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Fluorescence *In-Situ* Hybridization Detects Increased Sperm Aneuploidy in Men with Recurrent Pregnancy Loss

Ranjith Ramasamy, MD[1,2], **Jason M. Scovell, BA**[1], **Jason R. Kovac, MD, PhD**[1,2], **Peter J. Cook, BA**[1], **Dolores J. Lamb, PhD**[1,2,3], and **Larry I. Lipshultz, MD**[1,*]

[1]Department of Urology, Houston, TX

[2]Center for Reproductive Medicine, Houston, TX

[3]Department of Molecular and Cellular Biology Baylor College of Medicine, Houston, TX

Abstract

Objective—To investigate, in men presenting with recurrent pregnancy loss (RPL), the prevalence of sperm autosome and sex chromosome aneuploidy.

Design—Retrospective Study.

Setting—Male infertility clinic at a tertiary referral center.

Patients—140 men with recurrent pregnancy loss provided semen samples and five normozoospermic controls provided 140 semen samples for comparison. RPL, documented in the female partners, was defined as a prior miscarriage and/or recurrent IVF/ICSI failure.

Interventions—Fluorescent In situ hybridization (FISH) was used to detect numerical abnormalities in sex chromosomes (X,Y) and autosomes (13, 18, 21) in ejaculated sperm.

Main Outcome Measures—Sperm aneuploidy in men with RPL and normozoospermic controls.

Results—Men with RPL had a greater percentage of sperm aneuploidy within the sex chromosomes, chromosomes 18 and 13/21 (1.04% vs. 0.38%; 0.18% vs. 0.03%; 0.26% vs. 0.08%). In total, 40% of men with normal sperm density and motility had abnormal sperm aneuploidy in the all the chromosomes analyzed. Men with abnormal sperm density and motility had a higher proportion of sperm sex chromosome aneuploidy than men with normal density/motility (62% vs. 45%). Men with normal strict morphology (>4%) had lower rates of sex chromosome and sperm aneuploidy than men with abnormal strict morphology (28% vs. 57%). There was no association between sperm DNA fragmentation and sperm aneuploidy.

*Correspondence: Larry I. Lipshultz, MD, 6624 Fannin Street, Suite 1700, Houston, TX 77030, Tel: 713 798 6163, larryl@bcm.edu.

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Conclusions—Men with RPL have increased sperm aneuploidy compared to controls. A total of 40% of men with RPL and normal sperm density/motility had abnormal sperm aneuploidy. Men with oligoasthenozoospermia and abnormal strict morphology had greater percentage of sperm aneuploidy compared to men with normal semen parameters.

Keywords

male infertility; genetics; aneuploidy; FISH

INTRODUCTION

Infertility is the inability to produce offspring despite actively attempting to do so for one year. It affects approximately 15% of all couples - with 50% of cases being ascribed to a male factor (1). Many factors that contribute to altered male factor fertility (i.e. oligozoospermia, asthenozoospermia) can be overcome with the use of assisted reproductive technologies (ART) such as *in vitro* fertilization (IVF) with intracytoplasmic sperm injection (ICSI). These technologies allow males previously considered to be infertile to now father children of their own.

Offspring conceived by ICSI have been shown to be at increased risk for aneuploidies, in particular of the sex chromosomes (2-4). Given that ICSI is a relatively new technology (<30 years old), the long-term effects are still poorly understood. Indeed, one of the primary shortcomings of ICSI is the mechanism by which sperm are selected (5). While close attention is paid to selecting a sperm that displays the best possible combination of sperm parameters (i.e. motility and normal gross morphology) from the patient's sample, this process does not ensure the genetic integrity of the sperm, and hence, the resultant embryo.

As a sub-population, men with normal semen parameters who are partners in a couple with recurrent pregnancy loss (RPL) or unexplained recurrent IVF failure are commonly overlooked. Sperm aneuploidies in these normozoospermic men could represent a significant, but clinically under-appreciated, cause of infertility. In this context, cytogenetic analysis of sperm using fluorescent in situ hybridization (FISH) can help evaluate potential causes of recurrent pregnancy loss or recurrent IVF failure. In this study, we investigated the incidence of autosome and sex chromosome aneuploidies in the sperm of men who are partners in couples with RPL and IVF failure.

