Article

Paternal gonadal mosaicism detected in a couple with recurrent abortions undergoing PGD: FISH analysis of sperm nuclei proves valuable

Charintip Somprasit received her medical degree in 1995 from Prince of Songkla University in Thailand and finished her residency training in obstetrics and gynaecology from Chulalongkorn University in 2000. Following graduation, she pursued a clinical career in the prenatal diagnostic field at Thammasat University. In 2003, she began her research fellowship training in preimplantation genetic diagnosis at Baylor College of Medicine (Houston, Texas, USA) under the direction of Dr Farideh Z Bischoff.

Dr Charintip Somprasit

Charintip Somprasit[1,3], Monica Aguinaga[1,4], Pauline L Cisneros[1], Sergey Torsky[1], Sandra A Carson[1], John E Buster[1], Paula Amato[1], Sallie Lou McAdoo[1], Joe Leigh Simpson[1,2], Farideh Z Bischoff[1,5]
[1]Department of Obstetrics and Gynecology; [2]Department of Human and Molecular Genetics, Baylor College of Medicine, Houston, Texas, USA; [3]Department of Obstetrics and Gynecology, Thammasat University, Pathumthani, Thailand; [4]Instituto Nacional de Perinatologa, Departmento de Gentica, México
[5]Correspondence: Department of Obstetrics and Gynecology, Baylor College of Medicine, 6550 Fannin Street, Suite 885, Houston, TX 77030, USA. Tel: +1 713 7988885, Fax: +1 713 7985579, e-mail: bischoff@bcm.tmc.edu

Abstract

Many couples are now seeking preimplantation genetic diagnosis (PGD) and fluorescence in-situ hybridization (FISH) as an alternative approach to avoid spontaneous abortion by ensuring transfer of presumed chromosomally normal embryos. This case report describes unexpected findings in a couple having three spontaneous abortions and two failed IVF cycles. In two IVF PGD cycles, four of 13 (30.8%) embryos (blastomeres) demonstrated duplication involving the Down syndrome critical region, detectable by a locus specific chromosome 21 probe. The same duplication was subsequently detected by FISH in 66 of 1002 (6.6%) sperm nuclei, demonstrating paternal gonadal mosaicism. Cytogenetic studies of peripheral blood revealed normal karyotypes in both the male and female partners. This identification of paternal germ cell or gonadal mosaicism suggests that analysis of sperm nuclei prior to undergoing IVF with PGD may be of value in patients with recurrent spontaneous abortions or multiple failed IVF.

Keywords: *FISH, gonadal mosaicism, PGD, recurrent spontaneous abortion, spermatozoa*

Introduction

Recurrent pregnancy loss (three or more losses before 20 weeks of gestation) occurs in approximately 0.5–1% of couples (Regan *et al*., 1989; Simpson and Elias, 2003). Depending on the number of previous pregnancy losses, recent data suggest that the likelihood of recurrent pregnancy loss is 20–35% (Katz and Kuller, 1994; Badawy and Westpfal, 2000; Petrozza and O'Brien, 2002). At least 50% of all first trimester losses are cytogenetically abnormal, half involving autosomal trisomy (Soares *et al*., 2001; Petrozza and O'Brien, 2002; Rubio *et al*., 2003). Following three or more spontaneous abortions, analysis of karyotypic abortuses and parents is typically warranted (Petrozza and O'Brien, 2002; Duzcan *et al*., 2003). Even if parental karyotypes are normal, couples may still be at increased risk for aneuploidy as a result of gonadal mosaicism (Robinson *et al*., 2001; Simpson and Elias, 2003). Indeed, cytogenetic studies on female partners have revealed a high percentage of aneuploidy in unfertilized oocytes, presumably as a result of aberrant maternal meiosis (Zenzes and Casper, 1992; Cowchock *et al*., 1993; Munné *et al*., 1995; Rubio *et al*., 1999). Maternal gonadal mosaicism has been demonstrated in ovarian biopsies from couples with recurrent spontaneous abortions (Nielsen *et al*., 1988; Sachs *et al*., 1990; Tseng *et al*., 1994; Conn *et al*., 1999; Cozzi *et al*., 1999). Interestingly, in a study of 1235 cleavage-stage embryos, 45% (n = 556) were identified as mosaic by fluorescence in-situ hybridization (FISH) and proposed to be due to maternal age effect (Munné *et al*.,

2002). Aneuploidy originating in a male partner is less common, but responsible for some trisomies. Investigations of male gonadal mosaicism have been more limited.

