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ASSISTED REPRODUCTION TECHNOLOGIES

The use of fluorescence in situ hybridization analysis on sperm: indications to perform and assisted reproduction technology outcomes

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Abstract

Purpose To determine the consequences of an altered sperm fluorescence in situ hybridization (FISH) result for ART outcomes and the indications for a sperm FISH analysis.

Methods Data from 439 infertile men were collected. Bivariate analyses were performed to determine the association of men's age, seminal alterations, and sperm FISH indication, with the incidence of X, Y, 13, 18, and 21 sperm chromosomal abnormalities. A multivariate logistic regression analysis was performed to establish the most predictive variables for altered sperm FISH. Results from the IVF/ICSI cycles were collected for 248 out of 439 patients. Two distinct groups were established: 151 couples that used their own oocytes and 97 couples involved in egg donation programs. In both groups, ART outcomes were compared between normal and altered sperm FISH.

Results Teratozoospermia and oligozoospermia were associated with sperm chromosome anomalies ($p < 0.05$). Indications for sperm FISH analysis with the highest predictability were teratozoospermia, male age, oligozoospermia, and implantation failure (AUC = 0.702). Embryo quality ($p = 0.096$), pregnancy rate ($p = 0.054$), and implantation rate ($p = 0.089$) were higher in own-oocytes couples with normal sperm FISH than in altered sperm FISH couples, although differences were not statistically significant. In donor-oocytes couples, in which high-quality embryos were transferred later than in own-oocytes couples (3.8 vs. 3.0 days), we did not identify differences in the ART outcome between normal and altered sperm FISH couples. In both groups, the possible interference of woman age was negligible.

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Conclusions Sperm FISH is indicated in middle-aged oligoteratozoospermic patients with implantation failures in previous IVF/ICSI cycles. Sperm chromosome anomalies have a moderate detrimental impact on embryo quality, implantation, and pregnancy rates.

Keywords ART outcome · Blastocyst transfer · Male age · Seminal parameters · Sperm FISH indications

Introduction

The first study that analyzed the chromosomal constitution of spermatozoa from infertile individuals using protocols of fluorescence in situ hybridization (FISH) was published in 1995 [1]. Since then, a large number of sperm FISH studies have been published, leading to the conclusion that the population of infertile individuals presents a significant percentage of patients with higher rates of sperm chromosomal abnormalities [2–6]. The sperm FISH methodology is based on the use of chromosome-specific DNA probes to hybridize the previously decondensed sperm chromatin [7]. Two different probe combinations are the most widely used for sperm chromosome analysis: one combines centromeric probes to study numerical anomalies for chromosomes X, Y, and 18, whereas the other combination uses specific locus probes for chromosomes 13 and 21. Its application allows the screening of a large number of cells from the same patient in a relatively short time, which allows the estimation of sperm aneuploidy and diploidy rates. Using this methodology, we previously demonstrated that sperm FISH analysis is indicated in individuals with a 46,XY karyotype and low sperm count [3]. In this same study, in which we analyzed more than three hundred infertile males, we also suggested that the evaluation of chromosomes 21, X, and Y is enough to identify the majority of at-risk individuals. Moreover, we also established that the identification of significant differences in the rates of sperm chromosome abnormalities with respect to controls should be taken into consideration regardless of the numerical value, suggesting a qualitative interpretation of the results in the detriment of quantitative interpretation.

Although it is undeniable that FISH studies on decondensed sperm nuclei have become a notable advance in male infertility counseling [8], the technique has some drawbacks that have hampered their incorporation in the clinical appraisal of infertile patients [6]. Some of these limitations come from the technique itself, which is highly laborious and time-consuming. Moreover, there is no consensus about the consequences that an altered sperm FISH result has in clinics, that is, what is their impact on ART outcomes. At this regard, eight studies [8–15] have analyzed the relationship between altered sperm FISH results and their effect on the ART outcome (Table 1). Considered globally, these studies show that an altered sperm FISH result mainly affects at the level of implantation and pregnancy rates (Table 1). Even so, an important consideration of all these studies is that the sample size is relatively small; therefore, the dataset is still limited in its ability to provide definite conclusions. Moreover, an additional handicap is the difficulty to exclusively analyze the effect of sperm chromosomal abnormalities on ART parameters, excluding the female contribution to the couple's infertility.

The objective of the present study was to evaluate the role of sperm FISH results in the context of assisted reproductive clinics with two specific aims: (1) to provide additional data to clarify under which indications it is recommended to perform a sperm FISH study and (2) to establish the reproductive consequences of an altered sperm FISH result on ART outcome. To minimize the female contribution to couple infertility, the second objective was developed separately in own-egg and egg-donation couples.

Material and methods

Study population

Clinical data from 439 men who consulted for infertility were retrospectively collected from seven assisted reproduction centers. Collected data included the following: patient age, seminal parameters, karyotype (46,XY in all cases), sperm FISH indication, sperm FISH results, women age, female partner karyotype (46,XX in all cases), and ART outcome (Table 2).

