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Disomy rates for chromosomes 14 and 21 studied by fluorescent in-situ hybridization in spermatozoa from three men over 60 years of age

Sophie Rousseaux[1], Mira Hazzouri, Roberte Pelletier, Michèle Monteil, Yves Usson and Bernard Sèle

Unité INSERM U309, Université Joseph Fourier Grenoble 1, Institut Albert Bonniot, Domaine de la Merci, 38706 La Tronche Cedex, France

[1]To whom correspondence should be addressed

In order to further investigate the paternal-age effect on meiotic non-disjunction rates for the chromosomes 14 and 21, we examined spermatozoa from three men aged >60, using multicolour fluorescent in-situ hybridization (FISH). More than 10 000 sperm cells were analysed for each of the three subjects (A, B and C), by simultaneously hybridizing two YAC probes specific for chromosomes 14 and 21 respectively using two-colour FISH. The results show that the disomy 21 rates observed in the spermatozoa of two out of the three men aged >60 years were higher (1.02 and 1.17% respectively) than the rates observed in eight control adults aged <30 years (mean frequency 0.48%) analysed under similar conditions. These results suggest that there may be a small effect of age on male non-disjunction rates for chromosome 21. However, before any firm conclusions could be drawn, a much bigger sample of older men would have to be compared with a paired control population using the same FISH experimental approach.

Key words: aneuploidy/fluorescence in-situ hybridization/paternal age/spermatozoa/trisomy 21

Introduction

Trisomy in human neonates has long been known to increase with maternal age. Although some studies have reported associations between paternal age and the risk of fathering an aneuploid offspring (Lorda-Sanchez *et al.*, 1992a; Petersen *et al.*, 1993), none of the available data clearly confirm or reject this hypothesis. The issue of whether older men have a higher risk of having aneuploid offspring is still controversial. Both epidemiological and molecular studies suffer from the relatively limited number of cases of trisomy of paternal origin.

The data from epidemiological studies analysing the relationship between paternal age and the incidence of trisomy 21 have led to considerable controversies in the literature, some studies suggesting that there is (Stene *et al.*, 1981) and others that there is not (Cross and Hook, 1987; Hook, 1987) a paternal age effect on the incidence of trisomy 21. More recently, Thepot *et al.* (1993, 1996), analysing 11 535 pregnancies conceived by artificial insemination using donor spermatozoa, showed that the mean frequency of trisomy 21 was 0.25% (25 cases from 9794 birth and 35 fetuses after prenatal diagnosis). The incidence of trisomy 21 was shown to be higher in the group of donors aged >38 years than in the group of donors aged <38 years. This study did not suffer from the bias induced by maternal age effect because there was no correlation between parental ages in contrast to natural procreation. According to the authors, this observation may 'encourage the lowering of the age limit for donors'. However, in this study as well as in the previous ones, the parent and cellular stage of origin of trisomy is unknown, which is a major limitation since most trisomies are now known to be maternally derived (Hassold *et al.*, 1996; Wyrobek *et al.*, 1996).

The analysis of DNA polymorphic markers of the extra chromosome in trisomic patients makes it now possible to determine the parental and meiotic origin of the non-disjunction responsible for the abnormality (Sherman *et al.*, 1991). Using these molecular studies it has been shown that overall, only 10–30% of autosomal trisomies (chromosomes 13, 14, 15, 21 and 22) originate in paternal meiosis, most of these arising during meiosis II. Moreover only 5% of trisomy 21 is of paternal meiotic origin, with 40% arising at paternal meiosis I and 60% at meiosis II (Hassold *et al.*, 1996; Wyrobek *et al.*, 1996). In contrast, the paternal contribution to aneuploidy of the sex chromosomes is more substantial; e.g. 100% of XYY constitutional karyotypes involve an extra paternal chromosome, half of XXY cases are due to errors of paternal meiosis I (Lorda-Sanchez *et al.*, 1992a), and 80% of the sex chromosome missing in Turner syndromes are paternal, due to meiotic non-disjunction or post-fertilization loss (Lorda-Sanchez *et al.*, 1992b; Ogata and Matsuo, 1995). Several groups have used molecular studies to directly address the issue whether paternal age is elevated in trisomies of paternal origin, and this has led to conflicting results. For example, investigating the parent and cell division of origin of the additional sex chromosome in 142 males with a 47,XXY constitution and 50 females with a 47,XXX constitution, McDonald *et al.* (1994) found that the additional chromosome was paternal in origin in 66 of the former and in five of the latter and showed no evidence of an increased paternal age in the aneuploidies of paternal origin. On the contrary, significantly increased paternal age had been found in the paternally derived cases with a XXY sex chromosome constitution (Lorda-Sanchez *et al.*, 1992a). Studying 36 cases of free

