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Skewed X Inactivation and IVF-Conceived Infants

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Abstract

Our objective was to investigate whether skewed X chromosome inactivation (XCI) is associated with in vitro fertilization (IVF). We performed a retrospective cohort study of 30 female infants conceived by IVF and 44 naturally conceived control infants matched for gestational age and sex. Cord blood DNA samples were obtained and XCI patterns were analyzed using a methylation-sensitive assay. Eight IVF samples and 13 control samples were excluded from the study because they were either homozygous or alleles were too similar for the assay to determine skewing. Mildly skewed XCI (80–90% inactivation of one allele) was present in 2 of 22 (9.1%) IVF samples and 2 of 31 (6.5%) control samples. Extremely skewed XCI (>90% inactivation of one allele) was found in 2 of 22 (9.1%) IVF samples and 0 of 31 control samples. Neither difference was statistically significant. However, the mean degree of skewed XCI in the IVF group was 72.0% and in the control group was 62.4% (p=0.002). Larger studies are needed to clarify the relationship between IVF and skewed XCI.

Summary—Some studies suggest that the process of IVF and embryo culture may affect genetic imprinting of genes in children born from this procedure. One type of imprinting is X chromosome inactivation which results in silencing of most genes on one of the two X chromosomes in females. Usually, this silencing occurs randomly so that about 50 % of cells have an active paternal X chromosome and 50% have an active maternal X chromosome. In some circumstances, there is skewing of the silencing pattern where an individual has predominantly one X chromosome (either maternal or paternal) silenced and this can lead to disease. We studied whether skewing of X chromosome inactivation occurs in female infants after IVF. There was no statistical difference in the percentage of female infants with skewed X inactivation after IVF compared to naturally-conceived controls. However, there was a trend toward a higher mean percentage of skewing of X chromosome inactivation among IVF conceived infants. Larger studies will be required to evaluate this further.

Keywords

IVF; embryos; X chromosome inactivation; ICSI; infertility

INTRODUCTION

There has been much debate in the literature as to the short-term and long-term safety of assisted reproductive techniques (ART). While the association between birth defects and in vitro fertilization (IVF) is controversial, recent evidence suggests a statistically significant

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increase in major birth defects among children conceived by IVF (Hansen et al., 2002, Merlob et al., 2005, Olson et al., 2005). Furthermore, the process of IVF may result in epigenetic changes, as evidenced by multiple case reports of children conceived by IVF who have imprinting defects causing genetic disorders such as Beckwith-Wiedemann syndrome and Angelman syndrome (Cox et al., 2002, Orstavik et al., 2003, DeBaun et al., 2003, Maher et al., 2003, Gicquel et al., 2003). A case-control study confirmed children conceived by IVF are more likely to have Beckwith-Wiedemann syndrome than children who are naturally conceived, and hypomethylation of a maternal allele is the cause of a majority of those cases. (Halliday et al., 2004).

X chromosome inactivation, the random silencing of genes on one X chromosome in somatic cells of female mammals, is another epigenetic or imprinting phenomenon. A growing body of literature has linked skewed XCI to a variety of conditions such as premature ovarian failure, recurrent miscarriage, X-linked mental retardation, Rett syndrome, scleroderma and autism (Sato et al., 2004, Plenge et al., 2002, Özbalkan et al., 2005, Krepischi et al., 1998, Talebizadeh et al., 2005). Since XCI is a type of imprinting that occurs in very early embryonic development, it is conceivable that ART could affect XCI. Our null hypothesis is that there is no increase in XCI in female infants conceived by IVF.

MATERIALS AND METHODS

Subjects and Samples

Informed consent was obtained from all parents of subjects before enrollment in the study. The institutional review board at the University of Iowa Hospitals and Clinics (UIHC) approved the study protocol. This study was part of a larger study of disease variability in the newborn which has a DNA repository of over one thousand samples from both term and premature infants born at UIHC since 2001. Permission to obtain DNA samples from infants is obtained from parents either in the prenatal period or shortly after delivery. Information about conception (natural versus medically-assisted by IVF) are obtained by questionnaire and included in the database for each infant.

The IVF database includes the hospital where the infants conceived by IVF were born. This database was merged with the DNA repository database to identify appropriate IVF-conceived infants whose DNA was available to study. Therefore, this group of IVF-conceived infants represents the small fraction (<2%) of the total infants conceived in our program who delivered at the University of Iowa and were enrolled in the larger disease variability study. Control samples were selected from the repository after matching for gestational age and female gender.

