ORIGINAL ARTICLE: ASSISTED REPRODUCTION

X-chromosome inactivation in female newborns conceived by assisted reproductive technologies

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Objective: To investigate X-chromosome inactivation (XCI) skewing in female newborns conceived by intracytoplasmic sperm injection (ICSI), in vitro fertilization (IVF), and naturally.
Design: Case-control study.
Setting: Research institution.
Patient(s): A total of 185 female newborns, including 60 conceived by intracytoplasmic sperm injection (ICSI), 73 by in vitro fertilization (IVF), and 52 naturally conceived (NC).
Intervention(s): DNA was extracted from umbilical cord blood after birth.
Main Outcome Measure(s): XCI skewing values determined by assaying allelic ratio of methylated alleles at the *androgen receptor* (*AR*), *fragile X mental retardation 1* (*FMR1*), and *DXS6673E* loci.
Result(s): In the comparison of the ICSI, IVF, and NC populations, the frequency of skewing $\geq$75% (7.0% vs. 5.7% vs. 2.0%, respectively) or $\geq$90% (0 vs. 1.4% vs. 2.0%, respectively) was not statistically significantly different. The mean level of skewing between the ICSI, IVF, and NC groups also did not differ (63.7% vs. 61.8% vs. 60.7%, respectively). Skewing variability was observed in the placentas of the two extremely skewed cases. The parental origin of the preferentially inactivated X chromosome in the extremely skewed IVF and NC cases were maternal and paternal, respectively.
Conclusion(s): The assisted reproductive technologies of ICSI and IVF do not appear to affect XCI skewing. Skewing variability within the placentas analyzed supports the theory that weaker selective pressures occur in the placenta that could result in skewed inactivation. Our study is the largest to date to investigate this epigenetic phenomenon in infants conceived by ICSI and IVF alongside age-matched NC controls. (Fertil Steril® 2014;■:■–■. ©2014 by American Society for Reproductive Medicine.)
Key Words: Assisted reproductive technology, epigenetics, ICSI, IVF, X-chromosome inactivation

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Abnormalities in imprinting, a type of epigenetic modification, have been associated with assisted reproductive technology (ART). Higher rates of the imprinting disorders Beckwith-Wiedemann syndrome and Angelman syndrome are observed in ART-conceived infants compared with spontaneously conceived infants (1–6). It is proposed that another epigenetic mechanism known as X-chromosome inactivation (XCI) may be altered during embryo culture in ART. X-chromosome inactivation is a mechanism that occurs early in embryo development to ensure the random silencing of one X chromosome in the somatic cells of females. Skewed XCI can lead to the uncovering of X-linked recessive traits in heterozygous women and has been associated with recurrent spontaneous abortions, cancer, and chromosome abnormalities (7–9). Skewed XCI can result from selection against a chromosome abnormality, mutations of genes involved in X inactivation, a reduced number of precursor cells being present at the time of inactivation, or from the selection of a small number of normal cells to contribute to the inner cell mass (ICM) of the blastocyst (8). In vitro fertilization (IVF) and intracytoplasmic injection (ICSI) populations are subject to the risk factors of advanced maternal age, sperm aneuploidy, superovulation, in vitro handling of sperm and egg, and embryo growth in culture. These risks could cause epigenetic alterations, slow embryo growth, and/or cause cell selection resulting in skewed XCI.

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To assess the risk of skewed XCI in females conceived by ART, we determined the frequency of mild skewing (≥75%) and extreme skewing (≥90%) as well as the mean level of skewing in populations of newborn females conceived by ICSI, IVF, and by natural conception (NC). Extremely skewed cases were further investigated to determine the parental origin of the skewed allele and the skewing patterns in the placenta. Two previous studies have investigated XCI skewing in ART-conceived infants (10, 11), but those studies looked at a limited number of samples of either IVF or ICSI samples versus controls. In addition, females aged 0–19 were used as NC controls in one of the studies (10). To our knowledge, our study is the largest to date to investigate XCI skewing in IVF and ICSI newborns alongside age-matched NC controls.

MATERIALS AND METHODS

Participants

Patients who conceived through IVF and ICSI were recruited from several IVF centers across Canada. Patients who had conceived naturally were recruited from hospitals across the Lower Mainland in British Columbia, Canada. Informed consent was obtained from each patient before sample collection. Ethics approval for this study was obtained from the University of British Columbia Research Ethics Board.

Genomic DNA was extracted from the umbilical cord blood of newborn females (n = 185). A karyotype or comparative genomic hybridization (CGH) analysis of the chromosomes was performed for all newborn cases. Cases were excluded if congenital and/or chromosome abnormalities were present. The infants conceived by ICSI (n = 60) included six pairs of female twins and two female infants from a set of mixed-gender twins. The infants conceived by IVF (n = 73) included six pairs of female twins, one female from a set of female twins where the other female twin was not assessed, seven female infants from a set of mixed-gender twins, and one female from a set of triplets. Infants conceived naturally (n = 52) included two pairs of female twins. Twins were not purposely selected for to be included in this study. Infants were included in this study regardless of whether they were a part of a single or a multiple pregnancy.

