5 and about 4.9, respectively (40% that of *GAPDHS* for *ZPBP*), with low variability among samples. Early embryogenic mRNAs of *SLFNL1*, *OXCT2* and *FAM187B* had relatively high NQs of 1.6 to 3.4, approximately 30% to 40% that of *GAPDHS*. The quantity of early embryonic marker *POU5F1* was notably lower with a NQ of 0.65 (about 13%) (fig. 6, B). When we compared these results with qRT-PCR data, we noted a similar pattern among the markers studied. However, RNA-seq values were higher for *PIP*, *MSMB*, *KIT*, *ZBTB16*, *KLHL10* and *TEX11* due to the absence of internally controlled gene normalization, which was done for qRT-PCR.

Discussion

Human male infertility is a poorly understood health condition with descriptive clinical semen analysis and poor prognosis for assisted reproductive technology.[7,8] Current genetic testing in infertile males, which is limited to karyotyping and Y chromosome microdeletion tests, is informative in about 10% of these men while most of them are diagnosed with idiopathic infertility. IVF and ICSI are among the few assisted reproductive technology solutions available for infertile men. However, success rates are 50% or less and decrease with patient age.[29] Sperm RNA is a promising diagnostic marker of sperm fertility that might eliminate these problems.[15] However, inconsistent quantity, insufficient purity and absent intact 18S and 28S rRNAs have hindered sperm RNA research.[12,13]

We present what is to our knowledge the largest study of mRNAs and rRNAs in semen and sperm to date. We report HQSR, which improves RNA quality by minimizing sperm damage, reducing isolation time, limiting exposure to chemically active compounds and maintaining semen at 37C. Comparison of WS, SU and PG RNAs indicated that the least damage was observed in WS and SU while PG samples showed relatively poor RNA quality as previously observed. Intact, high quality RNAs (up to 4,000 nt) were naturally present in greater than 80% of WS and SU samples irrespective of the clinical semen diagnosis. The contribution of somatic cells to RNA yield and RIN was mostly minimal except in severely oligozoospermic samples. While sperm separation by standard Percoll gradient eliminates somatic round cells, it removes almost 60% of sperm and perhaps contributes to RNA fragmentation, particularly to the ribosomal 18S and 28S RNAs that are naturally present.

We performed RNA-seq and functional annotation of the normal sperm transcriptome and identified many genes related to mature sperm. Importantly more genes were associated with DNA replication, transcriptional and translational regulation, cell cycle and cell signaling, indicating possible roles in fertilization, blastocyst formation and embryonic division. qRT-PCR analysis with 14 stage specific mRNA markers revealed that most mRNAs originated from spermatocytes and mature sperm while somatic and spermatogonial cells left only residual mRNAs. The most consistent amounts were noted for the fertilization and embryogenic markers *FAM187B*, *SLFNL1*, *OXCT2* and *POU5F1/OCT3* in WS and SU specimens regardless of diagnosis. The first 3 mRNAs are associated with KRAB domain containing proteins and involved in DNA methylation during early embryogenesis.[28]

We noted that with time, under stress or at sub-optimal temperature ribosomal RNAs were more susceptible to degrading than mRNAs. We hypothesize that intact rRNA and mRNA degrade at different rates in sperm due to mRNA specific preservation.[13,30] RNA binding proteins protect certain mRNAs in cytoplasmic structures that serve as sperm RNA depots (spermatozoal cement or the chromatoid body) while rRNAs are present in unprotected ribosomes and degrade quickly.[12] This could explain the difference in the degradation rate and quality between mRNA and rRNA in the same specimen. We speculate that the ratio of full-length 18S to 28S rRNA could be a strong predictor of sperm integrity due to the likelihood of speedy degradation of rRNA. Therefore, rRNA integrity could be a good indicator of viability and fertilization potential in clinical testing and may dramatically improve the success rate of IVF and ICSI. We speculate that certain mRNAs involved in fertilization and early embryogenesis are specifically preserved and could serve as versatile diagnostic markers of white blood cells, epithelial cells, immature germ cells and ultimately of viable mature sperm.

