



## In Silico Analysis of Sperm From Ejaculates with Different Semen Characteristics

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### Abstract

**Background:** Male infertility is associated with altered characteristics of the sperm within the ejaculate. It is possible to find molecular explanations for the observed phenotypes and their consequences. This study aimed to analyze, using a specialized software, a gene set of transcriptomic data from different types of ejaculates.

**Methods:** Data from ejaculate samples categorized as normal, oligospermia, and teratozoospermia were obtained from Gene Expression Omnibus (GEO). After normalization, the data average for each sample category was calculated and analyzed independently using Ingenuity Pathway Analysis (IPA).

**Results:** Five important canonical pathways are involved in normal and altered semen samples (Oligospermia and teratozoospermia) except sirtuin signaling and mitochondrial dysfunction pathways. The five most important biological processes are identified in all semen phenotypes, but the only difference is the genes connected with initiation of RNA transcription in oligospermic and asthenospermic samples.

**Conclusion:** Surprisingly, different types of ejaculates share many pathways and biological processes; sperm proteomics as a new global approach gives clues for the development of strategies to explain the reason for observed phenotypes of ejaculated spermatozoa, their possible effect on fertility, and for implementing research strategies in the context of infertility diagnosis and treatment.

**Keywords:** Fertility, Male, RNA, Semen, Spermatozoa, Transcriptome.

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### Introduction

The evaluation of male fertility potential is based on physical exam, hormone, and semen analysis (1-3). Semen analysis is quick and relatively inexpensive but only describes the parameters of the cells within the ejaculate. As a consequence, it helps to find causes of infertility and allows to define therapeutic interventions although the origin of the observed phenotype is not explained. It is necessary to look for new approaches to identify the etiologies of male infertility that help us to understand the reproductive biology of a clinical condition (4). Clinically, male infertility is confirmed when poor sperm quality is observed including low concentration, motility, and morphology, each one alone or combined; it

has been shown that semen analysis describes the cellular aspects of the ejaculate but rarely predicts the functioning or fertilizing capacity of the sperm.

Mammalian spermatozoa are produced in testis after a complex process called spermatogenesis, which includes spermatogonial mitosis for germ line preservation before entering meiosis. Terminal differentiation of sperm precursors (5, 6), final maturation process in the epididymis, and alterations of this process are the causes of poor sperm quality and male infertility.

From the genetic point of view, the spermatogenic process in mammals requires a spatiotemporal expression of different genes, some of them

specific of the testis. Based on knockout mice models, it has been reported that some of these genes produce molecules that affect sperm quality (7-9). Though the common belief is that the unique function of the sperm is to deliver its DNA to the egg and also that it is transcriptionally inactive, the discovery of mRNAs in mature sperm questions this view (10). Indeed, the evidence suggests that some transcripts must be used for the fertilization and early embryonic development, evidenced by the fact that some sperm mRNAs found in the zygote are functionally important (11). Several recent studies have associated the presence of mRNAs in human sperm with sperm quality and fertility (12), and proposed these molecules might have clinical application as markers for predicting spermatogenesis status and the fertility potential of the patient (6).

The Gene Expression Omnibus (GEO) is a data repository that provides information for *in silico* analysis of the gene expression of a given cell or tissue (13), and recently it has been reported that many new candidate genes affect male fertility (14). This fact associated with the use of specific and integrative software allows researchers to analyze different aspects of a given phenomenon. Ingenuity Pathway Analysis (IPA) (5) is a software designed based on data derived from omics experiments; it integrates data related to specific conditions and helps to analyze, integrate, and interpret the data coming from different nucleic acid sources (RNAseq, small RNAseq, microarrays including miRNA and SNP), proteins, and metabolites. IPA goes beyond pathway analysis by identifying key regulators to explain expression patterns, predicting downstream effects on biological and disease processes, providing targeted data on genes, proteins, chemicals, drugs, and building interactive models of experimental systems (15).

Transcriptomic analysis of sperm and ejaculates has been performed in different species, such as humans (16), bull (17), boar (18), and sheep (19). Specifically, in humans, it has been described in different clinical conditions such as oligozoospermia (20), asthenozoospermia (21, 22), and teratozoospermia (23); in bovines, it has been used to identify potential markers of fertility (24), freezing tolerance in boar (18), seasonal changes in RNA production (25), or expression control in sheep (19). The study of pathways associated with gene expression in a given physiological process allows to investigate the nature of a phenomenon

in detail, and the comparison of them in different phenotypes helps at least to propose theories that explain the nature of their occurrence.

This work aimed at analyzing transcriptomic data of sperm cells from three different ejaculates (Normal, oligospermia and teratozoospermia) using IPA software as a way to understand and explain the observed differences in the phenotype of the ejaculates.

