Original Article

Profound Transcriptomic Differences Found between Sperm Samples from Sperm Donors vs. Patients Undergoing Assisted Reproduction Techniques Tends to Disappear after Swim-up Sperm Preparation Technique

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Abstract

Background: Although spermatozoa delivers its RNA to oocytes at fertilization, its biological role is not well characterized. Our purpose was to identify the genes differentially and exclusively expressed in sperm samples both before and after the swim-up process in control donors and infertile males with the purpose to identify their functional significance in male fertility.

Materials and Methods: This was a nested case-control study. Ten sperm samples were obtained from infertile patients [n=5 (two aliquots each from five samples; one before the swim-up process and one after)] and donors [n=5 (two aliquots from five samples, one before the swim-up process and one after)]. Oligonucleotide microarrays were employed to study the genome-wide expression of pooled samples from infertile patients vs. donors. A total of four microarrays were perfomed: two with sperm sample aliquots before swim-up and two with sperm samples aliquots after swim-up, from both the case and control groups. The results were evaluated to detect which genes expressed differentially [fold change (FC)>5 and $p<0.05$] and which genes were exclusive to each of the groups, both before and after swim-up.

Results: Profound differences were detected between the fresh sperm samples of donors vs. infertile patients with respect to both differentially and exclusively expressed genes. Nevertheless these differences seemed to decrease after the swim-up selection process.

Conclusion: There are important differences between the expression profiles of sperm samples of fertile donors vs. infertile patients who require assisted reproduction techniques (ART). These differences are potential forecasters of fertility success, although their reliability needs to be explored further.

Keywords: Gene Expression, Infertility, Microarray Analysis, Spermatozoa

Introduction

Male fertility has been diagnosed for several decades according to sperm count and motility. This type of sperm analysis is an easy, inexpensive and useful tool to determine the fertile status of a male. However, a significant number of men in whom a basic sperm analysis gives a normal result are unable to produce a full-term pregnancy (1). This fact clearly indicates the need to develop new male infertility tests to accurately diagnose the sperm samples of such individuals. Current work regarding sperm mRNA contents has detailed the importance of sperm transcripts in key reproductive events such as fertilization and early embryo development in several species (2).

Today it is well known that, as well as supplying DNA, sperm also provides the egg with paternal centrioles, proteins and RNAs (3). Although the functional significance of mRNA in mature spermatozoa remains essentially unexplored (4), it is known that mRNA is necessary from the moment of the first embryo cleavages until the activation of the embryonic genome (5) and may influence the phenotypic traits of embryos (6) and offspring (7, 8).

The male gamete is transcriptionally silent as

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a consequence of the highly condensed architecture of its chromatin and because there is little or no cytoplasm capable of withstanding translation. It has been originally hypothesized that the RNA present in semen is related to contamination from somatic cells. However, RNA remains present after stringent washing through density gradients, which shows that it originates in the sperm fraction and is subsequently introduced into the oocyte during fertilization (9).

As previously mentioned, sperm cells from infertile males whose sperm count and motility are not significantly decreased and whose partner is apparently normal exhibit a profoundly variable set of mRNAs with respect to the sperm of donors who father healthy newborns (10). These differences could explain, to some extent, the causes of infertility in natural (but not assisted) reproduction.

Nevertheless, sperm cells from infertile males undergoing assisted reproduction must exhibit certain capacities depending on the technique to be employed. For instance, sperm cells employed in intracitoplasmic sperm injection (ICSI) treatments do not have to present molecular features implicated in sperm-oocyte recognition, while those for intrauterine insemination (IUI) do (11). In this way, for each assisted reproduction protocol an analysis of ejaculates is necessary to characterize the molecular parameters essential for successful treatment.

Therefore, an analysis of the behavior of ejaculates is needed to characterize the molecular parameters required to succeed in each assisted reproduction protocol.

The efficacy of semen analyses in assessing male fertility is the subject of a hot debate that revolves around a central concept: the necessity to develop new markers of sperm function (12, 13).

The development of microarray technology has made it possible to determine expression profiles of the whole genome, thereby permitting two different biological conditions to be compared (14), which is expected to be essential in the future development of research, diagnostic and therapeutic tools (15).

Our aim with the present work was to compare the transcriptomic profile of spermatozoa obtained from infertile males and fertile sperm donors, using microarray technology as the first step towards designing a microarray-based diagnostic tool for assisted reproduction technique (ART) success.

Materials and Methods

Human biological samples

Five semen samples were obtained from the male partners of selected couples undergoing ART for infertility treatment at the Instituto Universitario IVI. A further five samples were collected from previously fertile sperm donors.