Many couples are now utilizing preimplantation genetic diagnosis (PGD) FISH as an alternative approach to avoid spontaneous abortion in order to ensure transfer of presumed chromosomally normal (euploid) embryos. Multicolour interphase FISH is a well-developed technique for detecting chromosomal aneuploidy and unbalanced segregation products in preimplantation embryos (Abdelhadi *et al*., 2003).

This case report describes unexpected findings in a couple who had three spontaneous abortions, two failed IVF cycles, and normal peripheral blood karyotypes in both partners. PGD unexpectedly revealed a chromosome 21 structural duplication involving the Down syndrome critical region (21q22.13-q22.2), present in four of 13 (30.8%) embryos tested. FISH studies on spermatozoa revealed the same chromosome 21 structural abnormality in 6.6% of sperm nuclei, consistent with germ cell or gonadal mosaicism. FISH analysis of spermatozoa may provide valuable information with regard to expected PGD outcome and pregnancy success.

Materials and methods

Clinical information

In this case report, a 32-year-old female and a 33-year-old male of Caucasian descent presented with a history of three first trimester spontaneous abortions. No chromosome studies were available on any of the aborted fetuses; peripheral blood chromosomes (karyotypes) were reported to be normal for both partners. The sole abnormality reported was in semen analysis, which showed abnormal morphology (8% normal forms) based on Kruger's strict criteria (normal ≥14%). A paternal sister was reported to have a Down syndrome infant, but confirmation was not recorded. They had previously undergone two IVF cycles both of which failed to yield a pregnancy. The couple then underwent two cycles of IVF and PGD, both approved by Baylor College of Medicine's institutional review board with written informed consent.

Stimulation and embryo biopsy

Down-regulation was achieved with a gonadotrophin-releasing hormone agonist. After stimulation with human menopausal gonadotrophin, 16 oocytes were aspirated in the first IVF cycle and nine oocytes in the second cycle. Three days following oocyte retrieval and intracytoplasmic sperm injection (ICSI), 6- to 8-cell embryos were biopsied. One or two blastomeres were aspirated from each embryo using suction and a Humagen™ biopsy needle. In the first cycle, nine embryos and in the second, six embryos were biopsied, then rinsed and maintained in culture until transfer.

Blastomeres were washed in phosphate-buffered saline (PBS) for 2 min and transferred to a poly-L-lysine coated slide as described by Coonen *et al*. (1994). After air drying, the slides were washed in PBS and sequentially dehydrated in alcohol (70, 90 and 100%).

FISH

FISH was performed with two sequential hybridizations. In the first hybridization, chromosomes 13, 18, 21, X and Y were enumerated. In the second hybridization, chromosomes 16, 21 and 22 were enumerated. Analysis of chromosome 21 was repeated in the second hybridization to confirm the results of the first hybridization. The probe cocktail mixture using direct-labelled probes for the first hybridization has been previously described (Vysis Inc., Downers Grove, IL, USA) (Bischoff *et al*., 1998). A 4.5-µl aliquot was used per blastomere per slide tested. The probe mixture was denatured prior to hybridization. After removal of coverslips, two post-hybridization washes were performed: 0.4× SCC at 70°C for 2 min, followed by 2× SSC/NP40 at room temperature for 1 min. Nuclei were counterstained and viewed using a Zeiss Axiosko microscope (Carl Zeiss, Thornwood, NY, USA) (McKenzie *et al*., 2003).