Table I. Sperm parameters

Subjects	Age (years)	No. of spermatozoa (10^6/ml)	Motility after thawing (%)
Controls (aged <30 years)			
F20	28	20	40
F21	28	30	30
F23	28	40	20
F24	21	25	20
F25	23	78	30
F26	29	55	20
F27	29	89	30
F28	29	89	30
Subjects aged >60 years			
A	64	10	20
B	64	80	60
C	64	10	10

trisomy 21 in which the supernumerary chromosome was of paternal origin with DNA markers specific for chromosome 21, Petersen *et al.* (1993) observed 15 meiotic II errors, eight mitotic errors and seven meiosis I non-disjunctions. Their results suggest a possible paternal age effect among paternal meiosis I cases. In contrast, Zaragoza *et al.* (1994) observed no effect of increasing paternal age in a small series of paternally-derived acrocentric trisomies. Here again, molecular studies are limited by the relative rarity of paternally-derived cases.

An alternative approach to overcome this difficulty is the direct analysis of the product of male meiosis, the sperm nucleus. The development of fluorescence in-situ hybridization (FISH) on interphase sperm nuclei allows the analysis of disomy rates in large numbers of spermatozoa. Using this method, a meiotic non-disjunction frequency can be precisely determined for each chromosome in each individual. The use of two- or three-colour FISH makes it possible to differentiate the disomies (24 chromosomes) from the diploidies (46 chromosomes) and, for the sex chromosomes, the meiotic stage (I or II) of the non-disjunction (Williams *et al.*, 1993).

Using multicolour FISH, we have looked for the disomy rates of chromosomes 14 and 21 in the spermatozoa from three men aged over 60. More than 10 000 sperm cells were analysed for each of the three subjects (A, B and C), by the simultaneous hybridization of two YAC probes respectively specific for chromosomes 14 and 21 in two-colour FISH. For each subject the disomy rates for these chromosomes as well as the diploidy rate were compared with those observed in the spermatozoa of eight control donors aged <30 years.

Materials and methods

Experimental sperm donors

A total of 11 healthy males were studied, eight under 30 years of age (controls) and three over 60. The sperm parameters are shown in Table I. The FISH experiments were done simultaneously with the spermatozoa from the older subjects and the controls.

Sperm preparation

Semen samples were thawed and washed twice in 0.01 M Tris pH 8 for 5 min at 600 *g*. The in-vitro decondensation procedure was performed as described by Rousseaux and Chevret (1995). Briefly, the sperm nuclei were incubated for 3–10 min with 10 mM dithiothreitol at room temperature. Spermatozoa were then dropped onto clean dry slides and fixed with 3:1 ethanol:acetic acid. Slides were kept frozen until their use.

DNA probes

The YAC probes (cloned in yeast artificial chromosome) 746B4 and 745H11 respectively specific for chromosomes 14 (14q23–24) and 21 (21q22.2) were a gift from D.Lepaslier at the 'Centre d'Etude du Polymorphisme Humain' (CEPH). Yeast were cultured and their DNA extracted. Human specific sequences were amplified using an alu-PCR protocol described by Lengauer *et al.* (1992). They were labelled by nick-translation with digoxigenin 11 dUTP (21q probe) and biotin 16 dUTP (14q probe) and co-hybridized in two-colour on the sperm preparations, as described below.

Fluorescence in-situ hybridization

FISH was performed simultaneously on sperm slides from the older subjects A, B and C and from the control subjects. The method has already been described (Pinkel *et al.*, 1986a,b; Rousseaux and Chevret, 1995). Before hybridization, sperm DNA slides were treated with RNase A (100 µg/ml) for 1 h at 37°C, dehydrated in ethanol (70:90:100%), and denatured in 70% formamide (2 min at 70°C), dehydrated again and air-dried.

A 20 µl sample of hybridization mix [50% formamide/dextran sulphate 10%/0.5× sodium chloride, sodium citrate (SSC)/0.5 SSPE], containing 100 ng of each probe, 10 µg of cot1 DNA and 10 µg of sonicated salmon spermatozoa, was heated at 75°C for 5 min to denature the probe, and preincubated at 37°C for 10 min for prehybridization.