The Ethics Commission on Human and Animal Experimentation of our center approved the study. In every instance, the centers provided all data while preserving patient anonymity.

Sperm FISH analysis

Each patient provided a semen sample after an abstinence period of 2–7 days. Samples were fixed with a 3:1 ratio of methanol to acetic acid and spermatozoa were spread on slides and processed for FISH. Two hybridizations were performed using two different probe combinations: a triple-color FISH with centromeric DNA probes for chromosomes 18 (CEP 18, locus D18Z1, Spectrum Aqua), X (CEP X, locus DXZ1, Spectrum Green), and Y (CEP Y, locus DYZ3, Spectrum Orange), and a dual-color FISH with locus-specific probes for chromosomes 13 (LSI 13, locus RB1, Spectrum Green) and 21 (LSI 21, loci D21S259, D21S341, D21S342, Spectrum Orange). FISH methodology was performed

Table 1 Compilation of published studies that evaluated the consequences of an altered sperm FISH result on ART outcome. *N*, number of individuals

	Fertilization rate	Embryo quality rate	Pregnancy rate	Implantation rate	Abortion rate
No effect	Colombero et al. 1999 [9] (*N* = 47) Burrello et al. 2003 [11] (*N* = 48) Nagvenkar et al. 2005 [12] (*N* = 30)	Burrello et al. 2003 [11] (*N* = 48)	Colombero et al. 1999 [9] (*N* = 47)	–	–
Detrimental effect[a]	Petit et al. 2005 [13] (*N* = 19) Mehdi et al. 2006 [14] (*N* = 12)	–	Calogero et al. 2001 [10] (*N* = 18) Burrello et al. 2003 [11] (*N* = 48) Nagvenkar et al. 2005 [12] (*N* = 30) Petit et al. 2005 [13] (*N* = 19) Nicopoullos et al. 2008 [15] (*N* = 56)	Burrello et al. 2003 [11] (*N* = 48) Vialard et al. 2008 [16] (*N* = 35)	Burrello et al. 2003 [11] (*N* = 48)

[a] Decrease in fertilization, pregnancy, and implantation rates. Increase in abortion rate

following the standard five steps of the procedure: sperm chromatin decondensation (5 mM dithiothreitol solution), DNA denaturation (70% formamide solution), probe and target DNA hybridization (overnight at 37 °C), and post-hybridization washes (sodium citrate solution) [7].

A minimum of 500 spermatozoa was manually analyzed per probe combination giving a minimum number of 1000 spermatozoa per patient. Expert personnel in interphase FISH performed the analyses following standard assessment criteria [22]. For each patient, we determined the incidence of disomy for chromosomes X, Y, 13, 18, and 21, and the incidence of sperm diploidy. To determine whether the disomy and diploidy rates in each infertile patient were different from those in the control population, we applied Fisher's exact test. p values less than 0.05 were considered statistically significant. Those patients with at least one category with a number of disomies or diploidies significantly above the cutoff values ($p < 0.05$) were classified with "altered FISH result." On the contrary, when all categories fitted into the results described in controls ($p > 0.05$), patients were classified with "normal FISH result." According to this procedure, 117 out of the 439 patients were classified with altered FISH (26.7%), and 322 out of the 439 samples were classified with normal FISH (73.3%).

It is important to highlight that each of the seven assisted reproduction centers analyzed their own control population, which was constituted by fertile individuals with normal karyotypes and normal seminal parameters. Accordingly, internal cutoff values were set for every chromosome anomaly in each assisted reproduction center (Supplemental Table 1). The use of internal cutoff values and the application of the same experimental FISH procedure guaranteed the reliability of the results allowing the compilation of data from different laboratories.

Semen analysis

Semen samples were also used to perform a seminogram following the criteria of the World Health Organization 2010 [17]. According to the results, samples were classified in the following categories (Table 3): asthenoteratozoospermia (AT; $n = 12$), asthenozoospermia (A; $n = 77$), normozoospermia (N; $n = 197$), oligoasthenoteratozoospermia (OAT; $n = 22$), oligoasthenozoospermia (OA; $n = 68$), oligoteratozoospermia (OT; $n = 14$), oligozoospermia (O; $n = 34$), and teratozoospermia (T; $n = 15$).

Sperm FISH indications and statistical analysis

To determine the sperm FISH indications preferably associated with increased frequencies of sperm aneuploidy/diploidy, we annotated the percentage of patients with an altered FISH result according to the indication. Results were assessed in contingency tables using a chi-squared test. To avoid cross-effects, differences were evaluated taking only into account those couples with a single indication.

Mann-Whitney rank sum tests were used to determine whether the presence of an altered sperm FISH result depends on sperm count, sperm motility, or sperm morphology. The same test was used to find out the association between male age and the occurrence of sperm aneuploidy/diploidy. To avoid the negative effect of data over-dispersion, when appropriate, variables were logarithmically transformed: log ($x + 1$). In those cases where logarithmic transformation was inadequate to normalize the data, variables were recoded into three categories using the quantiles and compared using a chi-squared test.