Patient inclusion criteria were couples whose male partner presented more than 25% of motile sperm (10 million/ml), a total number of inseminated sperm higher than 3 million, whose female partner did not have endometriosis, polycystic ovarian syndrome or evidence of endometriosis, exhibited tubal patency (determined by hysterosalpingography), and aged less than 36 years old. All males maintained 3-5 days of sexual abstinence before sample recovery. Sperm donors, aged between 18-35 years, presented the same inclusion criteria as infertile patients. Furthermore, controls all had their own children, including healthy newborns produced through our sperm donation programme.

This study was approved by the Institutional Review Board on the use of human gametes in research at the Instituto Valenciano de Infertilidad, and it complies with the Spanish Law of Assisted Reproduction and the Spanish Law of Biomedical Research. All patients signed an informed consent form approved by the Ethical Committee of this institution and based on the abovementioned laws and that of Human Rights in Research.

Semen analysis

Semen parameters were evaluated after 10 min liquefaction at 37ºC and 5% CO_2. Samples were examined for sperm concentration and motility in a Mackler® Chamber (Sefi Laboratories, Tel Aviv, Israel) following WHO guidelines (16).

Sperm swim-up

All samples were processed by swim-up. In short, ejaculates were diluted 1:1 (v/v) with sperm medium (MediCult, Jyllinge, Denmark) and centrifuged at 400g for 10 minutes. The supernatant was then discarded. Aliquots of 0.5 to 1 ml of fresh medium were overlaid on the pellet and incubated at 37ºC for 45 minutes with the tubes inclined at an angle of 45 degrees (6).

Isolation, quantification and storage of mRNA

Aliquots of the sperm samples were washed

in 3 ml of phosphate buffered saline (PBS). and centrifuged at 400g for 10 min. After discarding the supernatant, the pellet was resuspended in 1ml of TRIzol (Invitrogen) and immediately frozen by direct immersion in liquid nitrogen and stored in a nitrogen tank until mRNA extraction (less than ten days) when the total numbers of samples programmed for this study were obtained and experiments were performed.
Total RNA was extracted using the TRIzol method according to the manufacturer's recommendations (Life Technologies, Inc., Gaithersburg, MD) (17). The total amount of RNA was quantified using a BioRad (Durviz, Valencia, Spain) spectrophotometer. Only samples with a ratio cover of 1.5-2.0 (A260/A280) ratio for nucleic acid purity) were included for microarray analysis. In total, 20 samples were analyzed in duplicate.

Microarray analysis
The CodeLink Expression Analysis System was employed for microarray analysis according to the manufacturer's instructions. The Human Whole Genome Bioarray contains probes for more than 55,000 gene targets. Comparisons of the two groups were performed in duplicate. Spot intensities were normalized and analyzed using CodeLink Expression Analysis v4.1 software (18).
Sperm samples from five infertile patients (Group IM; n=5) and five fertile sperm donors (Group D; n=5) were analysed prior to swim-up process.
The same sperm samples were also analysed after swim-up. We carried out two microarray analyses of the same sperm sample; one with a pool of fresh samples (before swim-up) and the other with post-swim-up samples. Equal amounts of RNA from the same group were pooled prior to analysis.
Pooled RNA samples extracted from different subjects were applied to a single microarray chip in order to compensate for the technical difficulty in obtaining sufficient RNA and to reduce the cost of the microarray experiments. Appropriate RNA pooling can provide equivalent diagnostic power and improve the efficiency and cost-effectiveness of microarray experiments with only a moderate increase in the total number of samples required (19). In addition, given the genetic variability that exists among humans, the pooling of samples from different patients allows us to contemplate common genetic variations that define "normal condition" as "disease condition" when a variation falls within the boundaries of "normal condition" (20).

Bioinformatic data analysis and design
Four microarrays were eventually performed; 2 (in duplicate) with fresh sperm samples [Group donors fresh sperm samples (DF) (n=5) infertile patients fresh sperm samples (IMF) (n=5)] and another 2 (in duplicate) with post-swim up processed sperm samples [Group donors post swim-up process sperm samples (DC) (n=5) and infertile patients post swim-up process sperm samples (IMC) (n=5)].
The gene expression profile was determined by comparing the donors (D) and infertile patients (IM) groups (2 by 2 comparisons) using non-parametric tests to define genes exclusive genes (EG) and those that had altered the mRNA abundance of the different sample sets differentially expressed genes (DEG).
Microarray results were obtained via the following different approaches:
1. List of DEG in the fresh sperm of IMF and DF and in the same samples after swim-up (IMC and DC; DEG IMF, DEG DF, DEG IMC and DEG DC).
2. Genes present in one group but not in the other: EG - in fresh and post-swim-up samples of patient and donor groups (EG IMF, EG DF, EG IMC and EG DC).
3. Spermatogenic and fertility-related genes included in the previous approaches.