X-chromosome Inactivation Assay

A DNA methylation-sensitive assay was used to determine the degree of XCI skewing as previously described elsewhere, but with modifications (7). Genomic DNA (100 ng) was digested with 1 U of *Rsa*I and either 2.5 U of *Hpa*II (for the *androgen receptor* [*AR*] and *fragile X mental retardation 1* [*FMR1*] assays) or 2.5 U of *Hha*I (for the *DXS6673E* assay) in a total volume of 20 μL. For each sample, an undigested control was similarly prepared with only 1 U of *Rsa*I. Both *Hpa*II and *Hha*I are methylation-sensitive enzymes and will only cut the unmethylated active X, leaving only the methylated inactive X available for polymerase chain reaction (PCR) amplification of the repeat region. *Rsa*I is not methylation sensitive and does not have restriction sites in *AR, FMR1*, or *DXS6673E*. However, *Rsa*I is thought to enable more efficient and complete digestion by making restriction sites more accessible for methylation-sensitive enzymes (12).

The samples were incubated at 37°C overnight, and complete digestion was confirmed by PCR amplification of the 5′ region of the *MIC2* gene. *MIC2* escapes XCI and is therefore unmethylated at the 5′ end of both X chromosomes and should be completely digested by *Hpa*II and *HhaI* (13). Two microliters of digested and undigested samples were amplified with 1X Invitrogen PCR buffer, 400 nM of each primer, 2 mM $MgCl_2$, 333 μM of each dNTP, 4% dimethyl sulfoxide (DMSO), and 0.4 U of *Taq* (Invitrogen) in a total volume of 15 μL. The PCR conditions were 95°C for 3 minutes (initial denaturation); 95°C for 1 minute, 52°C for 1 minute, and 72°C for 2 minutes for 35 cycles with a final extension at 72°C for 7 minutes. Primer sequences were as follows: forward 5′-AGAGGTGCGTCCGATTCTT-3′ and reverse 5′-CGCCGCA GATGGACAATTT-3′. We added 5 mL of 5X loading buffer to the PCR products, and all the samples were run on a 1% ethidium bromide or SYBR Safe (Invitrogen) stained agarose gel for 45 minutes at 130 V. Fragments were visualized on a UV transilluminator (BioDoc-It Imaging System; UVP). The presence of a 400-bp band in the undigested sample but not in the digested sample indicated complete digestion. After complete digestion was confirmed, the DNA was PCR amplified using primers that flanked the restriction sites and a region of repeats (7).

The *AR* assay, which relies on differential methylation of a CpG site near a polymorphic trinucleotide (CAG) repeat in the first exon of the X-linked AR gene, was first used (14). If the *AR* locus was uninformative, skewing was determined at the *FMR1* (15) or *DXS6673E* (16) loci. The assay was repeated, and the value of the two tests was averaged if a sample was found to have ≥75% skewing. We performed PCR of *AR* according to the modified protocol of and primers designed by Allen et al. (14). Digested and undigested samples were amplified with 400 nM of each primer, 2 mM $MgCl_2$, 333 μM of each dNTP, 4% DMSO, and 0.4 U of *Taq* (Invitrogen), in a total volume of 15 μL. The PCR conditions were 95°C for 3 minutes (initial denaturation); 95°C for 1 minute, 53°C for 1 minute, and 72°C for 2 minutes for 35 cycles, with a final extension at 72°C for 7 minutes. The *AR* PCR results in a ~280-bp amplification product (14).

The XCI skewing was estimated at the *FMR1* locus according to the modified protocol of Hecimovic et al. (15). Digested and undigested samples were amplified in 1X Expand long PCR buffer 1 (Roche), 10% DMSO, 350 μM dATP, 350 μM dCTP, 350 μM dTTP, 100 μM dGTP, 250 μM 7-deaza-GTP (Roche), 1 μM of each primer, and 1.25 U Expand enzyme mix (Roche) in a total volume of 10 μL. Cycling conditions were 94°C for 2 minutes (initial denaturation); 94°C for 10 seconds, 62°C for 30 seconds, and 68°C for 2 minutes for 10 cycles, 94°C for 15 seconds, 62°C for 30 seconds, and 68°C for 2 minutes with an additional 20 seconds per cycle for 30 cycles, with a final extension at 68°C for 7 minutes. The primer sequences were as follows: forward 5′-CTCAGCTC CGTTTCGGTTTCACTTCCG-3′ and reverse 5′-AGCCCCGCACT TCCACCACCAGCTCCTCC-3′ (17). The *FMR1* PCR results in a ~308-bp amplification product (15).