Table 2 Information gathered from couples' reproductive history

Data collected	Category	Description
Male information	Age	Male age when initiated the ART cycle
	Seminogram	Numerical data on sperm count (spermatozoa/ml), sperm morphology (percentage of normal forms) and sperm motility (percentage of progressive motility). Seminogram classification was established according to the World Health Organization guidelines, 2010 [17]
	Karyotype	Numerical and structural information of the complete set of chromosomes
	Sperm FISH indications	
	- Male factor	$< 15 \times 10^6$ spermatozoa/ml
		< 4% normal morphology
		< 32% progressive motility
	- Severe male factor	$< 5 \times 10^6$ spermatozoa/ml
		< 4% normal morphology
		< 32% progressive motility
	- Varicocele	Abnormal enlargement of the pampiniform venous plexus in the scrotum
	- Idiopathic male infertility	Infertile individuals with normal seminogram and karyotype
	- Poor embryo quality	Previous IVF/ICSI cycles with a high proportion of low-quality embryos according to the criteria described by the Spanish association for the study of the reproduction biology (ASEBIR, 2015) [18]
	- Recurrent implantation failure	Failure to achieve clinical pregnancy (no detectable HCG production or no detectable gestational sac by ultrasonography) after the transfer of at least four good-quality embryos in a minimum of three fresh or frozen cycles in a woman under the age of 40 years [19]
	- Recurrent miscarriage	Three or more consecutive spontaneous abortions as it is defined by the European Society for Human Reproduction and Embryology (ESHRE) and Royal College of Obstetricians and Gynaecologists (RCOG) [20, 21]
	Sperm FISH result	
	- Normal	The rates of aneuploidy (involving chromosomes 13, 18, 21, X, and Y) and diploidy were not statistically different compared with control values
	- Abnormal	The rates of aneuploidy (involving chromosomes 13, 18, 21, X, and/or Y) and/or diploidy were statistically higher than control values
Female information	Age	Female age when initiated the ART cycle
	Karyotype	Numerical and structural information of the complete set of chromosomes
ART information	Metaphase II oocytes	Number of oocytes obtained at the stage of metaphase II
	Transfer day	Number of days between the day of fertilization and the day of embryo transfer
	Fertilization rate	Percentage of zygotes with respect to the number of metaphase II oocytes
	Embryo quality rate	Percentage of A and B quality embryos with respect to the total number of embryos
	Pregnancy rate	Percentage of cycles in which human chorionic gonadotrophin produced by the embryo may be detected in the blood or urine [19]
	Implantation rate	Percentage of cycles in which the embryo has produced an intrauterine gestational sac, detectable by ultrasonography, usually about three weeks after oocyte retrieval or about five weeks of gestation [19]
	Abortion rate	Spontaneous termination of a pregnancy before 20-week gestation

Additionally, Fisher's exact test was applied to analyze seminal parameters in qualitative terms. For each of the three seminal parameters, individuals were classified as follows: "asthenozoospermia" or "non-asthenozoospermia"; "oligozoospermia" or "non-oligozoospermia"; and "teratozoospermia" or "non-teratozoospermia." These pairs of variables were separately analyzed in 2 × 2 contingency tables with the variables of "normal sperm FISH" and "altered sperm FISH."

Finally, and with the objective to establish which of the previous variables were independent predictors of an altered sperm FISH result, variables with a p value lower than 0.2 were included in a multivariate logistic regression model [23]. The final model was obtained considering variables with a p value < 0.1. Model fitting was assessed using the receiver operating characteristics (ROC) curve. The area under the curve (AUC) was indicative of the prediction potential of the

Table 3 Individuals with altered sperm FISH results according to their seminal parameters. Sperm FISH alteration was dependent on seminal parameters (Fisher exact test; $p < 0.001$)

Seminogram	Number of patients	Number of patients with an altered FISH result	Percentage of patients with an altered FISH result (confidence interval)
Asthenoteratozoospermia	12	6	50.0 (21.7–78.3)
Asthenozoospermia	77	19	24.7 (15.0–34.3)
Oligoasthenoteratozoospermia	22	10	45.5 (24.6–66.3)
Normozoospermia	197	39	19.8 (14.2–25.4)
Oligoasthenozoospermia	68	20	28.4 (18.6–40.2)
Oligoteratozoospermia	14	9	64.3 (39.2–89.4)
Oligozoospermia	34	7	20.6 (7.0–34.2)
Teratozoospermia	15	7	46.7 (21.4–71.9)
Total	439	117	26.7 (22.5–30.8)

variables evaluated, which was classified into excellent ($0.9 \leq AUC \leq 1.00$), good ($0.80 \leq AUC < 0.90$), fair ($0.70 \leq AUC < 0.80$), poor ($0.60 \leq AUC < 0.70$), or failed ($AUC < 0.60$).

