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The Relationship between Paternal Age, Sex Ratios, and Aneuploidy Frequencies in Human Sperm, as Assessed by Multicolor FISH

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Summary

We studied the frequencies of X- and Y-chromosome-bearing sperm, diploidy and disomy for chromosomes 1, 12, X, and Y in sperm from 10 normal men aged 21–52 years, to determine whether there was any relationship between donor age and any of these variables. Multicolor FISH was used to control for lack of probe hybridization and to distinguish diploid sperm from disomic sperm. A minimum of 10,000 sperm per donor was evaluated for each chromosome, for a total of 225,846 sperm studied. Sperm were considered disomic if two fluorescent signals were separated by a minimal distance of one signal domain. The mean frequencies of X- and Y-bearing sperm were 50.1% and 49.0%, respectively; not significantly different from 50%. There was no correlation between paternal age and "sex ratio" in sperm. Similarly, there was no association between the frequency of diploid sperm (mean, .16%; range, .06%–.42%) and donor age. For disomy frequencies, there was no relationship between donor age and disomy 12 (mean, .16%; range, .10%–.25%), XX (mean, .07%; range, .03%–.17%), and XY sperm (mean, .16%; range, .08%–.24%). There was a significant increase in the frequency of YY sperm (P = .04; mean, .18%; range, .10%–.43%) and disomy 1 sperm (P = .01; mean, .11%; range, .05%–.18%) with donor age. In summary, our results do not support a correlation between paternal age and sex ratio or diploidy. A relationship between paternal age and disomy was observed for disomy 1 and YY sperm but not for disomy 12, XX or XY sperm.

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Introduction

There has been an ongoing debate about the possibility of a paternal age effect (independent of maternal age) on the incidence of trisomy. Studies of human newborns and data from prenatal diagnoses have yielded both positive and negative results. Most studies have not demonstrated any association between paternal age and trisomy (Erickson 1978; Regal et al. 1980; Hook and Cross 1982; Roecker and Huether 1983; Cross and Hook 1987; Hatch et al. 1990); however, some studies have shown a positive association (Stene et al. 1977, 1981; Matsunaga et al. 1978; Robinson et al. 1993).

Since the great majority of chromosomally abnormal embryos are lost during development, studies of newborns and data from prenatal diagnoses or spontaneous abortions might not uncover an existing relationship between paternal age and aneuploidy. For this reason, we studied human sperm chromosome complements (after fusion with hamster oocytes) to provide a direct test of the effect of paternal age on the frequency of nondisjunction in human sperm (Martin and Rademaker 1987). We studied sperm karyotypes in 30 normal men, aged 22–55 years with a mean age of 34.7 years. The sample was stratified with five males sampled in each of six age categories (20–24, 25–29, 30–34, 35–39, 40–44, and 45+ years). There was no relationship between donor age and the frequency of numerical chromosomal abnormalities in sperm. However, there was a significant excess of hypohaploid complements compared to hyperhaploid complements, and since hypohaploid complements can arise by technical artefact, a more conservative approach is to analyze only hyperhaploid complements. There was a significant negative correlation between age and the frequency of hyperhaploid complements, with 3.7% hyperhaploidy in the youngest age group and 0 in the two oldest groups. There have been studies of aneuploid liveborns that have also detected a negative paternal age effect when the extra chromosome was of paternal origin (Carothers et al. 1978, 1984; Hook and Regal 1984).

In our study of sperm karyotypes, we were not able to assess the paternal age effect on hyperhaploidy for

specific chromosomes because the sample size was too small. The development of in situ hybridization for analysis of interphase human sperm has provided a new avenue for detection of aneuploidy in individual chromosomes (Guttenbach and Schmid 1990; Pieters et al. 1990; Wyrobek et al. 1990; Holmes and Martin 1993; Martin et al. 1993; Williams et al. 1993). Since in situ hybridization analysis is much simpler and faster than sperm karyotyping, large samples of sperm can be studied. We report here an analysis of the relationship between donor age and the frequency of hyperhaploidy for chromosomes 1, 12, X, and Y in >200,000 human sperm.