Differential gene expression
Gene expression is a highly complex and tightly regulated process that allows a cell to respond dynamically to environmental stimuli and to its own changing needs. This mechanism acts as both an "on/off" switch that controls which genes are expressed in a cell and as a "volume control" that increases or decreases the level of expression of particular genes according to necessity.
Three criteria were used to define DEG among the sample sets: genes that were common in both groups (DF-IMF or DC-IMC), with an absolute fold change (FC) of 5.0 or more and a corresponding FC p-value lower than 0.05. Positive FC values reflect an over-expression in DF or DC vs. IMF or IMC, and a negative value denotes higher expression in IMF or IMC than in DF or DC.

Exclusive genes
We defined genes or transcripts that were expressed in only one of the two groups (DF-IMF or DC-IMC group) as EG.

Table 1: Genes expressed differentially (DEG) among 10 most differentially expressed with a possible fertility role (FC: fold-change, D: donors group, IM: infertile male group, F: sperm samples before swim-up, C: sperm samples post-swim-up).

DF	IMF	DC	IMC
ID: NM_003007.2	ID: NM_005184.1	ID: NM_030925.1	ID: NM_018444.2
FC: 45.32	FC: -177.05	FC: 11,89	FC: -359,60
Name: semenogelin I (SEMG1)	Name: calmodulin 3 (phosphorylase kinase, delta) (CALM3)	Name: calcium binding protein 39-like (CAB39L)	Name: protein phosphatase 2C, magnesium-dependent, catalytic subunit (PPM2C), nuclear gene encoding mitochondrial protein
P-value< 0.05	P-value< 0.05	P-value< 0.05	P-value< 0.05

Two criteria were used to define EG: a spot intensity greater than the negative control intensity's media plus twice the standard deviation and expression in only one of the two groups.

Spermatogenic and fertility-related genes included in the previous approaches

We assessed the data for genes involved in biological processes that might be related to male fertility, searching systematically for the following key GO terms: acrosome (GO:0001669), acrosome reaction (GO:0007340), acrosomal vesicle (GO:0001669), sperm binding to zona pellucida (GO:0007339), copulation (GO:0007620), embryo implantation (GO:0007566), embryonic development (GO:0009790), female pregnancy (GO:0007565), fertilization (GO:0009566), genitalia development (GO:0048806), germ cell development (GO:0007281), gonad development (GO:0008406), insemination (GO:0007320), mating (GO:0007618), placenta development (GO:0001890), reproduction (GO:0000003), reproductive process (GO:0022414), sexual reproduction (GO:0019953), sperm chromatin condensation, sperm motility (GO:0030317), spermatid development (GO:0007286), spermatid nucleus differentiation (GO:0007289), spermatogenesis exchange of chromosomal proteins (GO:0035093) and spermatogenesis (GO:0007283).

We created a database of genes associated with reproduction-related biological processes detected by GO and compared it with our lists of DEG and EG for all analyzed groups.

Results

Sperm quality

No differences were found between groups with respect to any of the sperm parameters; namely sperm concentration, motility (more than 25% of motile sperm in both groups) and morphology (both groups presented with at least 50% or more normal spermatozoa). Moreover, the mean age of the groups was similar [donors: 27.2 years (SD=3.9); patients: 29.8 years (SD=2.4)].

1. DEG in the fresh sperm of IMF and sperm donors (DF) and in samples after swim-up (IMC and DC).

Table 2: Summarised table of number of Genes expressed differentially (p-value<0.05) and EG from donors (D) and infertile male patients (IM) before (F) and post swim-up (C).

Group	DEG	EG
DF	1644	983
IMF	1568	692
DC	10	9
IMC	14	14

Table 3: Exclusive gene (EG) with a reproductive role (D: donors group, IM: infertile male group, F: sperm samples before swim-up, C: sperm samples post-swim-up).