ART outcome analysis

In 248 out of the 439 patients, we also collected ART outcome data from the couple's IVF/ICSI cycles, including women age, number of retrieved metaphase II (MII) oocytes, fertilization rate, embryo quality, transfer day, pregnancy rate, implantation rate, and abortion rate. Of these 248 couples, two distinct groups were formed. The first group constituted of 151 couples that used their own oocytes in fresh embryo transfers without preimplantation genetic testing for aneuploidy screening. The second group was established from 97 couples involved in egg donation programs with fresh embryo transfers and without PGT. Only those couples with both members exhibiting a normal karyotype (46,XY and 46,XX) and with available age data (donor and recipient women in case of couples involved in an egg donation program) were included in the study.

To evaluate the effect of the sperm FISH result over the ART outcome, data of fertilization rate, embryo quality, pregnancy rate, implantation rate, and abortion rate were compared between normal and altered sperm FISH couples. Analyses were performed using *t* test or Mann-Whitney rank sum test. These analyses were performed separately for couples involved in IVF/ICSI cycles using their own oocytes (151/439), and for couples involved in egg donation programs (97/439). This last population is of special interest to evaluate the influence of sperm FISH studies on the ART outcome without any disturbance coming from the female partner.

To discard any possible influence on the results by female age, the number of MII oocytes, or transfer day, we checked whether these parameters matched between normal and altered sperm FISH couples. These analyses were performed separately in the own-egg and egg-donation groups using *t* test or Mann-Whitney rank sum test.

All statistical analyses were performed using SigmaStat (version 2.03, San Jose, USA), GraphPad software (QuickCalcs online, La Jolla, USA), and SAS v9.4 (SAS Institute Inc., Cary, NC, USA). As mentioned, the multivariate logistic regression model was obtained considering variables with a *p* value < 0.1. For all other statistical tests, the level of significance was set at $p < 0.05$.

Results

Indications and sperm FISH result

Percentages of altered sperm FISH results in couples with a single indication ($N = 394$) are detailed in Table 4. The percentage of patients with an altered FISH result was clearly dependent on the indication ($p < 0.001$; chi-square test). Thus, "severe male factor" exhibited the highest incidence of abnormal results (76.5%; 13/17). In "male factor," the incidence was 25.5% (47/184), followed by "recurrent miscarriage" (22.6%; 7/31). The indications least associated with the presence of chromosome anomalies in sperm were "recurrent implantation failure" (14.8%; 4/27) and "poor embryo quality" (12.5%; 1/8) (Table 4).

More than one sperm FISH indication was present in 47 couples (Table 4). The indication of "male factor" or "severe male factor" was present in 44 of these 47 couples, and an altered FISH result was reported in 47.7% (21/44). Out of 44 couples with "male factor" or "severe male factor," 23 couples also showed an indication of "recurrent implantation failure." In this group, 60.9% (14/23) had an altered FISH result. Moreover, ten individuals disclosed "varicocele" as an indication and 50% of them (5/10) showed altered sperm FISH results. Importantly, 80% of the patients with varicocele showed also a "male factor" indication.

Seminal parameters and sperm FISH result

The mean sperm count in patients with an altered FISH result ($35.04 \times 10^6 \pm 39.45$ spermatozoa/ml) was lower than the mean value observed in patients with a normal result ($44.7 \times 10^6 \pm 40.7$ spermatozoa/ml) (median 21×10^6 spermatozoa/ml vs. 37×10^6 spermatozoa/ml, respectively; $p = 0.005$). Since data exhibited a high degree of over-dispersion (even after logarithmic transformation), we compared the percentage of patients with normal/altered results between three different categories: (i) $< 20 \times 10^6$ spermatozoa/ml, (ii) between 20 ×

Table 4 Incidence of altered sperm FISH results in patients with different numbers of sperm FISH indications. In patients with a single indication, the frequency of an altered FISH result varied significantly according to the indication ($p < 0.001$; chi-square test)

Indication	Number of patients	Number of patients with an altered FISH result	Percentage of patients with an altered FISH result (confidence interval)
One indication ($N = 394$)			
Male factor	184	47	25.5 (20.6–31.2)
Severe male factor	17	13	76.5 (56.4–89.3)
Idiopathic male infertility	123	23	18.7 (13.6–25.2)
Poor embryo quality	8	1	12.5 (0.0–42.8)
Recurrent implantation failure	27	4	14.8 (6.4–29.6)
Recurrent miscarriage	31	7	22.6 (12.6–37.0)
Others[a]	4	3	75.0 (34.6–95.2)
Two indications ($N = 41$)			
Male factor + recurrent implantation failure	17	11	64.7 (44.8–80.6)
Male factor + recurrent miscarriage	9	2	22.2 (6.9–50.4)
Male factor + poor embryo quality	6	1	16.7 (2.3–51.8)
Male factor + varicocele	3	1	Not applicable
Male factor + one previous miscarriage	1	1	Not applicable
Severe male factor + recurrent implantation failure	1	1	Not applicable
Severe male factor + varicocele	1	1	Not applicable
Recurrent implantation failure + poor embryo quality	1	0	Not applicable
Recurrent implantation failure + varicocele	2	0	Not applicable
Three indications ($N = 5$)			
Male factor + recurrent implantation failure + varicocele	2	1	Not applicable
Male factor + recurrent implantation failure + one previous miscarriage	1	0	Not applicable
Male factor + recurrent implantation failure + recurrent miscarriage	1	0	Not applicable
Male factor + recurrent miscarriage + varicocele	1	1	Not applicable
Four indications ($N = 1$)			
Male factor + recurrent implantation failure + recurrent miscarriage + varicocele	1	1	Not applicable

