Do Alterations in the Sex Ratio Occur at Fertilization? A Case Report Using Fluorescent In Situ Hybridization

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Purpose: The mechanisms by which the sex ratio might be altered at fertilization were reviewed, following a case of preimplantation gender analysis revealing a significantly skewed proportion of male-to-female embryos.

Methods: The case of a known carrier of X-linked hydrocephalus with a history of three affected male pregnancies is presented. Her husband's family history consisted of a strong increase in the number of males relative to females. She had four cycles of stimulated in vitro fertilization, with sex chromosome analysis using fluorescent in situ hybridization (FISH) on suitable cleavage-stage embryos. The difference in the sex ratio of normal male-to-female embryos was compared using a significance probabilities test for sex ratio. The sex ratio of sperm from a semen sample from the male partner was determined by FISH.

Results: Fifty embryos were suitable for analysis. A significantly higher number of normal male ($n = 20$) than normal female ($n = 8$) embryos was obtained ($P < 0.05$). The FISH assessment of the husband's semen analysis revealed no alteration in the normal X:Y ratio.

Conclusions: As the sperm analysis revealed a normal X:Y ratio, an alteration in the embryo sex ratio might be explained by the preferential binding of Y-bearing sperm to the oocyte, an oocyte-related "discouragement" of binding of X-bearing sperm, or a postfertilization event.

KEY WORDS: fluorescent in situ hybridization; in vitro fertilization; preimplantation genetic diagnosis; sex ratio.

INTRODUCTION

Preimplantation genetic diagnosis (PGD), used to sex embryos prior to their transfer to the uterine cavity, is an established procedure for couples who carry X-

linked genetic disorders. Following the removal of one or two blastomeres from a cleavage-stage embryo, the nuclei can be examined by either fluorescent in situ hybridization (FISH) or polymerase chain reaction (PCR) DNA analysis, to determine the chromosomal sex of each embryo that has been biopsied. Recent multicenter data have revealed that several hundred pregnancies worldwide have been reported using this technique (1).

However, there are problems with PGD, in particular, the high embryo utilization per successful implantation. Ovarian stimulation and in vitro fertilization of multiple oocytes are required. Even in optimal circumstances, only 50% of the resulting embryos are likely to be of the desired sex, and in at least 20% of all cases in which good-quality embryos are obtained, a chromosomal anomaly will be present (2), precluding transfer of those embryos. As a result, the majority of patients having PGD have only enough embryos to allow for fresh transfer, with no additional embryos for cryopreservation.

Additionally, other unforeseen circumstances arise during management involving PGD. We present a case of a couple having PGD in whom a significant, repeated bias in the sex ratio of the resulting embryos was noted, resulting in an excess of the (potentially affected) sex, substantially limiting their options for embryo transfer. As this was in the light of a family gender history of a similar bias following spontaneous conception, we considered possible causes of a bias in the sex ratio.

CASE HISTORY

The case of a 31-year-old woman, a known carrier of X-linked hydrocephalus, is presented. This anomaly has been attributed to a mutation in the L1CAM gene, located on the long arm of the X chromosome (3). The patient's reproductive history consisted of three

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pregnancies, all affected males. Her first pregnancy ended in an unexpected stillbirth at term, affected by hydrocephalus. In her second pregnancy, hydrocephalus was diagnosed at a second-trimester ultrasound. This pregnancy, an affected male, was terminated at 20 weeks of gestation. Linkage analysis of the affected fetus and other family members was performed, leading to a diagnosis of X-linked hydrocephalus. During the third pregnancy, amniocentesis revealed a male fetus, with a high probability on linkage analysis of being affected; termination of pregnancy was performed at 15 weeks.

The patient's husband's family history consisted of a strong increase in the number of males relative to females. In addition to the three (affected) males he has produced, he has one sibling, a brother, and his father is one of four male siblings.

METHODS

The couple was referred to Sydney IVF for preimplantation sexing of embryos, followed by selective transfer of female embryos. She had a total of four stimulated cycles. A standard midluteal long down-regulation protocol using GnRH analogue was used in each cycle, followed by gonadotropin stimulation. Oocyte collection and in vitro fertilization were performed using routine Sydney IVF protocols.

Normally cleaving embryos were biopsied on day 3 after oocyte collection, with two blastomeres removed from each suitable embryo and fixed, followed by fluorescent in situ hybridization (FISH) performed on each fixed blastomere, as described by others (4). Centromeric probes for chromosomes X, Y, and 18 (Immunodiagnosics; Vysis, Naperville, IL) were used for analysis. Following analysis of hybridized blastomeres, normal female embryos were transferred to the uterine cavity late on day 3.