The mixture was then applied to each sperm nuclei preparation and slides were covered with a 24 mm^2 coverslip, sealed with rubber cement and hybridized in the dark at 37°C for 20 h. Coverslips were then removed and slides were washed three times for 5 min each in 50% formamide/2× SSC at 45°C, three times for 5 min each in 2× SSC at 45°C and once for 3 min in 0.1× SSC at 60°C.

Biotinylated and digoxigenin-labelled probes were simultaneously visualized with avidin–fluorescein isothiocyanate (FITC) (1/300, Vector Laboratories) and anti-digoxigenin-rhodamine Fab fragments (1/200, Boehringer) respectively. Nuclei were counterstained with 4′,6-diamino-2-phenyl-indole dihydrochloride (DAPI) (50 ng/ml) in an antifade solution (Vector Laboratories; Biosys SA, Compiègne, France). Slides were screened using a ×100 objective on a Zeiss Axiophot microscope equipped with an FITC/rhodamine/DAPI triple band-pass filter.

Only individual and well-delineated spermatozoa were scored. Two signals of the same colour were scored as two copies of the corresponding chromosome when they were of the same intensity and size, and were clearly distinct (Figure 1). The slides were scored by two independant observers in a double blind study. A minimum of 10 000 sperm nuclei on two or three slides from several experiments were scored for each subject.

Statistical analysis

As a preliminary assessment of the reproducibility of the technique, the intra-individual/inter-experimental variations of disomy rates were calculated by comparing frequencies observed in different slides from the same individual.

In a first approach, we compared the results of each of the older men with the mean values observed in the controls using *P* values calculated from a χ^2 test. Assuming that the controls were representative of a population with frequencies following a normal distribution,

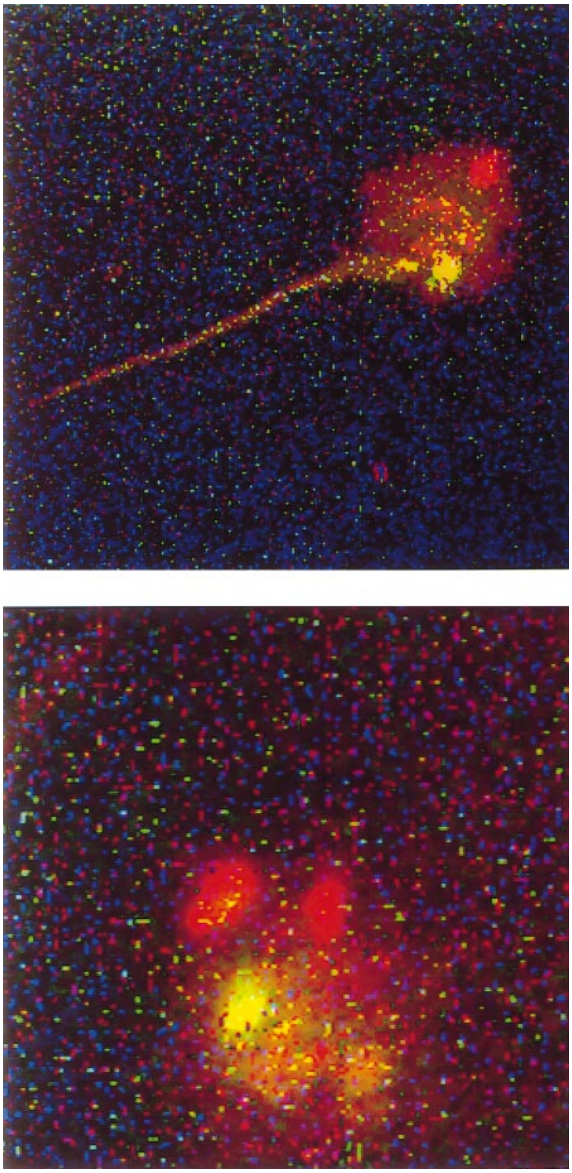

Figure 1. Spermatozoa hybridized with probes specific for chromosomes 14 (green), and 21 (red). Upper panel shows 14/21, lower panel shows disomy 21.

we then calculated the probability of each frequency observed in the older men to belong to the controls' normal distribution.