[a] This category includes one previous abortion (one couple), paternal age (one couple), chemotherapy (one couple), and previous surgical abortion due to genetic abnormalities (one couple)

10^6 and 49×10^6 spermatozoa/ml and (iii) $> 50 \times 10^6$ spermatozoa/ml. Results confirmed an association between sperm count and altered FISH results ($p = 0.009$): the proportion of individuals with altered sperm FISH results reached the highest values in the category of $< 20 \times 10^6$ spermatozoa/ml (47.9% of patients), while the categories of patients with 20–49×10^6 and $> 50 \times 10^6$ showed lower values (25.6% and 26.5% of patients, respectively).

Concerning the influence of morphology, the mean percentage of spermatozoa with normal forms in patients with an altered FISH result (8.2 ± 7.1%) was lower than the mean data observed in patients without sperm chromosome alterations (12.6 ± 9.1%) ($p < 0.001$). Finally, the parameter of sperm motility was equivalent between individuals with altered and normal sperm FISH results (35.8 ± 20.5% vs. 38.8 ± 19.1%; $p = 0.141$).

When individuals were grouped according to qualitative characteristics of the seminogram (Table 3), the percentage of patients with a sperm FISH alteration was clearly dependent on seminal parameters ($p < 0.001$). To go further, we determined if any of the seminogram components (sperm count, sperm motility, or sperm morphology) was preferentially associated with the presence of sperm chromosome anomalies. Results confirm a significant association between reduced sperm count and altered sperm FISH results ("oligozoospermia" (33.3%; 46/138) vs. "non-oligozoospermia" (23.6%; 71/301); $p = 0.037$) and between increased rate of abnormal forms and altered sperm FISH results ("teratozoospermia" (50.8%; 32/63) vs. "non-teratozoospermia" (22.6%; 85/376); $p < 0.0001$). Again, sperm motility was not associated with sperm FISH result ("asthenozoospermia" (30.7%; 55/

179) vs. "non-asthenozoospermia" (23.8%; 62/260; p = 0.124).

Age and sperm FISH result

The mean age of male patients included in the study was 37.8 ± 5.6 years, being 38.7 ± 5.7 years in the group of patients with an altered FISH result and 37.5 ± 5.6 years in the group with normal results. There were no age differences between these two groups of patients (median 38 years vs. 37 years, respectively).

Multivariate logistic regression analyses

Data from bivariate analyses were used to assess the potential of the variables assessed to predict an altered FISH result. The indication with the greatest predictability was sperm morphology ($p < 0.0001$). Male age ($p = 0.023$), quantitative oligozoospermia ($p = 0.028$), qualitative oligozoospermia ($p = 0.052$), and recurrent implantation failure ($p = 0.052$) were also predictive parameters, although with less weight. Taking into account all the significant variables, the accuracy of the prediction was fair (range 0.70–0.80), with an area under the ROC curve of 0.702.

ART outcome and sperm FISH result: own-oocytes couples

From the 151 couples included in this group, in 30 (19.9%; 30/151), an increased incidence of chromosomal anomalies was reported, while the remaining 121 patients exhibited normal sperm FISH results (80.1%; 121/151) (Table 5).

Women age, MII oocytes, and transfer day matched between FISH normal and FISH altered groups (Table 5). The mean value of the embryo transfer day was 3.0 days ± 1.1 (range 2–5). Couples exhibiting normal sperm FISH results showed increased values of embryo quality (median 50% vs. 38.5%; $p = 0.096$), pregnancy rate (mean 46.6% vs. 25.0%; $p = 0.054$), and implantation rate (mean 43.1% vs. 25.0%; $p = 0.089$) than those couples with altered sperm FISH result, although the differences were not statistically significant (Table 5). Effect size has a small or small/medium influence on the results (Table 5).

ART outcome and sperm FISH result: donor-oocytes couples

A percentage of 30.9% (30/97) of these couples showed an altered sperm FISH result, while 69.1% (67/97) of patients did not show increased incidences of sperm chromosome anomalies (Table 5). There were no significant differences between couples with normal and altered sperm FISH results regarding transfer day, fertilization rate, embryo quality, pregnancy rate, implantation rate, or abortion rate (Table 5). The number of metaphase II oocytes (9.6 vs. 8.5 oocytes; $p = 0.043$) and the age of the recipient women (41.5 years vs. 39.5 years; $p = 0.031$) were higher in couples with altered sperm FISH results (Table 5). Effect size has a very small, small, or small/medium influence on the results (Table 5).