Material and Methods

Donors

Semen samples were obtained from 10 normal, healthy men with no history of chemotherapy, radiotherapy, or chronic illness. The men were 21–52 years of age; six were of proven fertility and the remaining four were of unknown fertility. All donors gave their informed consent prior to the study, which was reviewed by our institutional ethics committee.

Preparation of Sperm Nuclei

Aliquots of the semen samples were washed three times with 10 mM Tris/150 mM NaCl solution (pH 8) and, after the final wash, were resuspended to a concentration of $\sim 50 \times 10^6$ sperm/ml. Two microliters of the washed semen sample were smeared on clean glass slides and allowed to air dry. To render the sperm chromatin accessible to DNA probes, the slides were treated as described by Williams et al. (1993) with dithiotreitol (DTT; Sigma) and lithium diiodosalicylate (LIS; Sigma).

Preparation of DNA Probes

Four DNA probes were used for this study: a chromosome 1–specific satellite II sequence, pUC1.77, kindly provided by H. J. Cooke, Edinburgh (Cooke and Hindley 1979; Tagarro et al. 1994); a chromosome 12–specific alpha satellite sequence, D12Z1, a gift from A. Baldini, Yale University (Baldini et al. 1990); an X-specific alpha satellite probe, XC, generously provided by E. Jabs, John Hopkins University (Jabs et al. 1989); and a Y-specific alpha satellite sequence, DYZ3 (ATCC).

Chromosome-specific DNA probes were labeled according to the manufacturers' instructions: either directly with a fluorochrome-conjugated nucleotide (fluorescein-14-dUTP, rhodamine-4-dUTP, or coumarin-4-dUTP [Amersham]), or indirectly with a hapten-conjugated nucleotide (biotin-14-dATP [Gibco] or digoxigenin-11-dUTP [Boehringer Mannheim]) by nick-translation.

Two- and Three-Color FISH

To determine the frequency of disomy for autosomes, two-color FISH was performed, where probes for chromosomes 1 and 12 were hybridized simultaneously. Slides of pretreated sperm nuclei were denatured in 70% deionized formamide/2 × SSC (pH 7.5) for 2 min at 70°C, passed through a cold ethanol series, and allowed to air dry. A 10-μl sample of hybridization mix (55% formamide, 10% dextran sulfate, 1 × SSC, and 500 ng sheared salmon DNA, pH 7) containing 20 ng of one probe and 20 ng of another probe was denatured at 70°C for 5 min and quickly cooled by plunging into ice. This hybridization mixture containing chromosome-specific DNA probes was applied to the slides, and the slides were incubated at 37°C for 12–26 h.

Post-hybridization was carried out at 45°C, and the slides were washed three times for 2 min each in 50% formamide/2 × SSC (pH 7). For hapten-labeled probes, detection was achieved by incubating the slide for 20 min at 37°C with avidin-Cy3 (3.3 μg/ml; Jackson ImmunoResearch) or avidin-fluorescein isothiocyanate (FITC) (15 μg/ml; Vector Labs) for biotin-labeled probes and with antidigoxigenin-FITC or anti-digoxigenin-rhodamine (13.0 μg/ml; Boehringer Mannheim) for digoxigenin-labeled probes. The slides were briefly counterstained with a dilute solution of propidium iodide (5–50 ng/ml; Sigma) and 4′,6-diamidino-2-phenylindole (DAPI) (1–100 ng/ml; Sigma) and were then mounted with 8.5 μl of antifade solution (0.5 μg/ml p-phenylenediamine, Sigma).

Three-color FISH was performed to determine the frequency of disomy for the sex chromosomes using three direct-labeled probes: Fluorogreen-X (XC), Fluorored-Y (DYZ3), and Fluoroblue-1 (pUC1.77) (Amersham). The protocol for hybridization was the same as for two-color FISH, with the exception of an extra 20 ng of the third probe in the hybridization mixture.

Data Collection and Analysis

The slides were examined with a Zeiss Axiophot microscope fitted with a FITC/rhodamine double bandpass filter set and a DAPI single bandpass filter set. For two-color FISH studies, the dual bandpass filter set was mainly used to count, and, when necessary, the single bandpass filter set was used to clarify the position of the sperm nuclei. For three-color FISH, the dual bandpass filter set was used to count the presence/absence of X and Y signals. Sperm with two or no X/Y signals were checked for presence of a single autosome signal by using the DAPI filter set before being counted as disomic or nullisomic, respectively.

