ELECTRONIC LETTER

Cytogenetic analysis of spermatozoa from males aged between 47 and 71 years

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Trisomy is the most common chromosome abnormality in humans and occurs in approximately 4% of all clinically recognised pregnancies.[1] This numerical chromosome abnormality may lead to spontaneous abortions, polymalformation syndromes, mental retardation, and impairment of gametogenesis. Although the great majority of trisomies are the result of segregation errors during paternal or maternal meiosis, some trisomies are the result of postzygotic mitotic error.[2]

Parental age has been known to be the most important aetiological factor implicated in human trisomy formation. Advanced maternal age is a predisposing factor for most autosomal trisomies but maternal age effect shows considerable variation among chromosomes.[3] Data on human trisomy from spontaneous miscarriages and offspring suggested that non-disjunctions at maternal meiosis I was the most common cause of trisomies for acrocentric chromosomes. Two possible mechanisms may be responsible for non-disjunction of acrocentric chromosomes: premature division of sister chromatids and non-disjunction of bivalent chromosomes. The second mechanism increases with maternal age.[4 5] The majority of trisomy 18 are the result of an abnormal distribution of this chromosome during the second maternal meiotic division.[6] Maternal age has variable effects on maternally derived constitutional aneuploidy for different human chromosomes. The maternal age effect is very strong for chromosome 21 and demonstrable for chromosomes 15, 16, and 21.[3 7]

The paternal contribution to aneuploidy is low and the possible relationship between paternal age and trisomy has been the subject of controversy. Most of the studies evaluating this parameter concerned aneuploidy discovered after miscarriages, prenatal diagnosis, or at birth. However, cases with proven paternal origin have been rarely reported.[3 7] Using molecular analysis of trisomic patients, 10-30% of autosomal trisomies originate at paternal second meiotic division and chromosomes 13, 14, 15, 21, and 22 have been implicated.[4] In trisomy 21, the extra chromosome 21 has been shown to have a paternal origin in about 5% with 60% the result of error at meiosis II[8] with a probable paternal age effect.[7 9 10] However, paternal contribution to aneuploidy is more substantial for sex chromosomes: 84% of XYY males result from paternal meiosis II non-disjunction,[11] 50% of cases of Klinefelter syndrome are the result of paternal meiosis I error,[12 13] and 80% of cases of Turner syndrome originate from loss of the paternal sex chromosome.[14 15] In cases of paternally derived Klinefelter syndrome, a paternal age effect was observed in a study performed in 47,XYY patients.[12] However, data on 151 non-mosaic 47,XXY males from Sardinia showed a significant increase in risk of 47,XXY livebirths at advanced parental ages, maternal age alone was sufficient to explain the effect, and no independent effects of paternal age were observed once maternal age had been taken into account.[16] No effect of increased paternal age was observed in the analyses of paternally derived XXY, XXX, and acrocentric trisomies.[4 13 17] More recently, in a study carried out in 38 fathers of boys with Klinefelter syndrome, paternal age was correlated with the frequency of XY sperm.[18]

Analysis of sperm chromosome constitution can overcome difficulties encountered with epidemiological studies in evaluating a paternal age effect. Spermatozoa karyotypes performed by the human sperm/hamster oocyte fusion assay has shown conflicting results; Martin *et al*[19] and Prestes Sartorelli *et al*[20] observed a significant negative correlation between age and the frequency of hyperhaploid complements. In contrast, Rosenbusch *et al*[21] found a positive correlation between donor age and total number of metaphases with numerical and structural abnormalities. Data from fluorescence in situ hybridisation (FISH) studies on sperm nuclei showed an age effect for the rate of YY disomy[22–24] and in the frequency of XY sperm nuclei,[25 26] except for one result carried out in three males over 80 years of age.[27] In this study, sperm disomy frequency for chromosomes 1 and 21 was positively correlated to donor age.[22 28]

However, extreme variability in the results was observed and was probably the result of not only the methodology used but also the number of subjects included. In most studies, the number of males aged over 45 years did not exceed 10 subjects.[22–30]

In the present study, we included a larger number of males aged between 47 and 71 years to evaluate the frequency of aneuploid spermatozoa for chromosomes 4, 15, 18, 21, 22, X, and Y and show that advanced age is a predictive factor for aneuploidy in spermatozoa. Disomy rates were compared with those evaluated in spermatozoa of 10 control donors aged between 28 and 40 years. The aim of our study was to show that advanced male age leads to an increased frequency of meiotic errors.

Key points

- The relationship between paternal age and aneuploidy in offspring has led to considerable controversy.
- The present study evaluated aneuploidy frequency in spermatozoa in 16 males aged between 47 and 71 years (12 of them over 50 years) and provided evidence of a paternal age effect on disomy for chromosomes 4, 18, and 21 and for XY sperm.
- These results suggest an age related alteration of chromosome segregation at the first and second meiotic divisions in human males.