Discussion

Sperm FISH indications

The relationship between male infertility, semen quality, and increased rates of aneuploidies in spermatozoa has been widely described in previous studies [3, 5, 6]. In agreement with this, the present work showed that the highest incidence of altered results on sperm FISH analysis was recorded in individuals with the indication of "severe male factor" or "male factor." Supporting this association is the fact that 94% of individuals with multiple indications also presented "male factor" or "severe male factor" indication, as well as increased rates of sperm chromosomal abnormalities. Conversely, indications related to abnormalities in post-fertilization stages, such as "poor embryo quality," "recurrent implantation failures," and "recurrent miscarriages," showed lower incidences of altered sperm FISH results. A similar situation was observed in idiopathic male infertility patients. These results suggest a poor association of these indications with sperm chromosome instability, at least when presented as a single indication (Table 4).

The analysis of the results in cases of more than one indication deserves additional commentaries in the case of "recurrent implantation failure" and "varicocele." "Recurrent implantation failure" in combination with other indications (Table 4) presented high values of patients with altered FISH results (14/26; 53.85%). Considering all patients with "recurrent implantation failure" ($n = 53$; Table 4), 18 individuals (18/53; 33.96%) exhibited an altered sperm FISH results. The combination of "varicocele" with other indications (Table 4) also reaches high values of affected patients (5/10; 50%). Although these results should be interpreted cautiously due to the presence of cross-effects between indications, "recurrent implantation failure" and "varicocele" could be also considered indications associated with meiotic chromosome instability in infertile patients. In any case, the analysis of additional series of patients is mandatory to clarify this point.

The strong association between seminal alterations and sperm chromosomal abnormalities suggests that the molecular factors leading to reduced numbers of sperm in the ejaculate, and to a detrimental effect on sperm morphology, also affect chromosome segregation during meiosis. Regarding sperm count, the results of the present study are consistent with the broad consensus on the observation of higher rates of

Table 5 Assisted reproductive techniques outcome versus sperm FISH result in own-oocytes couples and donor-oocytes couples

Category	Own-oocytes couples			Donor-oocytes couples		
	Normal sperm FISH (n = 121)	Altered sperm FISH (n = 30)	p value (effect sized)	Normal sperm FISH (n = 67)	Altered sperm FISH (n = 30)	p value (effect sized)
Women age[a]	35.1 ± 3.7 (25–45)	35.9 ± 5.0 (27–50)	0.336 (0.20)	39.5 ± 3.8 (30–48)	41.5 ± 4.3 (33–50)	0.031* (0.51)
Donor women age[a]	Not applicable	Not applicable	–	25.6 ± 3.9 (18–34)	24.4 ± 4.2 (18–34)	0.179 (0.30)
Metaphase II oocytes[b]	6 (6)	8 (6)	0.557 (0.11)	8 (5)	10 (3)	0.043* (0.38)
Transfer day[b]	3 (1)	3 (3)	0.467 (0.38)	3 (2)	3.5 (2)	0.267 (0.33)
Fertilization rate[b]	75 (44)	67 (33)	0.551 (0.04)	76 (30)	72 (23)	0.163 (0.31)
Embryo quality rate (A + B)[b]	50 (62.4)	38.5 (37.8)	0.096 (0.36)	50 (61)	37 (50)	0.273 (0.05)
Pregnancy rate[c]	46.6 (54/116)	25.0 (7/28)	0.054 (0.17)	61.2 (41/67)	53.3 (16/30)	0.467 (0.07)
Implantation rate[c]	43.1 (50/116)	25.0 (7/28)	0.089 (0.15)	44.8 (30/67)	43.3 (13/30)	0.895 (0.01)
Abortion rate[c]	16.0 (8/50)	0.0 (0/7)	0.357 (0.15)	20.0 (6/30)	30.8 (4/13)	0.458 (0.12)

[a] Mean ± SD (range); t test

[b] Median (interquartile range (IQR)); Mann-Whitney rank sum test

[c] Percentage (fraction); Fisher exact test

[d] Effect size; Cohen's d for mean/median values (very small, 0.01; small, 0.20; medium, 0.50; large, 0.80; very large, 1.20; huge, 2.00); phi coefficient (r) for fractions (small, 0.10; medium, 0.30; large, 0.50)

*Statistically significant

aneuploid spermatozoa in oligozoospermic samples [3, 24–29]. In this sense, an inverse correlation between sperm count and increased rates of chromosome abnormalities has been reported [3, 30]. Spermatogenesis is a highly regulated process in which chromosomes are synapsed, recombined, and segregated. Previous studies have demonstrated an association between sperm count and genetic defects of key chromosomal processes during spermatogenesis: synapsis and recombination [31–37], Sertoli cell polarity [38], centriole duplication [39], and spindle formation [40]. These defects have been related to germ cell apoptosis through the activation of meiotic checkpoints [41–45], leading to a reduction in the number of spermatozoa, which is visualized as a complete or partial absence of sperm in the ejaculate. In addition, inefficient control mechanisms could explain the higher incidences of chromosomal abnormalities in the spermatozoa of these patients; there may be errors in the identification of abnormal cells, or a malfunction in the process of cell elimination, or even the number of abnormal cells could be too high to be completely removed by control mechanisms.