PATIENTS AND METHODS

A total of 140 male partners of couples who presented with RPL were analyzed. We defined RPL as recurrent miscarriage and/or the inability to achieve a pregnancy with IVF/ICSI. We included men who had: (1) Sperm aneuploidy testing with FISH, (2) At least two semen analyses on two separate days, (3) At least one strict morphology (assessed with Kruger criteria) and (4) a sperm DNA fragmentation assay (assessed with TUNEL).

Sperm density and motility were averaged from the semen parameters available. Abnormal sperm density was defined as < 15million sperm/cc and abnormal motility as < 40%; according to the WHO 2010 guidelines. A total of 140 semen samples from five

normozoospermic men were used as controls for comparison. We excluded men with known causes of infertility such as Kleinfelter syndrome and Y-chromosome microdeletions and men who did not have at least two semen analyses for evaluation. The institutional review board at the Baylor College of Medicine approved the study.

FISH assay

Semen samples obtained for FISH testing were centrifuged for 10 minutes at 1900 rpm to separate seminal fluid from cells (within 24 hours). The supernatant was removed and 5mM KCl was added and sample was incubated for 25 minutes in 37C. Carnoy fix (3 parts methanol plus 1 part acetic acid) was added and sample was re-centrifuged. The step was repeated until the pellet is white and supernatant is clear. About 5ml of the sample is fixed on a slide and slide is stored at −20C prior to processing for FISH.

Three-color FISH was performed on ejaculated sperm in order to help define the numerical abnormalities in chromosome 18 and the sex chromosomes (X and Y). Two-color FISH was used to detect chromosome abnormalities in chromosomes 13 and 21. A minimum of 20,000 sperm were scored for each man. The VYSIS AneuVysion DNA Probe Kit (Catalogue # 35 – 161075) was used (incorporating CEP probes for chromosomes 13 and 21 and LSI probes for chromosome X, Y and chromosome 18).

Scoring of sperm nuclei was performed under a Olympus BX51 fluorescent microscope using an aqua filter (C43494 excitation peak=433nm; emission peak=480nm) for chromosome 18, a green filter (U-MNB2 excitation peak=330-385nm; emission peak=420nm) for chromosomes X and 13, a red filter (U-MGN2 excitation peak=530-550nm; emission peak=590nm) for the Y and 21 chromosome and filter U-M@U2 for DAPI (excitation=330-385nm; emission peak=420nm). Images were captured using the Image Pro software.

Sperm were scored as disomic if the same-colored signals were of similar intensity, size, and shape, and if both signals were clearly visible within the sperm. In addition, positively scored cells had a clearly defined border and were not overlapping. One technician specifically trained in FISH analysis performed all tests described. We defined an abnormal FISH parameter as having an aneuploidy percentage >2 standard deviations from the mean of our normozoospermic controls. Results can be standardized in our patient samples given that the proportion of aneuploidy in our control population is similar to that reported in the published literature on aneuploidy in the general population (6). We tested normality of the distribution using a quantile-quantile plot (QQ plot) and Shapiro Wilk test and ascertained that the data were normally distributed.

Statistical analysis

Statistical analysis was performed using SPSS (IBM, NY v22). Means were compared using an independent sample t-test, and frequencies were compared using a Fisher’s exact test. A $p<0.05$ was considered significant in all circumstances.