DNA was extracted from umbilical cord blood in all the subjects except for 1 infant in the IVF group in whom a cheek swab was used and 2 infants in the control group in whom whole blood was obtained.

X Chromosome Inactivation Analysis

X inactivation studies were performed on 30 DNA samples from female infants conceived by IVF and 44 DNA samples from naturally conceived female infants matched for gestational age.

The method for determining XCI has been previously described (Kimani et al., 2007). The method relies on the highly polymorphic short tandem repeat (STR) in exon 1 of the human androgen-receptor locus on the X chromosome as well as HpaII enzyme cleavage sites near these STRs. HpaII digests only unmethylated (active) DNA. By including the HpaII sites within the PCR amplification unit, only the methylated (inactive) X chromosome will be

amplified if the template DNA is digested with HpaII prior to amplification. Assuming that the maternal and paternal allele have different numbers of STRs, the more commonly inactivated (methylated) allele will be preferentially amplified and the degree of skewing of XCI can be determined by comparing the strength of the signal for each allele. Since methylation of these sites correlates with X inactivation, a product was obtained only from inactive X chromosomes, though without parental samples, it is impossible to tell which allele was maternal or paternal. The total peak areas for both alleles in the digested and undigested samples were used to determine the percentage of each X allele that was inactive. A correction factor was applied to compensate for unequal amplification of alleles. Completely random XCI would be expected to result in 50% inactivation of each X chromosome. By convention, mildly skewed XCI was defined as 80–90% skewed, and extremely skewed XCI was defined as >90% skewed (Kimani et al., 2007, Beever et al., 2003)). DNA from the same adult female and adult male was used as a control in each run of the assay to assess inter-assay variability. All samples were analyzed in duplicate, utilizing the mean value. The samples were genotyped using an ABI PRISM® 3100 or 3730 Genetic Analyzer and GeneMapper® Software version 3.0 or Peak Scanner® Software version 1.0 (Applied Biosystems, Foster City, CA).

Statistical Analysis

Fisher's exact test and Student's t-test were used to compare XCI and percent skewing between the IVF and control groups. P values of <0.05 were considered statistically significant.

RESULTS

In total, XCI studies were performed on 30 DNA IVF samples and 44 control samples. Eight IVF samples were excluded from this study: 3 were homozygous at the (CAG) polymorphism in the androgen-receptor locus, making the results uninformative. Five samples had such similar (CAG) polymorphisms at the androgen-receptor locus that the alleles could not be completely separated. Of the 44 control samples, 8 were homozygous at the (CAG) polymorphism in the androgen-receptor locus, and 5 had overlapping alleles. When assayed in duplicate, the measurement of skewing was highly repeatable with a coefficient of variation of less than 5%.

The frequency of skewing was similar in IVF and control groups. Two of the 22 (9.1%) IVF samples and 2 of 31 (6.5%) control samples displayed mildly (80–90%) skewed XCI (p=1.0). Two of 22 (9.1%) IVF samples and none of 31 control samples displayed extreme (>90%) XCI (p= 0.19). Thus, the total numbers of infants with any degree of skewing was 4 of 22 (18.2 %) IVF-conceived infants and 2 of 31 (6.5%) naturally-conceived infants (RR= 2.82; 95% CI= 0.56–14.06, p= 0.22). All four infants conceived by IVF who demonstrated XCI skewing resulted from eggs fertilized by intracytoplasmic sperm injection (ICSI) and transfer of fresh cleavage-stage embryos on day 3.

The mean degree of skewed XCI in the IVF group was 72.0% and in the control group was 62.4% (p=0.002). Among the infants born after IVF, there was a trend towards a greater degree of XCI skewing among infants conceived by ICSI (n = 13) as compared to nine infants conceived by insemination in culture (75.7% vs 65.4%; p=0.06). The mean degree of XCI skewing of infants born after day 3 embryo transfer (n=11) was not different than the degree of XCI skewing found in eleven infants conceived after day 5 (blastocyst stage)embryo transfer (75.5% vs 68.0%; p=0.16). Finally, among IVF-conceived infants, singletons had a slightly higher degree of XCI skewing than infants born from multiple gestations (79.2% vs 68.6%, p=0.04). When looking at all infants (IVF-conceived and

controls), there was no difference in XCI skewing between singletons and multiple gestations.