A relationship between polymorphic teratozoospermia and altered FISH results was also found, supporting previously published studies [26, 46–54]. Nevertheless, the data found in the literature were sometimes contradictory, since some authors failed to find this relationship [3, 24, 25]. Controversial results could be explained by the diversity of the morphological sperm alterations included in "terato" seminal alteration. We know little of the molecular pathogenesis of polymorphic teratozoospermia and its association with sperm chromosome anomalies. Recent high-throughput studies have revealed an association between polymorphic teratozoospermia and pathways related to cell cycle progression [55, 56]. The detrimental effect of some genes on cell cycle could compromise cell division (for instance, lack of coordination between karyokinesis and cytokinesis, lack of coordination between meiotic progression and spermiogenesis), including chromosome segregation, leading to the presence of increased incidences of chromosome sperm anomalies. Actually, in teratozoospermic patients, a relationship has been shown between chromosomal aberrations and abnormal morphology in the same spermatozoon, suggesting the same molecular basis [52].

On the contrary, the asthenozoospermia seminal alteration was not related to chromosomal stability, since we failed to find a relationship between sperm chromosomal abnormalities and progressive sperm motility. This result agrees with some previously published data [3, 24, 26, 57], but disagrees with others [25, 58–62]. Controversial results could be explained by methodological biases among studies (e.g., number of patients, statistical analysis) rather than real differences. In the present study, although some individuals with low sperm motility showed a significantly higher incidence of aneuploidies in their spermatozoa, no differences were observed between the "asthenozoospermic" and "non-asthenozoospermic" groups of patients. In agreement, the increased rate of sperm motility abnormalities was not described as a predictive parameter to obtain an altered sperm FISH result in the multivariate analysis. Actually, several regulatory pathways unrelated to chromosome stability were related to

asthenozoospermia: calcium pathway, cAMP-dependent protein kinase pathway, and function of reactive oxygen species, among others [63].

Concerning age indication, although it is known that advanced maternal age is a risk factor for giving birth to children with aneuploidies, there are few proven links between advanced paternal age and specific birth defects [64]. In the clinical context, paternal age is not usually considered as an indication to perform a sperm FISH analysis. Nevertheless, data about age-dependent frequency of aneuploidy in sperm are contradictory and hardly conclusive and, in many cases, were conditioned to the chromosome analyzed [3, 5, 65, 66]. In the present study, differences in age between the group of patients with an altered FISH result and the group of individuals with a normal result failed to reach statistical significance; however, male age was one of the predictive parameters when evaluated for the multivariate logistic regression analysis. One could argue that the association found between age and sperm chromosome anomalies in the regression analysis could be related to the decline in semen quality over time [67]. That is, advanced paternal age is associated with sperm chromosomal anomalies because patients have a tendency towards seminal alteration with age, and seminal alteration is clearly related to sperm chromosome anomalies [2–6]. Nevertheless, no correlation was found in our population between sperm count or sperm morphology with age (data not shown). That is, according to our results, the weak association between age and sperm aneuploidy depends on age, irrespective of the seminal condition of the patients, although the molecular basis remains to be determined.

What patients would benefit from sperm FISH analysis?

The results of the multivariate analysis confirmed the results obtained in bivariate analyses. The profile of patients with altered sperm FISH results was middle-aged (from 35 to 60 years old) oligoteratozoospermic men, who had experienced implantation failures in previous IVF cycles. Accordingly, FISH analysis is clearly indicated in patients with teratozoospermia, oligozoospermia, middle age, and implantation failure. Among them, the indication that is most closely related to the increased rate of chromosomal abnormalities in spermatozoa was the "terato" condition.

Sperm FISH effects on ART outcome in own-oocytes couples

It is highly probable that a chromosomally abnormal spermatozoon has a detrimental clinical impact on fertilization and on the subsequent embryogenesis [68]. Fertilization and early embryogenesis were inferred in this work by "fertilization rate" and "embryo quality." Our study did not show a negative effect on the fertilization rate when an altered sperm FISH result was obtained. Although the literature shows discrepancies between studies, our results are consistent with the series that analyzed a high number of individuals [9, 11, 12] (Table 1).

Actually, there is no reason to believe that there is a dependence between sperm chromosome anomalies and fertilization rate if the characteristics of the samples allow for the selection of appropriate spermatozoa for ICSI; this would be the case for most of the samples included in this work. Moreover, the fertilization rate would also be unaffected by the use of morphologically normal sperm (selected for ICSI) with a chromosome anomaly, since this rate is assessed at the zygote stage when the paternal genome has not yet reactivated [69].