Conclusions

High quality RNAs were present in 86% of whole semen samples and in 83% of mature sperm samples. This indicates that sperm RNA is mostly intact and can be consistently tested. We hypothesize that RNA could have great clinical usefulness for diagnostic purposes, such as testing whole semen and sperm, and assessing sperm quality, in patients undergoing IVF/ICSI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations and Acronyms

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HQSR	high quality sperm RNA isolation
ICSI	intracytoplasmic sperm injection
IPA	Ingenuity® Pathway Analysis
IVF	in vitro fertilization
KRAB	Kruppel-associated box
NQ	normalized supertranscript quantity
PG	Percoll gradient isolated sperm
qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction
RIN	RNA integrity number
RNA-seq	RNA sequencing
SU	swim-up isolated sperm
WS	whole semen

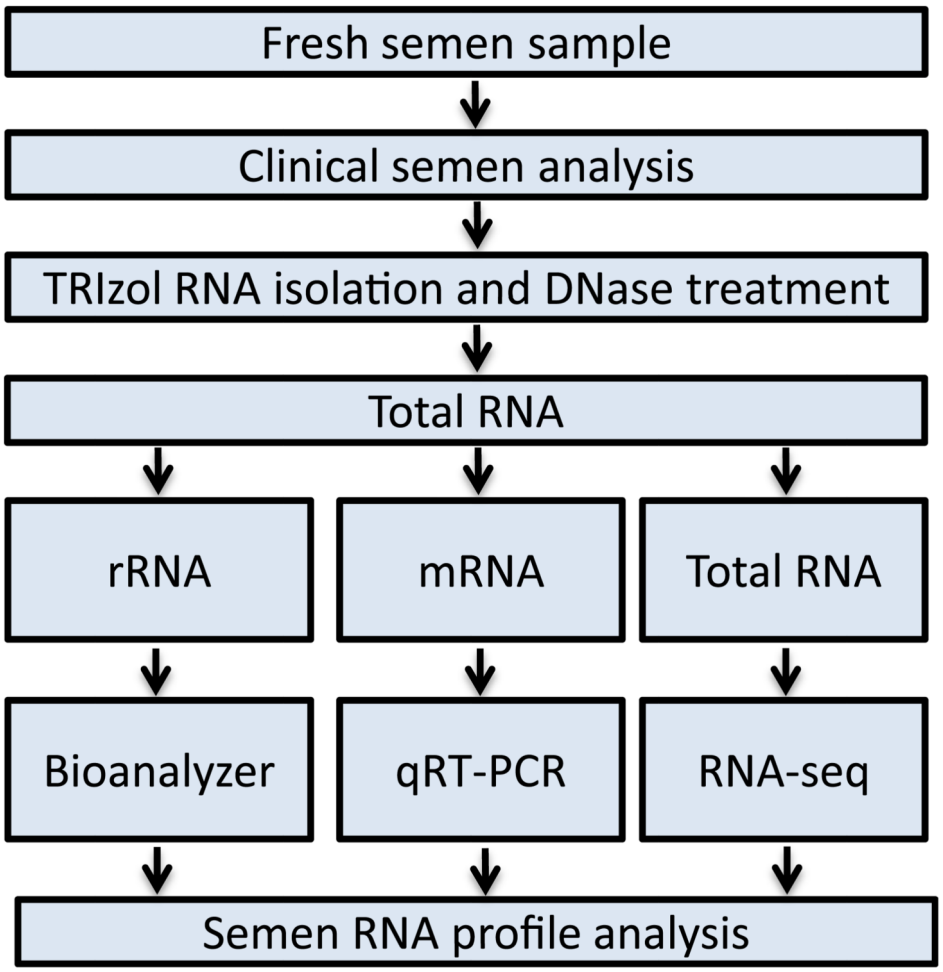

Figure 1.

Study design of human semen and sperm RNA profile analysis. Fresh semen samples were collected and analyzed according to 2010 WHO guidelines. Whole semen was kept at 37C. Ejaculate was immediately processed by TRIzol RNA isolation and deoxyribonuclease treatment. RNA from swim-up separated motile sperm and Percoll gradient separated sperm were also isolated. Total RNA was analyzed by 3 methods to obtain semen RNA profile. 1) rRNA was analyzed by bioanalyzer to identify degradation in sample. 2) mRNA was analyzed for relative transcript quantity by qRT-PCR for somatic and germ cell RNA markers. 3) rRNA depleted RNA was used for RNA-seq to identify full expression profile in mature sperm.