MATERIALS AND METHODS

Study population

Sixteen subjects, aged between 47 and 71 years, were studied. No subjects had had previous exposure to chemotherapy or radiotherapy or any chronic illness known to have an impact on male fertility. Ten subjects had fathered children previously

Table 1 Age and sperm parameters from 10 control subjects (C) and 12 subjects over 45 year old (E)

Subjects		Age (y)	Sperm concentration (10^6/ml)	Total count (10^6/ejaculate)	Motility (%)	Normal spermatozoa (%)
1	C	33	75	255	35	66
2	C	28	26	88.9	40	59
3	C	40	104	789	35	60
4	C	29	200	540.5	40	82
5	C	34	85	212.5	35	5
6	C	31	80	561.4	45	70
7	C	37	130	728.4	30	69
8	C	29	77	215.6	45	54
9	C	36	27	108.8	25	66
10	C	32	85	366.7	35	74
Mean (SEM)		33 (1.24)	88.90 (15.81)	386.70 (80.00)	36.50 (1.98)	60.50 (6.66)
11	E	71	470	517	35	58
12	E	47	34	163	40	4159
13	E	52	30	132	40	5453
14	E	52	130	390	45	4134
15	E	51	35	170	30	5941
16	E	47	46	37	45	7054
17	E	57	20	34	20	5555
18	E	49	48	197	35	4541
19	E	50	90	170	30	5645
20	E	46	84	369.6	35	5278
21	E	57	119	166.6	40	3456
22	E	65	2.5	1	50	5852
23	E	52	41	156	45	70
24	E	63	25	102.5	35	45
25	E	52	40	140	35	27
26	E	50	90	90	30	45
Mean (SEM)		53.81 (1.77)	81.53 (27.45)	177.23 (34.57)	34.06 (2.55)	50.81 (12.73)

and only one subject was referred to our centre for intracytoplasmic sperm injection because of oligoasthenospermia.

Ten males, aged between 28 and 40 years, recruited from the general population as sperm donors were included as a control group.

Sperm preparation

Semen samples were processed as described by Rives *et al*.[27] Briefly, simultaneous decondensation and denaturation of sperm nuclei were performed by incubation for 10 minutes with 3 mol/l NaOH at room temperature.

FISH procedure

Centromeric probes specific for chromosomes 4, 15, 18, X, and Y directly labelled with Spectrum Orange™ (chromosome X, CEP X; chromosome Y, CEP Y; chromosome 15, CEP 15) or Spectrum Green™ (chromosome 4, CEP 4; chromosome 18, CEP 18) (Vysis, Voisins Le Bretonneux, France) were co-hybridised in two (chromosomes 4 and 15) or three colour FISH (chromosomes X, Y, and 18). Whole chromosome paint probes for chromosomes 21 and 22 (WCP 21 Spectrum Orange™, WCP 22 Spectrum Green™, Vysis) were hybridised in dual colour FISH. Hybridisation and detection were carried out as described previously.[27] Slides were counterstained with a solution of 4′, 6-diamidino-2-phenylindole diluted in antifade mounting medium (Vysis).

Data collection and statistical analysis

The slides were examined using a ×100 magnification with an epifluorecence microscope (DMRD®, Leica, Germany). Preparations were scored with a triple band pass filter (FITC/rhodamine/DAPI) and a minimum of 10 000 sperm nuclei were evaluated per chromosome, except for one subject who showed sperm parameter alterations. Only individual and non-overlapping sperm heads were included in the count. Hyperhaploidy was scored but not nullisomy, since absence of signal could be the result of failure of hybridisation as well as non-disjunction.

Age and sperm parameters were compared between the two groups using the Mann-Whitney test and χ^2 test was performed to check for homogeneity of disomy frequencies in each group and compare the frequency of each hybridisation pattern between the two groups. A value of $p<0.05$ was considered significant (Statview® for Windows 95, Abacus Concepts, Inc, Berkeley, CA).

RESULTS

Sperm parameters and age are summarised in table 1. Disomy frequencies for the autosomes and for the sex chromosomes studied are reported in table 2. The mean efficiency of hybridisation was 99.83% (SD 0.12) for the control group and 99.87 (SD 0.04) for subjects aged between 47 and 71 years with no significant statistical difference between the two groups.

The mean age in the studied group was significantly higher than in controls ($p<0.001$). The same results were observed for the total sperm count ($p=0.02$) and for the percentage of morphologically normal spermatozoa ($p=0.016$). However, sperm number and motility did not differ between the two groups.

The sex ratio did not vary from the expected 1:1 ratio in the two groups. In the control group, homogeneity of disomy frequencies was observed for most of the chromosomes, except for chromosomes X and 15 disomy ($p<0.0007$). In the elderly male group, disomy and diploidy frequencies were heterogeneous ($p<0.0001$).