Group	Gene accession number	Gene description
EG DF	NM_053280	outer dense fiber of sperm tails 3 (ODF3)
	NM_024532	sperm associated antigen 16 (SPAG16)
	NM_001340	cylicin, basic protein of sperm head cytoskeleton 2 (CYLC2)
	NM_198393	testis expressed sequence 14 (TEX14), transcript variant 1
EG IMF	NM_002776	kallikrein 10 (KLK10), transcript variant 1
	NM_003542	histone 1, H4c (HIST1H4C)
EG IMC	NM_003529	histone 1, H3a (HIST1H3A)

Table 4: GDE and EG from all groups described in reproductive related processes. Column 1: Gene name. Column 2: Reproductive related processes. (DF: Donors fresh sperm samples; DC Donors after swim-up sperm samples; IMF: Infertile male fresh sperm samples; IMC: Infertile male after swim-up sperm samples)

		Gene	Reproductive related processes
Genes differentially expressed (GDE)		**SEMG1**	copulation, insemination, matting, reproduction, reproductive process
		SOX15	gonad development, reproduction, reproductive process
		OAZ3	gamete generation, male gamete generation , reproduction, reproductive process, spermatogenesis,
		DNALI1	fertilization, reproduction, reproductive process
		SPAG6	gamete generation, germ cell development, male gamete generation, reproduction, reproductive process, spermatid development, spermatogenesis.
		GNAS	female pregnancy, reproduction, reproductive process
		KHDRBS3	gamete generation, male gamete generation, reproduction, reproductive process, spermatogenesis
		PPAP2A	gamete generation, reproduction, reproductive process .
		RPL39L	gamete generation, male gamete generation, reproduction, reproductive process, spermatogenesis,
		HBXIP	reproduction, reproductive process
		ANKRD7	gonad development, reproduction, reproductive process
		ODF1	gamete generation, male gamete generation, reproduction, reproductive process, spermatogenesis
		EIF2B4	gonad development, reproduction, reproductive process
		RPL29	embryo implantation, female pregnancy, reproduction, reproductive process
		WDR33	gamete generation, male gamete generation, reproduction, reproductive process, spermatogenesis
		NCOA4	gonad development, reproduction, reproductive process
		SPATA4	gamete generation, male gamete generation, reproduction, reproductive process, spermatogenesis
	DF	**DUSP13**	gamete generation, male gamete generation , reproduction, reproductive process, spermatogenesis
		ADAM29	gamete generation, male gamete generation, reproduction, reproductive process, spermatogenesis
		TXNDC2	gamete generation, male gamete generation, reproduction, reproductive process
		SBDS	embryonic development
		LIG3	male gamete generation, reproduction, reproductive process
		RACGAP1	embryonic development, gamete generation, male gamete generation, reproduction, reproductive process, spermatogenesis,
		KLF4	embryonic development
		CCNA1	gamete generation, male gamete generation, reproduction, reproductive process, spermatogenesis
		SERPINA5	fertilization, fusion of sperm to egg plasma membrane, gamete generation, male gamete generation, reproduction, reproductive process
		NME5	gamete generation, germ cell development, male gamete generation, reproduction, reproductive process, spermatid development, spermatogenesis
		ADAM21	fertilization, reproduction, reproductive process
		YBX2	gamete generation, male gamete generation, reproduction, reproductive process, spermatogenesis, ,
		RPL10L	gamete generation, male gamete generation, reproduction, reproductive process, spermatogenesis
		DDX25	gamete generation, germ cell development, male gamete generation, reproduction, reproductive process, spermatid development, spermatogenesis
		NDRG3	gamete generation, male gamete generation, reproduction, reproductive process, spermatogenesis
		ACR	acrosome reaction, fertilization, reproduction, reproductive process
		SORD	sperm motility
		CCNI	gamete generation, male gamete generation, reproduction, reproductive process, spermatogenesis
		USP9X	gamete generation, reproduction, reproductive process
		SPANXB1	gamete generation, germ cell development, male gamete generation, reproduction, reproductive process, spermatid development, spermatogenesis
		TESK1	gamete generation, male gamete generation, reproduction, reproductive process, spermatogenesis
	IMF	**PSG6**	female pregnancy, reproduction, reproductive process
		Cep290	embryonic development
		BRD2	gamete generation, male gamete generation, reproduction, reproductive process, spermatogenesis
Exclusive Genes (EG)		**LEFTY2**	gamete generation, germ cell development, reproduction, reproductive process
		TSG101	reproduction, reproductive process
		FLT1	female pregnancy
		ABCE1	embryonic development
	DF	**RRAGA**	reproduction, reproductive process
		PRLR	embryo implantation, female pregnancy
		AMH	reproduction, reproductive process
	IMF	**TDGF1**	embryonic development
	IMC	**HPGD**	female pregnancy, reproduction, reproductive process

By means of microarray analysis, we identified 1644 DEG in the sperm of DF and 1568 in that of IMF. Nevertheless, when samples were analyzed after swim-up we found only 10 DEG in the DC group and 14 in the IMC group. Due to the extensive nature of these lists, Table 1 shows only one of the ten most differentially expressed genes of the four groups with a possible fertility role.