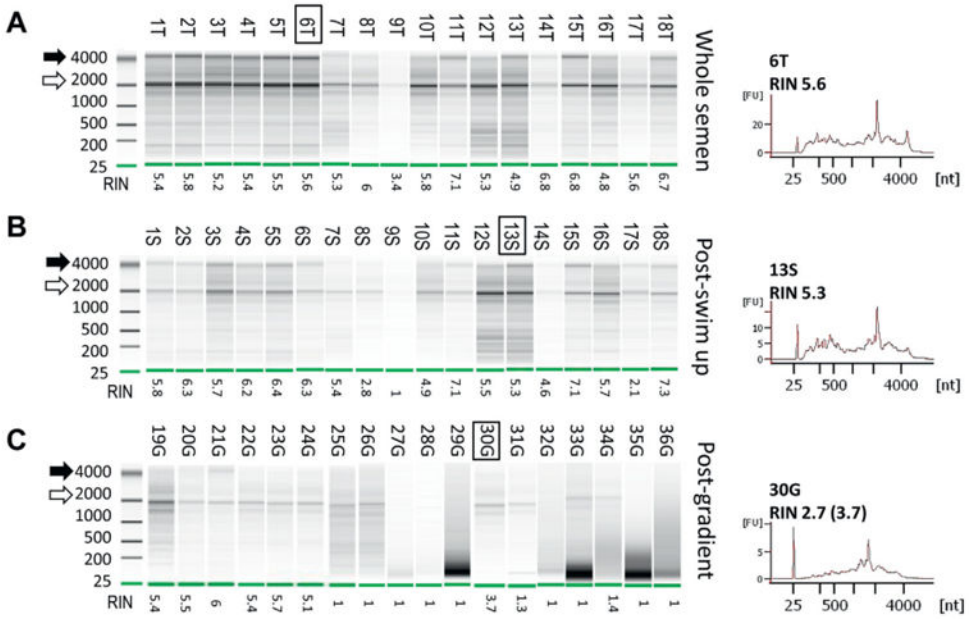

Figure 2.

Electrophoretic profile comparison of RNA from whole semen, swim-up separated mature sperm and gradient purified mature sperm. *A*, total RNA isolated from 18 whole semen samples was of high quality with intact 18S (white arrows) and 28S (black arrows) rRNA of about 2,000 and about 4,000 nt, respectively. *B*, RNA of 18 post swim-up separated mature sperm samples was also high quality. Most samples had full-length 18S and 28S rRNAs. *C*, RNAs from 18 mature sperm samples prepared by Percoll gradient were poorer quality. Most samples had 18S rRNAs but few intact 28S rRNAs and 6 were fully degraded with all RNA fragments below 200 nt. Black boxes represent samples with RIN closest to average RIN of entire group. Graphs show spectral profiles of these RNAs.

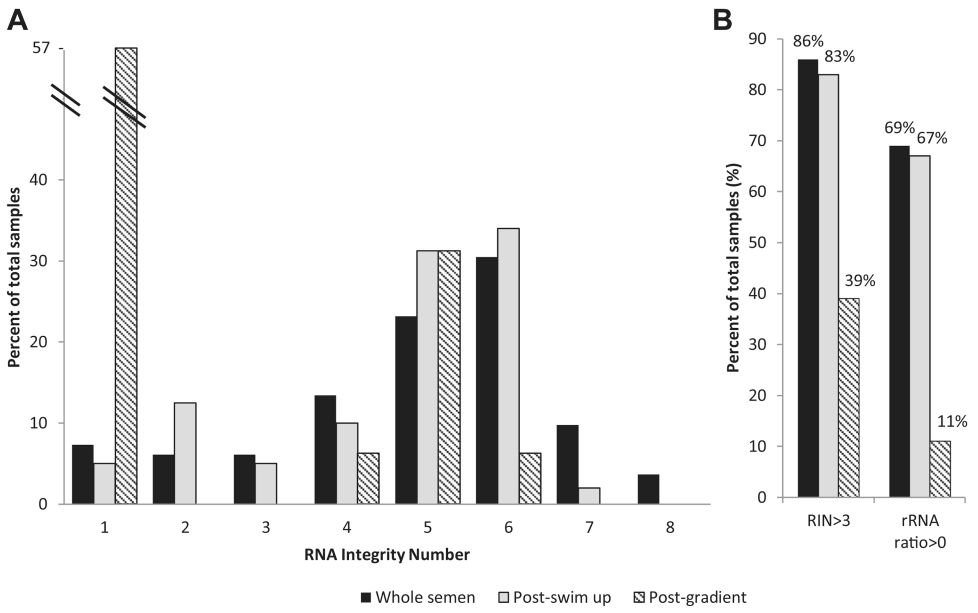

Figure 3.

RNA quality comparison of 83 whole semen, 18 swim-up separated mature sperm and 18 gradient purified mature sperm samples. *A*, RIN of more than 50% of gradient separated sperm RNA was 1 and RIN of most SU and whole semen RNAs was 5-6. *B*, overall rate of RNA samples with RIN greater than 3 and 28S/18S rRNA ratio greater than 0 as alternative indicator of 18S and 28S rRNA presence. RIN greater than 3 was found in 71 of 83 whole semen RNAs (86%) and 15 of 18 of SU RNAs (83%) but only 7 of 18 gradient prepared sperm RNAs (46%) had identifiable 28S rRNA. Of 83 whole semen samples 56 and 12 of 18 post-SU RNA samples had 28S/18S rRNA ratio was greater than 0. Two of 18 gradient separated mature sperm samples (11%) had rRNA ratio greater than 0.