### Methods

Data were obtained from GEO from the subset GSE 26881 that is part of a big series with accession number GSE26982. It includes 18 ejaculates, classified according to sperm count, motility, and morphology as normal (n=12), oligospermic (n=3), and teratospermic (n=3). In this study, the data deposited in GEO and reported by Pacheco et al. (21) were applied. Each ejaculate was processed through an optimized Percoll gradient (GE Healthcare, Sweden) to eliminate debris, non-sperm cells, and dead sperm.

After normalization of the signals, the data average of each sample's phenotype was calculated and analyzed independently using IPA (Qiagen, Germany). Next, top canonical pathways and the involved biological processes were analyzed.

### Results

Three different types of ejaculates (Phenotypes) were analyzed as normal, oligospermic, and teratospermic using the data deposited in GEO. The expression pattern of each phenotype was independently analyzed and later compared for discussion.

The top canonical pathways analysis is shown in table 1. Altered semen samples, oligospermic and teratozoosperm, shared the same canonical pathways, while normal samples showed sirtuin signaling and mitochondrial dysfunction which were different from the altered ones. Regarding the biological process, all semen samples shared cell death and survival, proliferation and cytostasis, export and transport of RNA molecules and proteins, protein synthesis and catabolism, but different genes were involved in initiation of RNA transcription in oligospermic and asthenospermic semen samples (Table 2).

### Discussion

After analyzing the gene expression in different sperm phenotypes, it was found that there are practically no differences in metabolic pathways,

**Table 1.** List of the top canonical pathways in three analyzed phenotypes

Teratospermic (n=3)	Normal (n=12)	Oligospermic (n=3)
Protein ubiquitination	Sirtuin signaling	Protein ubiquitination
Regulation of eIF4 and p70S6K signaling	mT or signaling	Regulation of eIF4 and p70S6K signaling
eIF2 signaling	eIF2 signaling	eIF2 signaling
mT or signaling	Regulation of eIF4 and p70S6K signaling	mT or signaling
Huntington's disease	Mitochondrial dysfunction	Huntington's disease

**Table 2.** List of top biological processes involved comparing three different phenotypes

Name	p-value	Overlap %	Overlap/mol
<b>Normal (n=12)</b>			
Cell death and survival	1.85E-24	27.2	302/1110
Proliferation and cytostasis	1.05E-17	25.6	266/1040
Initiation of RNA transcription	1.43E-17	28.5	186/653
Protein synthesis and catabolism	1.26E-10	30.2	90/298
Export and transport of RNA proteins and molecules	1.57E-09	27.0	111/411
<b>Oligospermic (n=3)</b>			
Cell death and survival	7.26E-06	10.3	114/1110
Proliferation and cytostasis	8.57E-05	9.9	103/1040
Protein synthesis and catabolism	3.16E-04	12.4	37/298
Formation of vesicles	1.78E-03	20.0	10/50
Export and transport of RNA proteins and molecules	2.11E-03	10.0	44/411
<b>Teratospermic (n=3)</b>			
Cell death and survival	3.07E-07	10.3	114/1110
Proliferation and cytostasis	1.78E-04	9.2	96/1040
Formation of vesicles	2.60E-04	22.0	11/50
Export and transport of RNA proteins and molecules	1.05E-03	10.5	43/411
Protein synthesis and catabolism	2.88E-03	10.7	32/298

biological processes, and networks between the three phenotypes analyzed. Unlike other studies, this study investigated similarities or differences in pathways or biological processes or networks of sperm from different phenotypes and did not evaluate the specific genes (26) associated with three different observed phenotypes that many researchers have treated as markers.

In IPA, to our surprise, the top canonical pathways from oligo and teratospermic samples have the same pathways, and a few are different from the normal semen samples. The most important network in altered phenotypes is protein ubiquitination, a process associated with degradation in all eukaryotic cells. Its most important function is to act as a signal for 26S proteasome-dependent protein degradation and it works as a cascade of three different enzymes including E1 (Ubiquitin-

activating enzyme), E2 (Ubiquitin-conjugating enzyme), and E3 (Ubiquitin ligase). Each E3 is specific to its substrate, or proteins to be targeted by ubiquitination (27). In the normal samples, the most important pathways are sirtuins (SIRT), which are protein deacetylases, phylogenetically conserved from microorganisms to humans and they are nicotine adenine dinucleotide dependent. In humans, there are seven types of SIRT found in a wide variety of subcellular locations which function as regulators of transcriptional repression. SIRT do not function alone as they need other proteins to form complexes. Moreover, they have a key role in pathophysiological processes associated with neurodegeneration, muscle differentiation, inflammation, obesity, and cancer (28). Activation of SIRT3 helps to prevent the death of granulosa cells and oocytes (28). In mice testis,

abnormal expression of SIRT1 alters gene expression in spermatogenesis and sumoylation of proteins (29). Liu et al. (30) reported an accumulation of acetylated LC3 in the spermatid nucleus, which affected the recruitment of several acrosome biogenesis-related proteins as a consequence of the alteration of SIRT1.