The second hybridization was performed for chromosomes 16 and 22 and, as a confirmation, chromosome 21. Slides containing nuclei identified as normal at the first hybridization were washed in 0.4× SSC at 70°C (to remove any remaining first hybridization probes). These slides were then transferred to 2× SSC followed by progressive dehydration in 70, 90, and 100% ethanol. For the second hybridization, a 10 µl probe cocktail mixture was used, consisting of 1 µl of probe for chromosome 22 (locus-specific; SpectrumGreen direct labelled), 1 µl of probe for chromosome 21 (locus-specific; SpectrumOrange direct labelled), 0.3 µl of probe for chromosome 16 (centromere alpha-satellite; 1:1 mixture SpectrumGreen: SpectrumOrange direct labelled probe detected as yellow fluorescence). Slides subjected to the second hybridization were then processed in the same fashion as those of the first hybridization. After post-hybridization washes, nuclei were counterstained and viewed under fluorescence microscopy.

Sperm FISH

A semen sample was obtained and fixed in methanol: acetic acid (3:1) and processed for FISH analysis. Details of sperm fixation, nuclear decondensation, and FISH processing were as described previously by Vidal *et al*. (1993). Centromeric alpha-satellite DNA probe for chromosome 18 (SpectrumGreen) and locus specific DNA probe for chromosome 21 (SpectrumOrange) (Vysis) were used. Analysis of sperm nuclei was performed using an Olympus BX 60 epifluorescence microscope equipped with a triple-band pass filter for DAPI/Texas red/fluorescein isothiocyanate (FITC) and single-band pass filters for FITC and Texas red.

Embryo transfer

Embryos having two signals for each autosome plus either XX or XY were selected for transfer 4 days following oocyte retrieval. Luteal support with intramuscular progesterone was maintained until a pregnancy test was performed 2 weeks later.

Results

PGD cycles

Fertilization rate was 64.3% (nine of 14) in the first cycle and 88.9% (eight of nine) in the second. Of the eight fertilized embryos in the second cycle, only four could be subjected to FISH. Two of the remaining four embryos failed to divide, and hence were unsuitable for biopsy; two other embryos were biopsied, but the recovered nuclei failed to hybridize with any of the probes, probably indicating the presence of empty nuclei.

PGD results are summarized in **Table 1**. In addition to various aneuploidies, an unexpected duplicated region hybridizing to chromosome 21 was detected in three of nine embryos in the first cycle and in one of four embryos in the second cycle (**Figure 1**). The close proximity of the two chromosome 21 signals indicated duplication within this region. This pattern was detected in four of 13 (30.8%) blastomeres. Because these findings indicated mosaicism, a germ cell origin was postulated. Among 178 PGD blastomeres previously analysed by FISH, a chromosome duplication pattern involving chromosome 21 has never been observed.

Overall, only two of the total 13 embryos were chromosomally normal by FISH, and therefore suitable for transfer. No pregnancy was achieved.

Sperm and whole blood FISH

Conventional cytogenetic and interphase FISH analysis of peripheral blood failed to detect the chromosome 21 duplication pattern. Reasoning that the mosaicism might have arisen in the father, FISH analysis was performed on decondensed sperm nuclei from a fresh semen sample. Hybridization efficiency was 98.5%, based on presence of one signal for the control chromosome 18 probe (**Table 2**).

Among 1002 sperm nuclei, 930 (92.8%) showed the expected normal (haploid) distribution of one signal for each chromosome 18 and chromosome 21. An abnormal chromosome 21 pattern (two closely positioned signals) was detected in 66 (6.6%) sperm nuclei (**Figure 2**). These data support gonadal mosaicism involving an abnormal duplication within the Down syndrome critical region on chromosome 21.

Table 1. Results of FISH analysis on biopsied blastomeres.