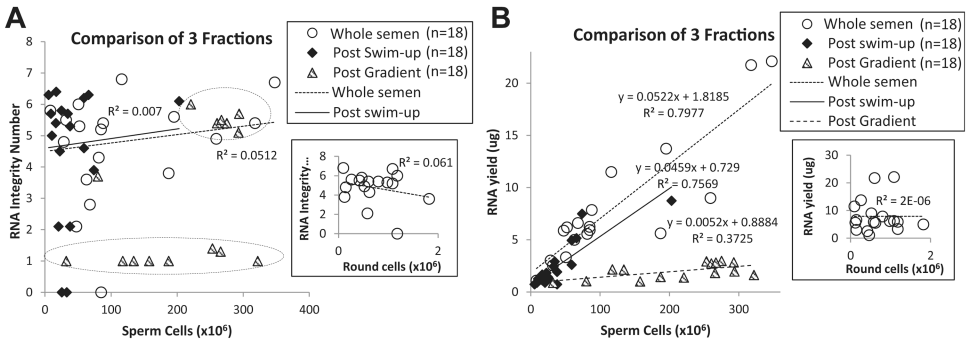

Figure 4.

Relationships of sperm, round cells and RNA calculated by simple linear regression. *A*, total sperm and RIN in 18 samples each of whole semen, post swim-up mature sperm and post-gradient mature sperm. Inset, RIN relationship to total number of round cells in semen samples. *B*, sperm count and RNA yield in 18 samples each of whole semen and sperm separated by swimup and gradient methods. Inset, RNA yield relationship to total number of round cells in whole semen samples. Circles represent whole semen RNA. Dotted lines indicate its correlation. Diamonds represent swim-up separated mature sperm RNA. Solid line indicates its correlation. Triangles represent post-gradient mature sperm RNA. Dashed line indicates its correlation.

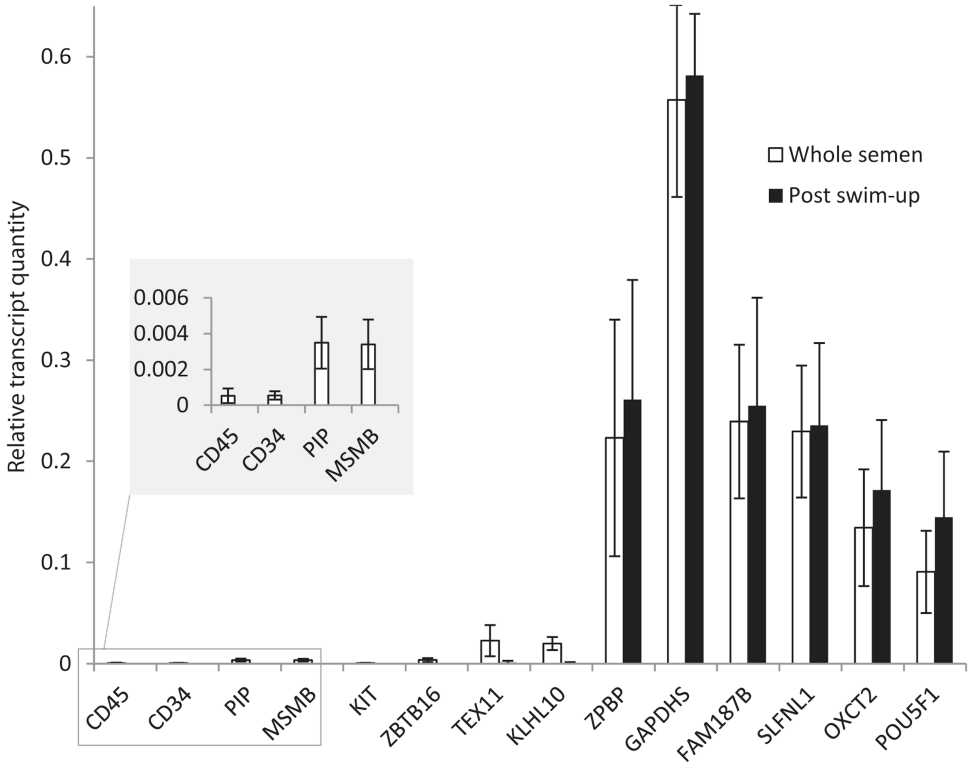

Figure 5.