RESULTS

The patient underwent four stimulated cycles between November 1995 and April 1997. Over the four cycles, 76 oocytes were collected and 56 fertilized (74%). At assessment on day 3, 50 embryos had developed normally and were biopsied. The results for hybridization of all embryos in all cycles are given in Table I. A normal signal response (i.e., normal male or normal female along with two signals for chromosome 18) was noted in blastomeres obtained from 28 embryos.

Table I. Overall Results for Embryo Biopsy in Four Stimulated Cycles

Result	No. embryos	%
Normal signal	28	56
Abnormal signal	7	14
No signal	5	10
Lysed blastomeres or fixation failure	10	20
Total	50	100

The proportion of normal male and normal female embryos, relative to overall results, is shown in Table II. In each cycle there was a relative excess of male-to-female embryos. When the total ratio was compared over the four cycles, a significant increase in male embryos (20 in total) to female embryos (8 in total) was noted ($P < 0.05$), using a significance probabilities test for sex ratio (based on chi-square goodness of fit) (5).

Given that a number of chromosomally normal male embryos developed, high-quality male embryos were cryopreserved, as linkage analysis PCR might be possible on these embryos in the future, to determine the probability of carriage of the gene mutation.

As the difference in the embryo sex ratio became clear, an analysis of the husband's sperm was performed using FISH for chromosomes X, Y, and 18. Forty-seven percent of sperm were X bearing and 38% Y bearing, with an uncertain or abnormal reading in a further 15% of sperm (total sperm counted, $n = 2980$).

None of the 46XX embryos transferred resulted in implantation.

DISCUSSION

This case report raises the possibility that some couples might skew the sex ratio of their offspring as a result of a mechanism operating prior to embryo implantation. Differences in male and female embryo

Table II. FISH Results for Successfully Biopsied Embryos

Cycle No.	Normal male (No.)	Normal female (No.)	Abnormal (No.)	No signal (No.)	Total (No.)
1	6	3	1	1	11
2	2	0	1	1	4
3	8	2	2	2	14
4	4	3	3	1	11
Total	20*	8*	7	5	40

* $P < 0.05$.

development in vitro have been reported in a number of species. In general, male embryos have been noted to cleave at a faster rate (6) and demonstrate higher levels of glucose uptake (7). Interestingly, a higher implantation rate of male embryos has been postulated, and yet the sex ratio at birth is unaltered from normal, suggesting some form of intrauterine correction following implantation (8).

However, if a paternal influence upon the sex ratio is possible, then this must occur either at or prior to fertilization. A higher proportion of one sperm population (either chromosome X bearing or chromosome Y bearing) would explain such a difference, although this was not the case here. Normal sperm ratios using FISH analysis have previously been reported in couples with multiple children of one sex (9).

Given that the FISH analysis of the husband's sperm revealed a normal sex ratio, a second possibility by which a factor on the male side could influence the embryo sex ratio would be preferential binding of Y-bearing sperm during the process of fertilization. This possibility has been alluded to in the literature (10) but has not been assessed definitively. Finally, the entry of a Y-bearing sperm into the oocyte might influence a post fertilization event resulting in preferential development of male embryos, in couples with a similar skewed sex ratio in their offspring.

A maternal influence upon preimplantation sex ratio is possible through an oocyte-related "discouragement" of binding of either Y-bearing or X-bearing sperm, but such a mechanism has not been observed or reported in the literature.

The relatively low number of normal female embryos available for transfer has limited this couple's options and would appear to contribute to their lack of success in attaining a pregnancy to date. Two other management strategies are possible in future attempts that might be of value. The first is to consider fluorescent-activated cell sorting to separate X-bearing from Y-bearing sperm, thus increasing the proportion of X-bearing sperm used in a subsequent assisted conception cycle (11). However, sperm density and motility are significantly reduced following this procedure and intracytoplasmic sperm injection would usually be required to achieve fertilization. This additional procedure might compromise success rates, as two manipulations of the oocyte/embryo would then become

necessary, one to achieve fertilization and the second to remove blastomeres.

The second strategy would be continue cryostorage of male embryos and await the reliable development of PCR-based linkage analysis for this particular disorder. However, this would require a second embryo biopsy. Alternatively, once linkage analysis is available, blastomeres obtained in future cycles could be tested for the gene mutation alone or in combination with testing for the Y chromosome, using PCR analysis.

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