DISCUSSION

Based on our data, we can state that skewed XCI is not a common occurrence after IVF. Although we found no statistically significant increase in the number of infants with skewed XCI after IVF, it remains possible that this small study had insufficient power to detect a true difference. One finding of interest is the slightly higher average degree of XCI skewing found in infants conceived by IVF and the non-significant trend towards greater XCI skewing when ICSI was utilized. Larger studies will be required to investigate this observation more fully.

One prior study evaluated XCI in 44 females conceived by ICSI compared to control samples obtained from girls age 0–19 (Robinson et al., 2005). This study found no difference in the mean degree of skewing (65.1 vs 69.8%) or in the frequency of extreme skewing (4.6% vs 10.8%) when comparing these groups. Differences between the studies include the populations studied and the age of the control groups. Our study adds to these initial results by including infants conceived by IVF but not ICSI.

Imprinting is an epigenetic phenomenon which affects the expression of genes, but not the DNA sequence itself. While most genes in the genome are expressed from both parents, there is a subset of genes that is expressed from either the maternal allele or the paternal allele. Imprinting is significant because it affects lineage-specific and age-specific gene expression patterns. During meiosis, the germ cells need to erase pre-existing methylation to ensure that the resulting gametes will have the proper female or male methylation patterns. Imprinting in the male appears to be complete by the time male germ cells become haploid (Thompson et al., 2005). In females, this is believed to occur during the last phase of oocyte growth and meiotic maturation after ovulation. In addition, following fertilization, epigenetic reprogramming occurs in the preimplantation period of embryo development and imprinting can be altered by in vitro culture conditions for the early embryo. Reports of rare, but serious, imprinting defects occurring more frequently in children conceived by IVF suggest that something about the IVF process, including ovulation induction or embryo culture, could disturb normal imprinting patterns. (Reik et al., 2001).

XCI is a type of imprinting that is necessary for normal function. In female cells, most genes on one X chromosome must be silenced or female cells would have twice the amount of X chromosome-encoded protein as male cells containing just one X chromosome. Studies in the mouse suggest that the paternal X chromosome is initially imprinted to undergo inactivation, and this remains true in placental tissues where it is always the paternal X that is inactivated. However, cells destined to form the inner cell mass and embryo reverse the initial paternal X chromosome inactivation and subsequently the maternal or paternal X chromosome is inactivated randomly. This final inactivation step likely does not occur until around or just after embryo implantation. However, some recent evidence suggests that the first steps of random inactivation begin very early in development, perhaps as early as the 2–4 cell embryonic stage in mice (Hajkova et al., 2004). Any event that reduces the number of embryonic cells present at the time of XCI increases the chance of skewed XCI (Lau et al., 1997), suggesting that in vitro culture conditions might affect XCI by altering embryonic cell numbers. Once XCI is decided, all daughter cells will have the same X chromosome inactivated as the parent cell. Although the exact stage at which human embryonic XCI occurs is unknown, it is a reasonable hypothesis that ART with embryo culture could affect this process.

In some individuals, for unclear reasons, one X chromosome is preferentially silenced leading to skewing of gene expression to either the paternal or maternal X chromosome, depending on the direction of the skewing. A study of cord blood from 590 newborns determined that 4.9% of newborns had >80% skew (Amos-Landgraf et al., 2006). Skewed XCI can have adverse consequences in some individuals. For example, a recent study described a female patient with X-linked alpha-thalassemia/mental retardation (ATRX). The *ATRX* gene is on the X-chromosome, and previously this syndrome had only been described in males. Further investigation of this patient revealed a de novo *ATRX* mutation located on the maternal allele. This mutation combined with complete inactivation of the paternal X chromosome (100% skewing) led to the phenotypic expression of this genetic disease in a female. Since this child was conceived by IVF, the authors proposed a link between ART and the unexpected methylation pattern (Badens et al., 2006).

We found 18.2% of our IVF population and 6.5% of our naturally-conceived population had >80% skewing of XCI, differences that, again, were not statistically significant in this small study. While these results are reassuring, the mean degree of skewing was significantly higher in the IVF group. Further investigation of XCI in IVF conceived females is warranted.

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