Embryo no.[a]	*Nucleus no.*	*FISH results*	*Interpretation*
Cycle 1			
1	1	XY, +dup (21), +13	Mosaic trisomy21[c]
1	2	XY, +dup (21)	
2	1	Y, –13, –13, –18	Haploid monosomy13
2	2	Y, –13, –13, –18, –21	
6	1	X, –13, –18, –21	Haploid nucleus
6	2	X, –13, –18, –21	
8	1	XY, –18	Monosomy 18
8	2	XY, –18	
10	1	XX, –13, –13, –21	Monosomy 21
11	1	XY	Normal XY[c]
12	1	XX	Normal XX
15	1	Complex	Polyploid
16	1	Complex	Polyploid[c]
Cycle 2[b]			
5	1	XX, +13, +13, +18, +21, +21	Tetraploid[c]
8	1	XY, –21	Monosomy 21
10	1	XX	Mosaic haploid
10	2	X, –13, –18, –21	
11	1	Y, –13, –13, –18, –21	Haploid
11	2	Y, –13, –13, –21	

[a]At biopsy, two blastomeres were recovered from each of four embryos in cycle #1 (embryo #1, 2, 6, 8,) and two embryos in cycle 2 (embryo nos. 10, 11).
[b]Of six embryos subjected to biopsy, four blastomeres were suitable for FISH analysis.
[c]Embryos containing abnormal chromosome 21 duplication pattern (dup 21).

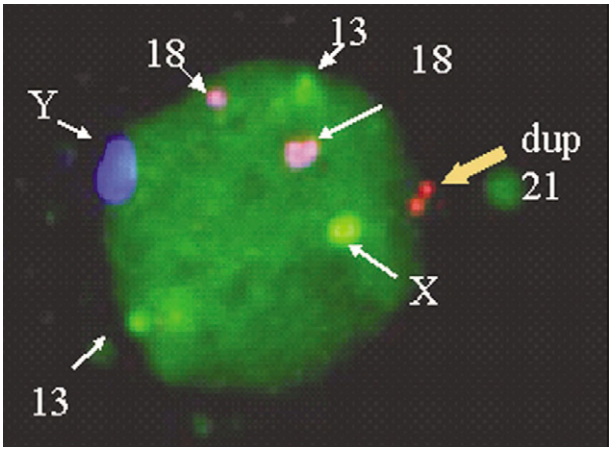

Figure 1. Fluorescence in-situ hybridization (FISH) on a blastomere nucleus showing an abnormal chromosome 21 duplication pattern (yellow arrow). No normal signal for chromosome 21 (one spot) was detected. Other probes (X, Y, 13 and 18) detected in the first hybridization are shown by white arrows.

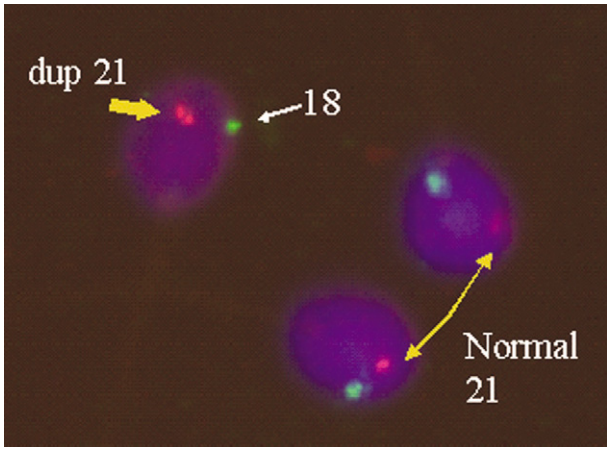

Figure 2. Interphase FISH analysis of patient's sperm sample. Yellow arrow indicates detection of the abnormal chromosome 21 duplication, but normal chromosome 18 signal (green), in one nucleus. The other two sperm nuclei show normal presence of one signal for each the 18 and 21 probes.

Table 2. Results of sperm FISH analysis.