Results

For subjects A, B and C respectively, the disomy 14 rates were 0.48, 0.40 and 0.56%, the disomy 21 rates were 0.67, 1.02 and 1.17%, and the diploidy rates were 0.54, 0.66 and 1.10%. In the men under 30 years of age, the mean frequencies of disomy 14, disomy 21 and diploidies were respectively 0.48, 0.48 and 0.56% (Table II). Within each individual, the variations of frequencies obtained from different experiments and by different observers did not exceed 20% (data not shown).

Compared with the mean values of the control population aged under 30, the frequencies of normal and abnormal spermatozoa observed in the three older men were significantly different for subjects B and C ($P < 0.001$, χ^2 values 22.3 and 50.2 respectively) but not for subject A.

A more detailed analysis of the data consisted in testing the hypothesis that each frequency observed in the older men belonged to a normal distribution of the corresponding value calculated from the control population. Within a 95% confidence interval (for a 5% threshold, $u = 1.96$), this hypothesis was rejected for frequencies observed in subject B and C. Indeed both subjects B and C had significantly lower proportions of normal spermatozoa than the controls ($P < 0.05$, $u =$ 1.99 and 4.69 respectively) and for subject C the disomy 21 rate and diploidies were significantly higher than in the control population ($P < 0.05$, $u = 2.37$ and 3.61 respectively).

Discussion

Some of the data using multicolour FISH on interphase spermatozoa suggest that aneuploidy of sex chromosomes increases with age of the male in the human (for XX and YY disomies, Wyrobek *et al.*, 1994; Robbins *et al.*, 1995; for XX, YY and XY, Griffins *et al.*, 1995; and for YY, Martin *et al.*, 1995a; Kinakin *et al.*, 1997) but in fact there is still controversy regarding the age-effect on sex chromosomal aneuploidy rates. For instance, for XY spermatozoa (which represents meiosis I error and may give rise to children with Klinefelter syndrome), Griffin *et al.* (1995), but not Robbins *et al.* (1995) found an association with age in the human. No age effect on XY spermatozoa was found in mouse (Lowe *et al.*, 1995). Age effects have also been reported for autosomal sperm disomies. For instance, disomy 1 was reported to be increased in sperm of aged men (Martin *et al.*, 1995a; Kinakin *et al.*, 1997). So far, there has not been a study on the effect of age on disomy 21 rates observed by FISH on interphase spermatozoa.

The present study was a first attempt to detect an age-effect on disomy 21 rates in spermatozoa using dual-colour FISH with probes specific for chromosomes 14 and 21. The data show that the disomy 21 rates observed in the spermatozoa of two of the three over 60 years old men were double the mean rate observed in eight controls aged under 30 analysed under similar conditions using the same probes. Our statistical approach confirmed that the proportions of normal spermatozoa (i.e. spermatozoa with no diploidy, no disomy 14 or 21) were significantly lower in subjects B and C, whereas the disomy 21 and diploidy frequencies were significantly increased in subject C. It suggests there may be a small effect of age on male non-disjunction rates for chromosome 21.

However, FISH studies have limitations. Disomy rates observed in the young donor group were higher in comparison with those observed in previous FISH studies (Blanco *et al.*, 1996; Spriggs *et al.*, 1996, see below). Moreover, in an earlier study using the same combination of probes on the spermatozoa of another healthy subject, we observed lower disomy rates for both chromosomes 14 and 21 (0.13% and 0.10% respectively) (Rousseaux *et al.*, 1995). These discrepancies are generally attributed to many factors such as different sperm decondensation techniques, interindividual variations and/or application of different scoring criteria (Guttenbach *et al.*, 1997a). In general, FISH results on sperm aneuploidy rates vary between laboratories and therefore each laboratory should establish its own 'reference' values of aneuploidy rates for each chromo-

Table II. Number of spermatozoa considered to be haploid, disomic for chromosomes 14 or 21, or diploid in control males and men aged >60 years. Figures in parentheses are percentages