2. Genes from fresh and post-swim-up samples of both patient and donor groups (IMF, DF, IMC, DC) that are present in one group but not in the others (EG).

We detected 985 exclusive genes in the DF group and 692 exclusive genes in the IMF group. However, when samples were analysed after swim-up only 9 exclusive genes in the DC group and 14 in the IMC group were identified (Table 2).

As these lists are extensive, table 3 only shows those with a possible fertility role.

3. Spermatogenic and fertility-related genes included in the previous approaches.

A thorough and systematic search of the lists of DEG and EG from DF-IMF or DC-IMC groups using the database of genes involved in the biological processes that have been described in the Material and Methods section was undertaken.

A total of 38 genes involved in some of these "reproductive-related processes" among the DEG of the DF group, 1 among the DEG of the IMF group, 7 among the EG of the DF group and 3 among the EG of the IMF group were identified.

After analyzing the lists of genes after swim-up (DEG and EG) only one gene involved in some reproductive related processes was detected. The results of this systematic search are shown in table 4.

According to the data shown in table 4, emphasis can be placed on some genes known to be closely related with male fertility like SEMG1 (that participates in the formation of a gel matrix entrapping the accessory gland secretions and ejaculated spermatozoa and contributes to the activation of progressive sperm movements), SOX15 (involved in male sex determination) or ODF1 and ODF4 (that both are components of the outer dense fibers (ODF) of spermatozoa which could be involved in sperm tail structure and sperm movement). All are DEG from the DF group.

Discussion

According to our results, it can be deduced that sperm transcriptome differs considerably between sperm samples that lead to pregnancy (sperm donor samples) vs. those that do not (infertile male patients). These findings support the hypothesis that microarray-based diagnostic tools can predict the potential of sperm to initiate a pregnancy and explain idiophatic male infertility.

Hundreds of genes were differentially up- or down-regulated in the samples of both study groups, confirming the huge mRNA differences between sperm of fertile males and that of infertile men as referred to in our previous reports (10). These variations could be responsible for the possibility of conceiving or the failure to do so. A significant number of genes are expressed exclusively in sperm donor samples or in samples that fail to result in pregnancy, thereby confirming once again the potential diagnostic value of microarray analysis for the successful outcome of ART.

Nevertheless the great heterogeneity involved in infertility status must be considered. Absence of precise selection leads to a great heterogeneity in RNA differences among different individuals. These differences may be masked, added or diluted when mixed sperm RNA samples from different individuals are hybridized on a single microarray, and thus the next step is to perform individual microarray analysis for each patient/donor sample.

It should be stressed that all these differences among the groups disappeared when post-swim-up samples were analysed (DEG DC, DEG IMC, EG DC and EG IMC lists). This suggests that sperm preparation is effective in obtaining healthy sperm cells, as the profound differences between fresh sperm from donor and infertile males practically disappeared after swim-up. In spite of this fact, some differences were still found (10 DEG in group DC; 14 in the MC group; 9 EG in the DC group and 14 in the IMC group). Those differences could play a role in male reproductive success.

This is the first step towards developing a newly designed test to predict the success of ART based on a more accurate sperm diagnosis and a better understanding of sperm function via microarray analysis, which should, in turn, improve the chances of achieving pregnancy.

The development of microarray technology has made it possible to determine the cell and tissue expression profiles of the whole genome, thereby enabling a comparison of two different biological conditions (14). This is expected to be essential

in the development of research, diagnostic and therapeutic tools (15).
In this sense the present work complements, from a clinical point of view, the pioneering works of mRNA presence and function in sperm cells and their relationship with fertility.

Conclusion

Sperm mRNA variations between donors vs. infertile patients after swim-up sperm preparation are subtle, therefore it can be concluded that this selection method technique is an effective selective method in ART that may allow us to select the healthiest sperm cells.
Nevertheless these variations tend to disappear after the swim-up sperm preparation technique. Some differences between donors vs. infertile patients have been detected that could be potentially employed to detect fertility success markers.

Supplementary material

The complete lists of DEG and EG of both groups before and after swim-up are available from the corresponding author.

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