Further evidence of the safety of assisted reproductive technologies

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Worldwide, it is estimated that more than 3 million babies have been born as a consequence of the application of some form of assisted reproductive technology (ART). Although the majority of children born after ART are healthy, some concerns remain regarding the safety of these novel procedures. Several studies have indicated that epigenetic programming may be disrupted after ART, because the incidence of genomic imprinting defects appears to be higher in ART offspring compared with the general population in some studies (1). Moreover, some animal studies and long-term follow-up studies of ART children have implied that there may be an increased incidence of genetic, physical, or developmental abnormalities, although several reports contradict these findings (2). Likewise, some investigators have suggested that intracytoplasmic sperm injection (ICSI), by virtue of its invasive nature, is more likely to result in genetic and/or developmental abnormalities. However, it is difficult to assess whether the purported abnormalities are a consequence of ICSI or rather result from the underlying defects responsible for the infertility. Moreover, many studies have not reported increased abnormalities after the application of ICSI (3). To clarify this issue, in a recent issue of PNAS, Caperton *et al.* (4) explored the influence of ART procedures on the creation of *de novo* point mutations in ART offspring. The incidence of point mutations in ART offspring has not been previously explored in either animals or humans. This important study provides much-needed insight on the impact of ART procedures on genomic alterations.

To date, chromosomal evaluation has been one of the main assessment tools of genetic integrity. It is reassuring that the rate of chromosomal abnormalities after ICSI does not seem to be increased over that of the general population (3). In addition, when *in vitro* fertilization (IVF) and ICSI are compared, no difference in autosomal chromosomal abnormalities has been noted (5). In contrast, the prevalence of sex chromosomal abnormalities appears to be increased after ICSI in comparison with IVF (6). This finding is likely related to the underlying chromosomal defects often observed in males with the most severe semen abnormalities.

Chromosomal evaluation only identifies significant structural aberrations, including divergence of ploidy and large deletions, insertions, or rearrangements. The influence of ART on more subtle changes in genetic composition, such as point mutations, has been largely ignored. Point mutations are in fact very common, and their existence is completely overlooked when only chromosomal assessment is performed.

Point Mutations

Point mutations may arise spontaneously during DNA replication. They include single-base substitutions, as well as small deletions, insertions, or rearrangements that cannot be identified by karyotypic analysis. The rate or type of mutation may be increased by mutagens, which can be chemical or physical in nature. It is possible that the *in vitro* culture environment or physical manipulation during IVF or ICSI could likewise be mutagenic. In the current work, Caperton *et al.* (4) attempt to address whether there is a global increase in *de novo* mutations or whether a shift in the spectra of mutations results as a consequence of ART.

Given the rate of spontaneous point mutations and the size of the human genome, it is impractical to study the occurrence of *de novo* mutations in human offspring. Thus, Caperton *et al.* (4) used a transgenic mouse model in conjunction with a well established mutagenesis assay to study the effects of ART on *de novo* mutations. The transgenic mouse used by these authors carries a λ phage shuttle vector containing the *lacI* target gene, which has been extensively used as a target for identification of genomic mutations (7). Using this model, Caperton *et al.* identified a frequency of *de novo* mutations of $\approx 1 \times 10^{-5}$ in the study population. The authors found no alteration in frequency of mutations consequent to *in vitro* culture conditions or after several different ART procedures.

To confirm the existence of the *de novo* mutations identified as well as classify the types of mutations and determine their distribution, Caperton *et al.* (4) performed genomic sequencing on each of the mutant *lacI* genes recovered. The distribution or spectrum of mutations was similar in fetuses conceived naturally, those exposed to *in vitro* culture conditions, and those conceived by the investigated assisted reproductive techniques [IVF, ICSI, and ICSI with round spermatids (ROSI)].

Single-base changes are the most common *de novo* point mutations, with transition substitutions occurring much more frequently than transversions (8). Correspondingly, Caperton *et al.* (4) found transition substitutions to be the most frequent point mutation identified in all of the studied groups. Transition point mutations often are the result of deamination of methylated cytosine residues. When spontaneous deamination occurs, the resultant thymine is not recognized as an error, resulting in a C·G to T·A transition. In general, transitions occur with a much higher frequency than predicted by random expectation, with one-third occurring at C·G nucleotide pairs, thus confirming that C·G pairs are hotspots for mutations (9). To adequately assess the rate of spontaneous mutation, the genes being evaluated must contain hotspots such that there is sufficient opportunity for mutations to occur. Bisulfite genomic sequencing allowed Caperton *et al.* to determine that the *lacI* transgene is heavily methylated, which correlated with the high frequency of transitions in the distribution of point mutations.