Concerning embryo quality, results indicate that couples with normal sperm FISH produce more high-quality embryos than couples with altered sperm FISH values do it (Table 5). Although the obtained p value did not reach a significant result, it was close to being significant ($p < 0.1$). Although the lack of statistical power in this comparison cannot be ruled out due to limited sample size (size effect of 0.36; Table 5), results might also be related to the timing of early embryo development events. It is well-known that the presence of sperm chromosome anomalies increases the presence of embryo aneuploidies [70, 71]. Nevertheless, several works have suggested that the presence of chromosomal abnormalities in the embryo (either sperm- or oocyte-derived) does not affect embryo development until day 3 of development [72] when the embryo genome become activated. In our work, embryo transfer was performed in the population of own-oocytes couples around day 3 (mean 3.0 days). That is, in day 3 of development, any negative effect from the presence of aneuploidies on embryo quality probably is in a precocious phase, since the embryo genome is starting to activate.

Moving forward through the ART variables assessed, although the results of pregnancy and implantation rates did not reach significant values, several pieces of data support the association between altered sperm FISH results and a detrimental effect on these variables. First, in both cases, p values were close to the significance ($p < 0.1$) which suggests that small sample size has influenced the power of the statistical test performed. Second, a negative impact of the presence of sperm chromosome anomalies on pregnancy and implantation rate has been described before [10–13, 15, 16] (Table 1). Third, an increased incidence of embryo aneuploidies in couples with an altered sperm FISH result has been reported [70, 71], and it has been described that during the transition from cleavage to blastocyst stage embryos, there is a negative selection of aneuploid embryos [73–76]. Moreover, this selection against aneuploidy is constant along development; it has

been described that fetal aneuploidy is present at a frequency of up to 90% in losses aged 0–6 weeks of gestation, in about 50% of sporadic losses occurring at 8–11 weeks and 30% in tissues from losses at 16–19 weeks [77, 78]. Although in our study we did not find an increased rate of abortion when using semen with increments of chromosomal abnormalities, it is probably that the detrimental relationship observed between altered sperm FISH results and pregnancy or preimplantation rates, which are previous events, could mask any effect on the abortion rate (most aneuploid embryos have been already negatively selected). Moreover, the limited number of pregnancies and abortions in our series hampers the statistical analysis. In any case, the association between increases in miscarriage rates and sperm aneuploidy has been described in the literature [11] (Table 1). Even so, higher ratios of chromosomal anomalies in sperm have been described in couples displaying previous recurrent miscarriages [54, 57, 79–82].

Sperm FISH effects on ART outcome in donor-oocytes couples

As mentioned before, the group of couples that used oocytes from a donor was of special interest due to the possibility to evaluate more precisely the effect of the sperm FISH result on the ART outcome. In this group, none of the results of applying ART showed differences between the two groups established according to their results on sperm FISH analysis. Slight differences were observed regarding the age of the recipient mother, in the sense that the group of individuals with altered sperm FISH results was the group that included elder women. This difference cannot be responsible for the absence of differences in ART outcome between the groups of normal and altered sperm FISH results because if advanced maternal age had any effect, it would increase the differences between groups. Moreover, the group of individuals with altered sperm FISH results showed higher numbers of oocytes at MII stage obtained from donor women. The fact that having more oocytes at MII and probably of better quality because they were obtained from young women [83] could be a cause for the absence of differences between normal and altered sperm FISH result individuals. In this sense, it could be suggested that the presence of high rates of chromosomal abnormalities in spermatozoa, when this sperm is used to fertilize oocytes from young women, does not compromise the success of the reproductive outcome. In order to assess whether this statement is too speculative, ART outcome was compared between the group of couples using oocytes from a donor and the group of couples that use their own oocytes (data not shown). Interestingly, as well as foreseeable differences in pregnancy rate (58.8% in couples using oocytes from a donor vs. 42.4% in couples using their own oocytes; $p = 0.013$), differences were observed in the day of transfer (3.8 days in couples using oocytes from a donor vs. 3.0 days in couples using their own oocytes; $p < 0.001$). The differences in ART outcome in these two groups could originate from the fact that in the donor group, embryos were obtained from younger oocytes and transferred later. As we stated above, in the transition from cleavage to blastocyst stage embryos, there is a positive selection of high-quality euploid embryos [73–76]. In other words, in this group of couples, we have preferably transferred high-quality embryos at the blastocyst stage in which selection of euploid embryos has occurred. Consequently, these results suggest that the detrimental effect of sperm anomalies could be partially surpassed by the beneficial effect of culturing embryos from young women to the blastocyst stage. However, since embryo aneuploidy still persists at the blastocyst stage, the application of preimplantation genetic testing for aneuploidy in embryos from couples with an altered sperm FISH result should not be ruled out. In any case, an important consideration of this part of our result is that the sample size is relatively small; therefore, the dataset is still limited to provide definite conclusions.

In summary, sperm FISH is indicated in middle-aged oligoteratozoospermic patients with implantation failures in previous IVF/ICSI cycles. Sperm chromosome anomalies have a moderate negative impact on implantation and pregnancy rates. This detrimental effect could be overcome by the use of oocytes from young women, and embryo culture until the blastocyst stage.

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Compliance with ethical standards

The Ethics Commission on Human and Animal Experimentation of the Universitat Autònoma de Barcelona approved the study.

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