FISH signal distribution[a]	*Frequency (%)*	*Interpretation*
1 – green; 1 – red	930 (92.8)	Normal (haploid)
1 – green; 2 – closely positioned red signals	66 (6.6)	Normal 18, duplication 21
1 – green; 0 – red	4 (0.4)	Normal 18, nullisomy 21
0 – green; 1 – red	1 (0.1)	Nullisomy 18, normal 21
2 – green; 2 – red (far apart)	1 (0.1)	Diploid or disomic for 18, 21

[a]Number of signals detected following hybridization using chromosome 18 and 21 directly labelled probes (Vysis, Inc.) in spermatozoa from the male partner. Green signal connotes detection of chromosome 18 (control probe) and red signal denotes detection of chromosome 21.

Discussion

In this case report, paternal gonadal mosaicism involving duplication of a region on chromosome 21 was observed, both in PGD embryos as well as in spermatozoa. Among 13 embryos biopsied, only two were normal. Four of 13 embryos (30.8%) showed the abnormal chromosome 21, in a pattern indicative of duplication involving the Down syndrome critical region. This was subsequently observed by FISH analysis of spermatozoa, with 6.6% showing the same chromosome 21 duplication. The duplication might have arisen following a paracentric inversion in gonad or germ cell lineage. Analysis of testicular biopsy tissue would provide more accurate assessment of the level of mosaicism in this individual. Molecular studies are underway to delineate the size and precise location of the apparent duplication.

Aneuploidy most commonly arises during maternal meiosis. Maternal gonadal mosaicism is well known, and many reports of maternal gonadal mosaicism in couples with recurrent spontaneous abortion and recurrent aneuploidy exist (Nielsen *et al*., 1988; Sachs *et al*., 1990; Tseng *et al*., 1994; Conn *et al*., 1999; Cozzi *et al*., 1999; Bruyere *et al*., 2000; Robinson *et al*., 2001). Aneuploidy can also arise from errors in paternal meiosis. Approximately 5–10% of trisomy 21 cases are of paternal origin (Antonarakis, 1991; Yoon *et al*., 1996). Less common is paternal gonadal mosaicism (Soares *et al*., 2001). Of 78 Down syndrome cases studied by FISH in only peripheral blood, 33% were found to be mosaic (Modi *et al*., 2003). Given that the degree of phenotypic manifestations is related to the percentage of trisomic cells, it is plausible that normal individuals may in fact have low level mosaicism involving gonadal tissue. Blanco *et al*. (1998) reported mosaic chromosome 21 disomy in the semen of two men, each of whom had a Down syndrome offspring. Using FISH, sperm analysis of 12 couples with recurrent spontaneous abortions showed significantly increased incidence of sex chromosome disomy compared with donor spermatozoa (0.84 versus 0.37%) (Rubio *et al*., 1999). Based also on FISH studies, Spriggs *et al*. (1996) and Blanco *et al*. (1996) reported chromosome 21 disomy in normal (donor) spermatozoa to be only 0.29 and 0.38% respectively. In all these studies, the two

chromosome 21 signals were relatively far apart from each other, as expected with two free lying chromosome 21s. Sperm FISH in the present case showed 6.6% nuclei to have two chromosome 21 signals (**Figure 2**), 17-fold greater than reported for normal donor spermatozoa. Unlike previously reported cases, the presence of two signals in very close proximity indicated a duplication pattern involving 21q22.13-q22.2 loci in this case.

Although recurrent pregnancy loss in this couple plausibly might be due to the observed chromosomal abnormalities in spermatozoa, maternal contributions cannot be excluded. In fact, the percentage of abnormal chromosome 21 pattern was relatively poorly correlated between biopsied embryos (30.8%) and spermatozoa (6.6%). That only 6.6% of the spermatozoa were abnormal for chromosome 21 further suggests selection against abnormal spermatogonial cells during germ cell maturation (Johnson, 1995). Although there is no direct evidence of selection against these abnormal spermatozoa, FISH on testis tissue biopsy may more accurately reveal the level of mosaicism. Alternatively, spermatozoa bearing the apparent duplication may have a selective advantage involving morphology, given that fertilization was achieved through ICSI.