In Vitro Culture Conditions

The highlighted study was designed to assess the frequency and spectrum of point mutations created as a direct result of ART. Because of the unique design of the study, the authors were able to isolate the potential impact of several procedures encompassed within ART; *in vitro* culture, IVF, ICSI, and ROSI. Using a population of fertile animals allowed the authors to directly study genetic alterations without the potential bias associated with infertility.

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In vitro culture has been shown to influence the genetics and physiologic development of preimplantation embryos. *In vitro* culture of sheep and cattle embryos has been associated with increased birth weights as well as with abnormalities in physiologic function and organ development (10). Various mechanisms may account for these changes (11). It has been postulated that the loss of signaling cues from the *in vivo* environment could lead to aberrations in genetic expression. In addition, selective ingredients in culture medium, including serum and amino acids, have been documented to affect the postimplantation viability of murine embryos and subsequent fetal development. Furthermore, murine studies have shown that *in vitro* culture results in abnormal epigenetic modifications giving rise to dysregulation of imprinted genes, many of which are involved in the control of pre- and postnatal growth (12). It has even been suggested that subtle changes in culture conditions, such as the level of oxygen tension, may have an impact on embryonic metabolic function and gene expression (13).

Given the preponderance of evidence that *in vitro* culture affects embryo development, it could be assumed that the formation of point mutations may also be influenced by the *in vitro* culture environment. To investigate this potential link, Caperton *et al.* (4) chose to examine the potential effects of both Chatot, Ziomet, and Bavister (CZB) and Whitten's media, the latter of which has been directly implicated in the disruption of expression of imprinted genes (14). It is reassuring that the frequency and distribution of point mutations were not altered by either CZB or Whitten's medium.

Further support for the idea that culture medium influences embryo development is provided by the high incidence of monozygotic twinning after blastocyst (day 5) transfer (15). Alterations of apoptotic pathways during the course of prolonged incubation have been proposed as possible mechanisms for the increased twinning rate observed with blastocyst transfer. It is of interest to note that Caperton *et al.* (4) were unable to demonstrate an impact of the duration of incubation on point mutation frequency or distribution.

The most important innovation in assisted reproduction in the past 25 years has been the evolution of ICSI (16). ICSI's remarkable success in overcoming the most severe male infertility problems has engendered some concerns about its safety. ICSI is a more invasive procedure than IVF in that it requires physical insertion of a sperm into the oocyte. Theoretically, this technology could result in physical damage to or biochemical disturbances in the oocyte. For example, sperm injection could be associated with disruptions of subcellular compartments and changes in intracellular ion concentrations, resulting in the release of nucleases that could potentially damage DNA. Although in theory all of these mechanisms may contribute to the creation of point mutations, Caperton *et al.* (4) were unable to show that manipulation with IVF or ICSI led to an increased frequency or alteration in the spectrum of point mutations.

Intracytoplasmic sperm injection is a more invasive procedure than *in vitro* fertilization.

Similarly, no increase in point mutations was observed after ROSI. Although these positive results are encouraging, it should be emphasized that these immature sperm were derived from fertile males, and caution should be applied when extrapolating these results to an infertile population.

Although the ROSI procedure in mice appears to be successful in achieving viable pregnancies, only a few reports of human pregnancies have been published. In humans, even when fertilization occurs after ROSI, high rates of aneuploidy have been reported (17). The contribution of ROSI to the formation of chromosomal or other genetic abnormalities in viable human offspring remains unclear because of the sparsity of pregnancies after this procedure.

The findings of this study (4) suggest that neither the *in vitro* culture environment nor the physical manipulation associated with IVF, ICSI, or ROSI leads to alterations of *de novo* mutations. Furthermore, the report implies that mechanisms involved in gene repair were maintained under these conditions. The lack of *de novo* mutations in the study of Caperton *et al.* and the reports of epigenetic abnormalities with IVF suggest that the maintenance of genetic integrity is more tightly controlled than the maintenance of epigenetic regulation.

The potential impact of the various ART procedures on implantation rates and pregnancy loss should also be investigated. The work of Caperton *et al.* (4) specifically addresses genetic abnormalities in developing fetuses, but it does not attempt to quantitate abnormalities in fetuses that fail to develop. However, the authors observed differences in the proportion of transferred embryos that developed to midgestation among the various methods of ART. The specifics of these preliminary observations were not described but may be of great significance. Is it possible that embryos exhibiting an increase in point mutations—some possibly lethal—do not implant and the present study outcomes reflect changes that represent only the genetic integrity of surviving embryos? Therefore, future investigations should focus on whether these procedures increase point mutations involved in decisive developmental checkpoints that could result in implantation failure or pregnancy losses.

The investigation of Caperton *et al.* (4) supports the concept that the studied ART procedures do not increase *de novo* point mutations in surviving offspring. The lack of DNA damage observed should be reassuring to practicing physicians and to patients undergoing these treatments.

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