RESULTS

We performed two and three-color FISH analysis of sperm chromosomes 13, 18, 21, X and Y on ejaculated sperm from 140 men who had RPL. Average sperm aneuploidy was greater in sperm from men with RPL than in normozoospermic controls (Sex Chromosome: 1.04% vs. 0.38%, p=0.015, Chromosome 18: 0.18% vs. 0.03%, p<0.001, Chromosomes 18/21 = 0.26% vs. 0.08%, p=0.002) (Table 1). A higher proportion of men with RPL had sperm aneuploidy (> 2 standard deviations above the mean aneuploidy for controls) for the sex chromosome than did controls (53% vs. 3%, p<0.001). Interestingly, there was no difference between the proportion of men with RPL and aneuploidy and controls for either chromosome 18 (36% vs. 29%, p=0.25) or chromosomes 13/21 (50% vs. 53%, p=0.71).

A total of 40% of men with normal sperm density and motility had increased sperm aneuploidy in both sex chromosomes and autosomes (Table 2) compared to controls. On evaluating sperm aneuploidy in men with abnormal semen parameters, we found that a larger proportion of men with isolated low sperm density (<15 million/cc) and low sperm motility (< 40%) had greater sperm sex chromosome aneuploidy than did men with normal sperm density and motility (62% vs. 45%, p=0.042) (Table 2). Similarly, there was a greater proportion of men with abnormal strict morphology (<4%) who also had sex chromosome aneuploidy (57% vs. 28%, p=0.04) than of men with normal strict morphology (>4%) (Table 3). As expected, there was no association between abnormal DNA fragmentation (>30%) and severity of aneuploidy or aneuploidy rates (supplementary table).

DISCUSSION

With the increased use of IVF/ICSI for male factor infertility, it is important to identify reasons for failure. One of the greatest challenges with ICSI is the identification of “normal sperm” for micro-manipulation. Unfortunately, with current technologies, we can only identify sperm with grossly abnormal morphology rather than detecting underlying genetic abnormalities such as aneuploidy.

Controversy exists concerning safety of ICSI and whether using genetically defective sperm will lead to abnormal fetuses or IVF failure. Several studies have demonstrated an increased frequency of genetic abnormalities in men with spermatogenic impairment (7-9). Even though men with infertility have increased sperm aneuploidy (10, 11), most practical clinical genetic testing for men with infertility is currently limited to detection of chromosomal abnormalities using a karyotype and Y-chromosome microdeletion analysis (12).

Furthermore, research in male factor infertility predominantly is focused on men with abnormal semen parameters. Indeed, men with grossly normal semen parameters and RPL/IVF failure usually are not counseled on any particular causes and are not encouraged to undergo any further testing. It is important to realize that sperm aneuploidy rates can be high even in men with normal sperm morphology (13). And herein, we demonstrate that increased sperm aneuploidy is present in men with normal strict sperm morphology.

Additionally, the most interesting finding in our study was that up to 45% of men with normal sperm density and motility had abnormal FISH results. We believe that sperm

aneuploidy testing is indicated in this particular subpopulation of men – that is, in men with normal semen parameters and RPL or recurrent ART failure.

It is noteworthy that although the overall mean aneuploidy appears to be small (0.18 – 1.04%), it is up to 4 times higher than the aneuploidy observed in the controls (0.03 – 0.38%). We also demonstrated an increase in aneuploidy in both sex chromosomes and autosomes. It is expected that meiotic recombination errors would affect both sex chromosomes and autosomes equally. In fact, in a study of men with Klinefelter syndrome, sperm had increased disomy in chromosome 21 (14). The marked increase in disomy is concerning considering that trisomy 13, 18, and 21 results in Patau's syndrome, Edwards's syndrome and Down's syndrome respectively. XY disomies and aneuploidies will lead to Klinefelter syndrome (47 XXY) and Turners syndrome (46XO). Couples with abnormal sperm FISH should be counseled regarding these possibilities and be urged to make informed reproductive choices.

Our study has several strengths as well as limitations. The current report describes a very large series of men with RPL who have also had sperm aneuploidy testing. Furthermore, during the laboratory testing process, each patient sperm FISH sample was compared to a fresh semen sample from a control. Consequently, in spite of the fact that we only had five normospermic men to use as controls, our data are made more valid by the presence of inter-test controls. Unfortunately, none of our five-normozoospermic controls attempted a pregnancy.