Relative transcript quantity of somatic and germ cell markers detected by qRT-PCR in whole semen and swim-up mature sperm RNA. Quantity of somatic markers *CD45*, *CD34*, PIP and *MSMB* was relatively low in whole semen at less than 1% of that of mature sperm marker *GAPDHS* (inset). Levels of spermatogonial markers *KIT* and *ZBTB16* were similar to those of somatic markers. SU samples did not contain identifiable levels of somatic cell and spermatogonial markers, indicating that transcripts were not present in mature sperm cells. Spermatocyte markers *TEX11* and *KLHL10* showed low amounts of RNA by each method. Mature sperm and post-fertilization marker levels were high with uniform quantity among samples.

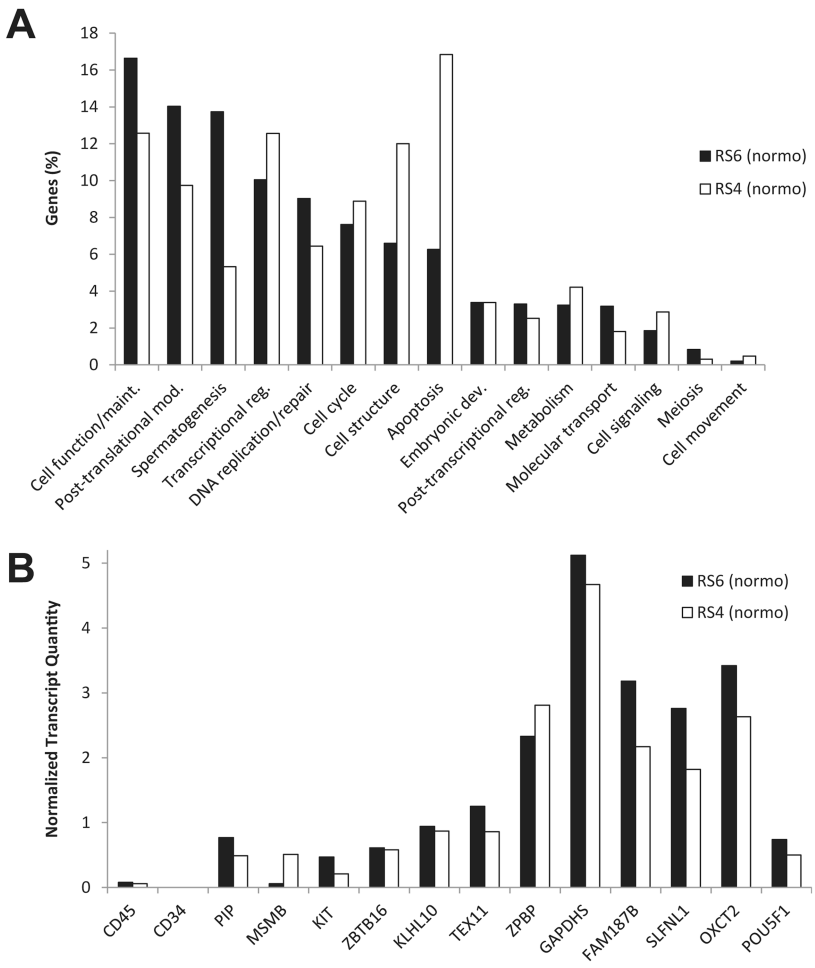

Figure 6.

RNA-seq of normozoospermic (*normo*) sperm RNA from 2 fertile males. *A*, functional annotation of significantly expressed genes in RS6 and RS4 normozoospermic sperm transcriptomes using IPA, which annotated 3,149 (RS6) and 5,009 (RS4) genes. Functional categories were cell function/maintenance (*maint*.) (cellular assembly and organization, function of cellular components and cell maintenance), post-translational modification (*mod*.) (protein synthesis, ubiquitination, folding, etc), spermatogenesis (germ cell and sperm differentiation and maturation), transcriptional regulation (*reg*.) (initiation, promoter binding, etc), cell structure (structural components and cytoskeleton development), embryonic development (*dev*.) (blastocyst formation, embryonic cell division, organ formation, etc), post-transcriptional regulation (RNA processing, splicing and RNA induced silencing complex), molecular transport (protein, hormone, ion transports, etc), cell signaling (signal transduction and cell-cell signaling) and cell movement (flagella/cilia organization and vesicle trafficking). Some genes were assigned to more than 1 functional category. *B*, normalized quantity of 14 transcripts expressed in somatic cells and spermatogenesis used in qRT-PCR.