Disomy frequencies for chromosomes 4 and 18 increased significantly in elderly males. Although the frequency of YY and XX disomic sperm nuclei was similar to that detected in the control group, the incidence of XY hyperhaploid spermatozoa was significantly increased ($p<0.0001$). The same data were observed for the disomy frequency of chromosome 21 ($p=0.0005$) and for the rate of diploidy ($p<0.0001$). The rate of disomic 15 and 22 spermatozoa did not significantly vary between the two groups.

Table 2 Aneuploidy frequency in sperm nuclei of 10 control subjects (C) and 16 elderly subjects (E) evaluated in two* and three† colour FISH

Subjects		Aneuploidy frequency for chromosomes (%)								Diploidy rate (%)
		4*	15*	18†	21*	22*	XX†	YY†	XY†	
1	C	0.09	0.11	0.03	0.21	0.23	0.18	0.14	0.19	0.07
2	C	0.14	0.26	0.01	0.19	0.27	0.02	0.05	0.11	0.10
3	C	0.07	0.11	0.01	0.19	0.29	0.07	0.07	0.14	0.06
4	C	0.08	0.20	0.02	0.15	0.21	0.04	0.08	0.10	0.05
5	C	0.04	0.08	0.04	0.28	0.19	0.07	0.08	0.12	0.06
6	C	0.05	0.16	0.04	0.21	0.22	0.13	0.09	0.20	0.05
7	C	0.03	0.08	0.01	0.28	0.25	0.08	0.03	0.12	0.07
8	C	0.05	0.12	0.01	0.27	0.27	0.06	0.05	0.17	0.08
9	C	0.09	0.08	0.00	0.29	0.27	0.07	0.05	0.09	0.05
10	C	0.04	0.15	0.01	0.16	0.27	0.08	0.05	0.16	0.07
Mean (SEM)		0.07 (0.01)	0.13 (0.02)	0.018 (0.004)	0.22 (0.02)	0.25 (0.01)	0.08 (0.01)	0.07 (0.01)	0.14 (0.01)	0.07 (0.01)
11	E	0.03	0.08	0.05	0.24	0.20	0.20	0.01	0.12	0.05
12	E	0.09	0.05	0.13	0.33	0.31	0.0	0.01	0.15	0.16
13	E	0.05	0.11	0.07	0.31	0.31	0.09	0.03	0.31	0.11
14	E	0.14	0.20	0.24	0.26	0.18	0.04	0.3	0.69	0.51
15	E	0.32	0.14	0.15	0.33	0.35	0.0	0.03	0.13	0.21
16	E	0.06	0.51	0.06	0.32	0.34	0.07	0.02	0.16	0.10
17	E	0.09	0.05	0.14	0.29	0.23	0.05	0.07	0.16	0.18
18	E	0.11	0.04	0.21	0.21	0.33	0.02	0.01	0.13	0.28
19	E	0.07	0.12	0.13	0.27	0.21	0.03	0.06	0.14	0.20
20	E	0.09	0.18	0.12	0.37	0.31	0.03	0.0.8	0.16	0.13
21	E	0.28	0.31	0.37	0.17	0.19	0.09	0.2	0.24	0.18
22	E	0.21	0.36	0.20	0.93	0.93	0.13	0.0	0.73	0.28
23	E	0.03	0.05	0.05	0.27	0.25	0.40	0.0	0.10	0.07
24	E	0.013	0.12	0.44	0.67	0.22	0.09	0.15	0.12	0.19
25	E	0.31	0.35	0.37	0.23	0.27	0.05	0.02	0.71	0.14
26	E	0.10	0.18	0.38	0.24	0.32	0.06	0.15	0.67	0.23
Mean (SEM)		0.13 (0.02)	0.18 (0.03)	0.19 (0.03)	0.34 (0.05)	0.31 (0.04)	0.05 (0.01)	0.07 (0.02)	0.29 (0.06)	0.19 (0.03)

DISCUSSION

Epidemiological studies concerning chromosome abnormalities discovered during prenatal diagnosis or at birth have provided limited support for a paternal age effect in their genesis. Cytogenetic analysis of male gametes is a direct approach to evaluate aneuploidy generated during meiotic divisions. In our study, aneuploidy for sex chromosomes and chromosomes 4, 15, 18, 21, and 22 was evaluated in spermatozoa from males aged between 47 and 71 years; 12 subjects were over 50 years of age (table 1). The results were compared to the rates observed in 10 control males with a mean age of 33 years (SD 1.24). Paternal age effect on chromosome meiotic segregation may be detectable at 39 years of age but more significantly after 41 years.[9] To our knowledge, no study has yet considered males with this mean age (53.81 years (SD 1.77)) and with such a large total number of subjects (n=16). Most studies have generally analysed a small number of males aged over 45 years (from three to 10).[22–30]

The expected 1:1 sex ratio was similar between the two groups of subjects. These results are in agreement with the fact that male age at conception does not affect sex ratio and confirms data performed by human sperm/hamster egg fusion assay[32] and by FISH.[22] Heterogeneity of disomy frequencies has been observed in the group of elderly males ($p<0.0001$) and has been reported in various series. This result could be related not only to the methodology or criteria used or interindividual variability,[33] but also to a variable rate of non-disjunction occurring during meiosis in elderly males.