Regrettably, there are no universally accepted standards for abnormal FISH results compared to those that exist for strict morphology and DNA fragmentation. We calculated mean aneuploidies from our normozoospermic controls and defined a cutoff for abnormal FISH as two standard deviations above this mean. Given that the sperm aneuploidy rates in our control population were similar to those rates in published studies, we are confident that our definition of abnormal FISH can be applied to other studies as well.

In summary, up to 45% of men presenting with RPL and normal sperm density, motility, and morphology can have abnormal sperm aneuploidy. Abnormal sperm aneuploidy can result in increased miscarriages and abnormal fetuses. There is a need to reduce the burden associated with repeated pregnancy attempts through either natural conception or ART. Therefore, men presenting with recurrent pregnancy loss or recurrent unexplained ART failure should consider sperm aneuploidy testing to determine an underlying etiology to enable better informed reproductive choices. Further controlled studies are necessary to determine the benefit of FISH testing in men with RPL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Baseline characteristics of men with recurrent pregnancy loss (RPL)

	Men with RPL	Control	p
N	140	140	-
Age	30.5	23.5	0.335
Sperm density (Million / cc)	37.5	116.8	<0.001
Sperm motility	46.7	62.2	<0.001
Sex Disomy (%)	1.04%	0.38%	0.015
18 Disomy (%)	0.18%	0.03%	<0.001
13 & 21 Disomy (%)	0.26%	0.08%	0.002
Men with Sex Chromosome Disomy (%)	52.9°% (74/140)	2.9% (4/140)	<0.001
Men with Chromosome 18 Disomy (%)	36.4°% (51/140)	29.3% (41/140)	0.252
Men with Chromosomes 13/21 Disomy (%)	50.0% (70/140)	52.9% (74/140)	0.720

Table 2

Prevalence of fragmentation, morphology, and abnormal fish in men with normal/abnormal semen parameters

	Abnormal Sperm Density & Motility	Normal Sperm Density & Motility	P – value
Age	38.0 +/− 6.6	37.4 +/− 5.4	0.535
SM%	1.45% +/− 1.47	2.56% +/− 1.93	0.001
Density	13.6 +/− 19.3	57.7 +/− 35.3	<0.001
Motility	30.5 +/− 19.4	59.9 +/− 11.2	<0.001
Sex Disomy (%)	0.01 +/− 0.01	0.10% +/− 0.80	0.361
18 Disomy (%)	0.23% +/− 0.53	0.14% +/− 0.34	0.267
13 & 21 Disomy (%)	0.36% +/− 0.91	0.18% +/− 0.29	0.132
Men with Sex Chromosome Disomy (%)	62.3% (n=40)	44.7% (n=34)	0.042
Men with Chromosome 18 Disomy (%)	37.5% (n=24)	35.5% (n=27)	0.861
Men with Chromosomes 13/21 Disomy (%)	56.3% (n=36)	44.7% (n=34)	0.235

Table 3

Comparison of sperm aneuploidy in men with normal and abnormal strict morphology

	SM < 4%	SM > 4%	p
Age	37.9 +/− 5.7	35.6 +/− 5.9	0.149
SM%	1.46% +/− 1.10	5.56% +/− 0.84	<0.001
Density	34.4 +/− 32.5	66.9 +/− 45.8	<0.001
Motility	45.0 +/− 21.0	60.6 +/− 13.8	0.001
Sex Disomy (%)	0.08% +/− 0.70	0.00% +/− 0.01	0.276
18 Disomy (%)	0.20% +/− 0.47	0.05% +/− 0.11	0.005
13 & 21 Disomy (%)	0.28% +/− 0.75	0.15% +/− 0.49	0.479
Men with Sex Chromosome Disomy (%)	56.6% (n=56)	27.8% (n=5)	0.038
Men with Chromosome 18 Disomy (%)	40.5% (n=99)	16.7% (n=3)	0.066
Men with Chromosomes 13/21 Disomy (%)	50.5% (n=50)	27.8% (n=5)	0.122