Based on our results, it is not possible to determine if the activation of the mitochondrial dysfunction pathway protects the sperm from potential injuries or if it is part of the normal function in the ejaculate. However, the production of reactive oxidative species, as the by product of oxidative phosphorylation, leads to mitochondrial dysfunction. Mitochondria that are selectively recognized and targeted for degradation are repaired in the ejaculate, in a process called mitophagy, which contributes to the maintenance of a healthy mitochondrial network and apoptosis. This could be induced by the loss of the mitochondrial membrane potential caused by kinase PINK1 and the E3 ligase Parkin and recruitment of mitophagy receptors. There are other types of mitophagy pathways that are independent from the PINK1/Parkin pathways which are mediated by LIR-containing receptors as an answer to different stimuli (27).

The analysis of various biological functions shows that the spermatozoa are cells that express mainly genes associated with cell death and survival due to their highly differentiated cell nature. They have been prepared only to stay alive while they meet the egg. The differences show that the normal ejaculate has sperm molecules associated with the initiation of transcription and abnormal ejaculates show only vesicle formation as a typical difference.

The network analysis does not necessarily show specific patterns for different types of ejaculates, as the analysis of casual networks only reveals expressed molecules. The most appropriate interpretation would be that in the ejaculates, the cells have different fates and if a normal pattern is assumed in the ejaculate, then the subsequent events in sperm biology are preparations for fertilization or embryo development.

Transcriptomics provides information about the RNAs located in the studied cells, but it does not necessarily guarantee that these molecules will function within the cell. In the case of sperm, from an efficiency point of view, the majority of the observed mRNAs will be used and needed after chromatin compaction. One example is that transcripts associated with DNA decondensation

must be used after fertilization. If the sperm had the same nature similar to other cells in the body, one would expect that in the ejaculate, there would only be the RNAs needed to move, and hopefully, to join the oocyte.

Many of the detected mRNAs are related to the normal function of any given cell, not precisely associated with a specific function of the sperm. Only 10% of the sperm proteome (628 proteins) have moderately to extremely high-level expression (>25 RNA transcripts/per sperm) (16). This paradoxical assertion could be associated with the mathematical nature of the analysis, and not with the biological response in any research. It is impossible to conceive an experiment for analyzing thousands of genes at the same time. Lalancette et al. (31), studying sperm RNA samples, report that the majority of them show at least 80% similarity. They also analyzed "pairs" and found that most of them belong to specific and different metabolisms, and probably the concept of "pairs" is used to avoid technical problems.

A spermatozoon is the result of a complex series of biological processes, including spermatogenesis, spermiogenesis, and the completion of the meiosis (32). According to RNA-seq and microarray studies, a diverse and complex population of spermatozoal RNAs exist (33). Sperm transcripts are rich in small non-coding RNAs, long non-coding RNAs, and mRNAs, indicative of their transcriptional history. The results from transcriptomic studies are usually associated with the possibility of analyzing all spermatogenic events that could be used in fertility research (34, 35). Ontology and pathway mapping of these differential transcripts showed the disruption of several pathways, and as a consequence, manifested their association with the abnormal parameters observed. Teratospermy has been associated with altered ubiquitin-proteasome and apoptotic pathways (23); in asthenospermia samples, changes in the ubiquitin biosynthesis pathway have been identified (22), and for oligozoospermia, changes in DNA repair and oxidative stress regulation have been detected (20); also, lack of translation in the sperm is regulated by the inactivation of the rRNAs (36).

Indeed, the heterogeneous nature of the human ejaculate has been a "problem" for the identification of markers or molecules responsible for a specific function or phenotype; for this reason, some researchers have questioned the usefulness of sperm analysis in human fertility research (37).

To date, motility, morphology, and the quantity of sperm are the parameters to determine fertility. In a strict sense, the spermogram is a description of the cellular composition of the ejaculate. If this finding is evaluated at the transcriptomic level, it could be expected that the transcripts observed in the sperm must reflect the preparations for its function, but the analysis carried out in this report did not reflect those differences. To date, most of the research has been focused on the function of sperm transcripts during fertilization and early development (38, 39), and additionally, researchers hope that the analysis of the transcripts describes sperm phenotypes. Garcia-Herrero et al. (40) studied differential gene expression in sperm from males in an artificial insemination program which revealed profound differences in sperm samples. It is clear from the analyzed transcriptomic data that the results are usually associated with cell function and not different steps or specific processes in the spermatogenesis.

### Conclusion

In conclusion, the use of transcriptomics and the analysis of different phenotypes of ejaculated spermatozoa give some clues to explain the nature of the observed phenomena and to develop new strategies for explaining their possible effect on fertility in order to implement new research strategies for diagnosis and treatment of male infertility.

### Conflict of Interest

The authors have no conflicts of interest to declare.

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