The rate of XY spermatozoa was significantly higher in males with advanced age ($p<0.0001$). Most of the previously reported studies have shown that non-disjunction for sex chromosomes mainly occurred at the second meiotic division.[22 24 34 35] One study found a slight increase of XY sperm nuclei in three males over 80 years old which suggested the occurrence of non-disjunction for sex chromosomes at the first meiotic division. However, one study found a relationship between male age and non-disjunction at the first and second meiotic divisions.[25] Lowe *et al*[18] conducted a study on spermatozoa from fathers of boys with Klinefelter syndrome and suggested that older fathers produce higher frequencies of XY sperm. Our data suggest that a male age effect on sex chromosome aneuploidy occurs at the first meiotic division. No significant increase was observed in the rate of XX and YY spermatozoa in our study. Although Bosch *et al*[30] did not observe an increase in disomic XX, YY, or XY spermatozoa in males aged more than 45 years, they reported a linear trend association between the global rate of sex chromosome disomy and age in human males. Molecular analysis of 47 subjects with an XXY constitution showed significantly increased paternal age in the paternally derived cases because of a meiotic I error. There was no evidence of recombination in the centromeric and pseudoautosomal regions between paternal X and Y chromosomes.[12] However, reduced recombinations have also been found to induce non-disjunction.[14 36 37]

The repercussions of male age on spermatic autosomal disomy have not yet been completely explored so cannot be proved or rejected. A significant increase of disomic 4, 18, and 21 as well as diploid spermatozoa was found in our elderly males. Martin *et al*[22] found a positive correlation between chromosome 1 disomy rate and male age. In contrast, Lahdetie *et al*[34] failed to find any age effect on disomy frequency for this chromosome. Disomy 1,[27] disomy 6,[30] disomy 8,[23] disomy 12,[22 26] disomy 13,[29] disomy 17,[27] disomy 18,[25–27] and 21[29 30] did not show an age dependent increase in males. However, Rousseaux *et al*[24] observed a small rise in the rate of disomic 21 spermatozoa in two 60 year old males, but not for disomy 14. In most of these studies, the number of males over 45 years was not very many and this might have underestimated the real effect of advanced age on the rate of aneuploid spermatozoa. In our study, 16 males over 45 years were analysed and 12 subjects were aged between 50 and 71 years. Prenatal diagnosis on 35 year old mothers showed that older fathers have an increase in trisomy 21 offspring and for paternal ages of 41

years upwards the age effect is quite strong.[9 38] These observations confirm that it would be interesting to study males over 50 years to explore a male age effect on meiotic non-disjunction.

There may be different explanations for the effect of male age on the rate of meiotic non-disjunction. Paternal age effect may probably vary from one chromosome to another because of chromosome length, chromatin characteristics, and rates of recombination.[39 40] Centromere structure plays a fundamental role during sister chromatid separation of somatic cell mitosis, but also during the second meiotic division. Ageing significantly accelerates premature division of sex chromosome centromere during lymphocyte mitosis[41 42] and this process could be responsible for chromosome non-disjunction in lymphocytes as well as in spermatocytes during meiotic divisions of males of advanced age. Progressive shortening of telomeres during ageing may induce aneuploidy in somatic cells. The role played by telomere length in the cellular ageing process may be more relevant in tissues with high cellular turnover.[43] Similarly, this role could be considered for spermatogonia or spermatocytes in human testis. It is possible that shortening of telomere sequences may not only disturb fixation of chromosomes on nuclear membrane at the zygotene stage, but also disturb initialisation of bivalent formation. Alterations in genetic recombination is also a major predisposing factor in both age dependent and age independent trisomies. Absent or reduced meiotic recombination is a common source of aneuploidy in humans and has been reported for paternally and maternally derived sex chromosome trisomies and for maternal trisomies 13, 16, 18, and 21.[44] Finally, alterations of mechanisms responsible for the ageing process are determined by myriad genetic factors that frequently interact with the environment.

In conclusion, our study shows a significant increase in chromosome non-disjunction during the two meiotic divisions in elderly males. Telomere exploration during spermatogenesis of males of advanced age could be a useful tool for the understanding of mechanisms involved in meiotic non-disjunction during ageing.

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