Slides were used for counting if the efficiency of hybridization was ≥98%. For each of the donors, one to three hybridizations were used to count a minimum of 10,000 sperm nuclei for every pair of chromosomes

Table 1

Donor Ages and Frequencies for X- and Y-Bearing Sperm and Diploidy

Donor	Age (Years)	Total	X (%)	Y (%)	Diploidy (%)
1	21	20,029	49.80	49.58	.24
2	27	20,658	50.92	48.53	.08
3	30	30,862	49.76	49.74	.23
4	33	20,670	49.50	49.39	.13
5	34	20,489	50.33	48.47	.17
6	37	20,718	49.57	49.61	.42
7	39	30,759	49.70	48.65	.06
8	44	19,996	50.57	48.70	.08
9	46	21,672	49.99	48.22	.09
10	52	19,993	50.90	49.32	.07
Mean	36	22,585	50.10	49.02	.16

studied. A sperm was considered to be disomic when two fluorescent domains of the same color were clearly positioned within the sperm head, comparable in brightness and size, and at least one domain apart. Certain populations of sperm nuclei were eliminated from scoring: overlapping nuclei where it was impossible to assign a signal to a given nucleus, disrupted nuclei with indistinct margins, very large nuclei with diffused chromatin possibly due to over-decondensation, or nuclei with no signals because of a failure to hybridize.

Results

The aneuploidy frequencies for chromosomes 1, 12, X, and Y were assessed for the 10 normal men in a total of 225,846 sperm. The frequencies of X- and Y-chromosome-bearing sperm and diploid sperm, as well as the ages of the donors, is presented in table 1. Individual nullisomy frequencies are presented in table 2, and disomy frequencies are in table 3.

A Pearson correlation coefficient was employed in order to study the relationship between the donor's age and the various abnormality frequencies and to determine whether the frequency of nullisomy was significantly increased compared to disomy. The *P*-value was calculated using a *t*-test for 0 correlation (with 9 df).

There was a significant excess of nullisomic sperm compared to disomic sperm for all chromosomes studied ($P < .03$). There was no correlation of donor age with the frequencies of X- or Y-bearing sperm, nullisomy for any chromosome, disomy for chromosomes 12, X, XY, or diploidy. However, disomy frequencies for chromosomes 1 ($P = .01$) and Y ($P = .04$) were significantly increased with donor age.

Discussion

There has been some suggestion that the secondary sex ratio decreases with paternal age. That is, in older men, the ratio of male to female births is decreased. For example, Erickson (1976) studied a population of 5 million and found a significant negative association of sex ratio with birth order and paternal age. Similarly, James and Rostron (1985) determined that advanced paternal age and increased parity resulted in a decline in the sex ratio. Ruder (1985) reported that this effect of paternal age on sex ratio was independent of birth order and maternal age. However, Curtsinger et al. (1983) found no parental-age effect on the sex ratio.

If paternal age does affect the secondary sex ratio, this effect might be caused by a change in the frequency of X- and Y-bearing sperm in older men. To investigate this possibility, we previously analyzed the frequency of

Table 2

Individual Donor Frequencies for Nullisomy in Sperm

	Nullisomy (%)		
Donor	1	12	X and Y
1	.16	.17	.22
2	.23	.69	.33
3	.12	.42	.25
4	.27	.29	.59
5	.55	.70	.78
6	.18	.43	.37
7	.28	1.11	1.33
8	.12	.35	.28
9	.57	.52	1.07
10	.06	.13	.36
Mean	.25	.48	.56

Table 3

Individual Donor Frequencies for Disomy in Sperm

	Disomy (%)				
Donor	1	12	X[a]	Y[a]	XY[a]
1	.09	.16	.08	.13	.19
2	.05	.17	.05	.10	.08
3	.06	.15	.04	.11	.11
4	.09	.15	.17	.14	.22
5	.08	.12	.10	.21	.11
6	.16	.25	.07	.14	.24
7	.06	.15	.03	.13	.16
8	.15	.20	.05	.23	.17
9	.15	.10	.10	.43	.18
10	.18	.13	.04	.22	.16
Mean	.11	.16	.07	.18	.16

[a] Disomy frequencies for the sex chromosomes (and autosomes) are based on all sperm.