FISH detection of common aneuploidies in decondensed sperm nuclei has been suggested prior to PGD in couples with recurrent abortions (Egozcue *et al*., 1997; Vidal *et al*., 2001). Considering that such couples are undergoing PGD FISH anyway, utility of FISH on spermatozoa would be to provide additional information that may alert the IVF–PGD team to possible unexpected findings, especially if few embryos are involved. The present study supports this recommendation, particularly in couples with failed IVF cycles who lack other identifiable factors.

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References

Abdelhadi I, Colls P, Sandalinas M *et al*. 2003 Preimplantation genetic diagnosis of numerical abnormalities for 13 chromosomes. *Reproductive BioMedicine Online* **6**, 226–231.

Antonarakis SE 1991 Paternal origin of the extra chromosome in trisomy 21 as indicated by analysis of DNA polymorphism. Down syndrome collaborative group. *New England Journal of Medicine* **324**, 872–876.

Badawy SZ, Westpfal EM 2000 Frequency of etiological factors and cost effectiveness of the work up for patients with history of recurrent pregnancy loss. *Early Pregnancy: Biology and Medicine* **4**, 253–260.

Bischoff FZ, Lewis DE, Nguyen DD *et al*. 1998 Prenatal diagnosis with use of fetal cells isolated from maternal blood: five-color fluorescent in situ hybridization analysis on flow-sorted cells for chromosomes X, Y, 13, 18, and 21. *American Journal of Obstetrics and Gynecology* **179**, 203–209.

Blanco J, Egozcue J, Vidal F 1996 Incidence of chromosome 21 disomy in human spermatozoa as determined by fluorescent in-situ hybridization. *Human Reproduction* **11**, 722–726.

Blanco J, Gabau E, Gomez D *et al*. 1998 Chromosome 21 disomy in the spermatozoa of the fathers of children with trisomy 21, in a population with a high prevalence of Down syndrome: increased incidence in cases of paternal origin. *American Journal of Human Genetics* **63**, 1067–1072.

Bruyere H, Rupps R, Kuchinka BD *et al*. 2000 Recurrent trisomy 21 in a couple with a child presenting trisomy 21 mosaicism and maternal uniparental disomy for chromosome 21 in the euploid cell line. *American Journal of Human Genetics* **94**, 35–41.

Conn CM, Cozzi J, Harper JC *et al*. 1999 Preimplantation genetic diagnosis for couples at high risk of Down syndrome pregnancy owing to parental translocation or mosaicism. *Journal of Medical Genetics* **36**, 45–50.

Coonen E, Dumoulin JC, Ramaekers FC, Hopman AH 1994 Optimal preparation of preimplantation embryo interphase nuclei for analysis by fluorescence in-situ hybridization. *Human Reproduction* **9**, 533–537.

Cowchock FS, Gibas Z, Jackson LG 1993 Chromosome errors as a cause of spontaneous abortion: the relative importance of maternal age and obstetric history. *Fertility and Sterility* **59**, 1011–1014.

Cozzi J, Conn CM, Harper J *et al*. 1999 A trisomic germ cell line and precocious chromatid segregation leads to recurrent trisomy 21 conception. *Human Genetics* **104**, 23–28.

Duzcan F, Atmaca M, Cetin GO, Bagci H 2003 Cytogenetic studies with reproductive failure. *Acta Obstetricia et Gynecologica Scandinavica* **82**, 53–56.

Egozcue J, Blanco J, Vidal F 1997 Chromosome studies in human sperm nuclei using fluorescence in-situ hybridization (FISH). *Human Reproduction Update* **3**, 441–452.

Johnson L 1995 Efficiency of spermatogenesis. *Microscopy Research and Technique* **32**, 385–422.