	Spermatozoa				
	Haploid 14/21	Disomy 14 14/14/21	Disomy 21 14/21/21	Diploid 14/14/21/21	Total
Controls aged <30 years					
F20	10 270 (98.91)	40 (0.39)	26 (0.25)	47 (0.45)	10 382
F21	10 043 (98.56)	74 (0.73)	4 (0.04)	69 (0.68)	10 190
F23	10 826 (98.45)	28 (0.25)	83 (0.75)	59 (0.54)	10 996
F24	10 373 (98.73)	67 (0.64)	17 (0.16)	49 (0.47)	10 506
F25	10 089 (98.43)	46 (0.45)	51 (0.50)	64 (0.62)	10 250
F26	10 829 (98.17)	58 (0.53)	76 (0.69)	68 (0.62)	11 031
F27	10 506 (98.54)	53 (0.50)	69 (0.65)	34 (0.32)	10 662
F28	10 282 (98.04)	40 (0.38)	82 (0.78)	84 (0.80)	10 488
Mean control	10 402 (98.48)	51 (0.48)	51 (0.48)	59 (0.56)	10 563
Men aged>60 years					
A	10 184 (98.31)	50 (0.48)	69 (0.67)	56 (0.54)	10 359
B[a]	10 553 (97.92)[b]	43 (0.40)	110 (1.02)	71 (0.66)	10 777
C[a]	10 147 (97.17)[b]	59 (0.56)	122 (1.17)[b]	115 (1.10)[b]	10 443

[a]Observed frequencies significantly different from the mean values of the control population ($P < 0.001$, $\chi^2 = 22.3$ and 50.2 for subjects B and C respectively).
[b]These frequencies are significantly different from the corresponding frequencies in the controls, assuming that the frequencies in the controls have a normal distribution (for a 5% threshold, $u = 1.96$).

some in a control population using the same probe with the same hybridization conditions and scoring criteria (Martin *et al.*, 1995b).

Another limitation is that aneuploidy frequencies in sperm cells are relatively low for individual chromosomes (Hassold *et al.*, 1996; Hassold, 1998) and age effects are only obvious and become significant when large numbers of spermatozoa from many individuals are scored in the same laboratory and under similar conditions. Moreover, there are interindividual variations in sperm aneuploidy rates (e.g. Robbins *et al.*, 1993; Bischoff *et al.*, 1994; Guttenbach *et al.*, 1997b, and the present study) which may be due to many factors such as genetic predisposition, exposure to chemicals, drugs and environmental pollutants, smoking and previous infections (Eichenlaub-Ritter, 1996). These rather large interindividual variations as well as the cumulative effects on sperm aneuploidy rates that these factors (at least some of them) are likely to have, contribute to the difficulty of analysing the effect of paternal age as a specific and independant factor.

It is worth noting that subject B had both an elevated disomy rate and exceptionally high sperm motility parameters. There seems to be no correlation between sperm functional parameters and sperm disomy rates. This is also suggested by FISH data from 45 infertile patients whose disomy rates of chromosomes 1, 7, 10, 17, X and Y were similar to those of healthy fertile males (Guttenbach *et al.*, 1997a).

In other FISH studies, the mean disomy 21 rates were 0.38% in the spermatozoa of nine individuals analysed by Blanco *et al.* (1996) (ranging from 0.20 to 0.54%), and 0.29% in the spermatozoa of five individuals analysed by Spriggs *et al.* (1996) (ranging from 0.22 to 0.33%). In both studies, disomy 21 frequencies were significantly higher than disomies observed for other autosomes under the same experimental conditions. FISH studies, as well as pooled data from human sperm karyotypes, show higher frequencies of non-disjunction during male meiosis for the sex chromosomes and for chromosome 21 than for other autosomes (Spriggs *et al.*, 1996). Although various theories were proposed to explain these differences, the mechanisms underlying non-disjunction of bivalents are not well understood. It could be that many factors including ageing preferentially affect the same chromosomes which have a higher tendancy of non-disjunction, but this remains to be explored.

The diploidy rates was also significantly higher in donor C than in the control population. However, this observation cannot be discussed in relation to age because the number of individuals is not sufficient given the considerable variation in the frequency of diploid spermatozoa among normal men (Rademaker *et al.*, 1997).

The results of the present study show that, if any, the age-effect on trisomy 21 of paternal origin is likely to be small and influenced by many other factors. This explains the difficulties of the previous epidemiological or even molecular studies to draw any firm conclusions on this issue. Although the present study has dealt with much larger numbers of meiotic segregations, there is no evidence yet that one should encourage the 'lowering of the age limit for donors' in the practice of artificial sperm insemination with donor. Moreover, the age-effect on aneuploidy rates is likely to be influenced by other factors, especially environmental factors. In order to confirm or reject a small effect of age on male non-disjunction rates for chromosome 21, a much bigger sample of older men would have to be compared to a paired control population, using the same FISH experimental approach.

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