X- and Y-chromosome-bearing sperm in 9,225 sperm karyotypes from 143 men, ranging in age from 21 to 55 years (Martin and Rademaker 1992). The percentage of X-and Y-chromosome complements was not significantly related to donor age. Similarly, in our present FISH study of >200,000 sperm from 10 men aged 21–52 years, there was no correlation between donor age and the proportion of X- and Y-bearing sperm. Therefore, it seems likely that, if there is a paternal age effect on sex ratio, it is mediated by some factor associated with paternal age such as coital frequency (Zarutskie et al. 1989) rather than a direct effect on the frequency of X- and Y-bearing human sperm.

There was no relationship between diploid sperm and the age of the donor. Also there was no paternal age effect on the frequency of nullisomic sperm. Since there was a significant excess of nullisomic sperm compared to disomic sperm, this might indicate a lack of probe hybridization or visualization for one of the two or three probes scored. However, our hybridization efficiency was >98% for all experiments. In our studies of human sperm karyotypes, we also found a significantly higher frequency of nullisomic sperm, which could be attributed to technical loss during slide preparation. Alternatively, it is possible that nullisomic sperm are produced by mechanisms other than nondisjunction, such as anaphase lag. It is also possible that our scoring criteria underestimated the disomy frequency, leading to the higher nullisomy frequency.

For disomy 12, XX, and XY sperm, we did not find a paternal age effect. However, there was a significant increase in the frequency of disomy 1 and YY sperm with donor age. A few other in situ hybridization studies have explored the relationship between disomy frequencies and donor age. Guttenbach and Schmid (1990, 1991) and Guttenbach et al. (1994*a*, 1994*b*) found no age effect for disomy frequencies for chromosomes 1, 3, 7, 10, 11, 17, 18, and Y in seven to eight donors aged 23–57 years. Similarly, Miharu et al. (1994) found no relationship between donor age and disomy frequencies for chromosomes 1, 16, 18, X, and Y in eight men aged 27–59 years. However, both of these studies employed small sample sizes of 1,000–2,000 sperm/DNA probe/donor. With an approximate disomy frequency of 1/1,000 sperm, it is clear this sample size could lack the power to detect a true paternal age effect on aneuploidy frequency. Two other FISH studies, reported as abstracts, have shown evidence of a paternal age effect. Griffin et al. (1994) found a significant increase in disomy for chromosome 18 and the sex chromosomes in 10 men aged 22–45 years, with 20,000 sperm analyzed per male. Wyrobek et al. (1994) reported a paternal age effect for XX and YY sperm with the strongest effect for disomy Y in 14 men of two age groups (mean 46.8 years vs. 28.5 years).

In our data, the strongest evidence of a paternal age effect was for disomy of chromosome 1. Trisomy for chromosome 1 has never been reported in spontaneous abortions or newborns, but it has been observed in an 8-cell human embryo derived from in vitro fertilization (Watt et al. 1987). Thus, it is likely that trisomy 1 embryos are selectively eliminated early in embryogenesis, and any paternal age effect for this chromosome would not have clinical significance.

We also found evidence for a paternal age effect for YY sperm, as did Wyrobek et al. (1994). This is interesting, since 47,XYY is the only aneuploidy with 100% paternal origin. Studies on the relationship between paternal age and 47,XYY offspring are rare. Carothers et al. (1978) found a small but significant inverse relationship between paternal age and the incidence of 47,XYY. Thus, the evidence at the present time is conflicting as to whether there is any association between paternal age and meiosis II nondisjunction of the Y chromosome.

In summary, our results do not support a correlation between paternal age and sex ratio, diploidy, or disomy for chromosomes 12, XX, and XY in human sperm. There is suggestive evidence of a paternal age effect for YY sperm and clear evidence for disomy 1 sperm. Future studies on a larger sample of men will allow us to determine whether there is a paternal age effect on the frequency of aneuploidy for specific chromosomes.

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