Katz VL, Kuller JA 1994 Recurrent miscarriage. *American Journal of Perinatology* **11**, 386–397.

McKenzie LJ, Cisneros PL, Torsky S *et al*. 2003 Preimplantation genetic diagnosis for a known cryptic translocation: follow-up clinical report and implication of segregation products. *American Journal of Medical Genetics* **121A**, 56–59.

Modi D, Berde P, Bhartiya D 2003 Down syndrome: a study of chromosomal mosaicism. *Reproductive BioMedicine Online* **6**, 499–503.

Munné S, Alikani M, Tomkin G *et al*. 1995 Embryo morphology, developmental rates, and maternal age are correlated with chromosome abnormalities. *Fertility and Sterility* **64**, 382–391.

Munné S, Sandalinas M, Escudero T *et al*. 2002 Chromosome mosaicism in cleavage-stage human embryos: evidence of a maternal age effect. *Reproductive BioMedicine Online* **4**, 223–232.

Nielsen K, Poulsen H, Mikkelsen M, Steuber E 1988 Multiple recurrence of trisomy 21 Down syndrome. *Human Genetics* **78**, 103–105.

Petrozza JC, O'Brien B 2002 Early pregnancy loss. *e-Medicine* **10**, 2002.

Regan L, Braude PR, Trembath PL 1989 Influence of past reproductive performance on risk of spontaneous abortion. *British Medical Journal* **26**, 541–545.

Robinson WP, McFadden DE, Stephenson MD 2001 The origin of abnormalities in recurrent aneuploidy/polyploidy. *American Journal of Medical Genetics* **69**, 1245–1254.

Rubio C, Simon C, Blanco J *et al*. 1999 Implications of sperm chromosome abnormalities in recurrent miscarriage. *Journal of Assisted Reproduction and Genetics* **16**, 253–258.

Rubio C, Simon C, Vidal F *et al*. 2003 Chromosomal abnormalities and embryo development in recurrent miscarriage couples. *Human Reproduction* **18**, 182–188.

Sachs ES, Jahoda MG, Los FJ *et al*. 1990 Trisomy 21 mosaicism in gonads with unexpectedly high recurrence risks. *American Journal of Medical Genetics* **7** (suppl.), 186–188.

Simpson JL, Elias S (eds) 2003 *Genetics in Obstetrics and Gynecology*, 3rd edn. Saunders, Philadelphia, pp. 101–132.

Soares SR, Templado C, Blanco J *et al*. 2001 Numerical chromosome abnormalities in the spermatozoa of the fathers of

children with trisomy 21 of paternal origin: generalised tendency to meiotic non-disjunction. *Human Genetics* **108**, 134–139.

Spriggs EL, Rademaker AW, Martin RH 1996 Aneuploidy in human sperm: the use of multicolor FISH to test various theories of nondisjunction. *American Journal of Human Genetics* **58**, 356–362.

Tseng LH, Chuang SM, Lee TY, Ko TM 1994 Recurrent Down's syndrome due to maternal ovarian trisomy 21 mosaicism. *Archives of Gynecology and Obstetrics* **255**, 213–216.

Vidal F, Moragas M, Catala V *et al*. 1993 Sephadex filtration and human serum albumin gradients do not select spermatozoa by sex chromosome: a fluorescence in situ hybridization study. *Human Reproduction* **8**, 1740–1743.

Vidal F, Blanco J, Egozcue J *et al*. 2001 Chromosomal abnormalities in sperm. *Molecular and Cellular Endocrinology* **183**, S51–S54.

Yoon PW, Freeman SB, Sherman SL *et al*. 1996 Advanced maternal age and the risk of Down syndrome characterized by the meiotic stage of chromosomal error: a population-based study. *American Journal of Human Genetics* **58**, 628–633.

Zenzes MT, Casper RF 1992 Cytogenetics of human oocytes, zygotes, and embryos after in vitro fertilization. *Human Genetics* **88**, 367–375.

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