Epigenetics, Assisted Reproduction, and Intracytoplasmic Sperm Injection: A Review of the Current Data

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Abstract

Since the birth of the first *in vitro* fertilisation baby in 1978, >5 million babies have been born worldwide using assisted reproductive technologies (ART). ART were initially considered safe, but, in recent years, concerns regarding the association between these procedures and the increasing incidence of imprinting diseases have developed. There are numerous steps involved in ART and there are many variables that must be considered; even parental infertility may play an important role in offspring epigenetic modifications. This review presents available data from the literature regarding the incidence of these epigenetic modifications after ART, with a primary focus on oocyte insemination methodology. The authors conclude that ART, especially intracytoplasmic sperm injection, may induce epigenetic changes that can be transmitted to the offspring, but additional data are necessary to evaluate the factors involved and to determine the safety of each ART step.

INTRODUCTION

Since the birth of Louise Brown, the first *in vitro* fertilisation (IVF) baby in 1978, >5 million babies have been born using assisted reproduction technology (ART).[1] Initially, ART procedures were considered safe, but, recently, reports have shown an increased prevalence of epigenetic anomalies after assisted reproduction.

In the authors' opinion, ovarian stimulation, oxygen tension, *in vitro* maturation of oocytes, the type of culture media, the way the oocytes are inseminated (IVF or intracytoplasmic injection), the duration of embryo culture (Day 3 versus Day 5 transfer), and the transfer of fresh or thawed embryos are all factors with epigenetic potential.

There are concerns that children born using ART may have increased frequencies of diseases known to have an epigenetic aetiology; however, the effect of ART on the epigenome is unclear. The data available regarding the epigenetic effects on the offspring following ART are heterogeneous,[2] potentially due to the wide range of genes studied and to differences in the function of imprinting genes.

This article reviews important data from the literature that highlight the epigenetic changes that can occur during ART procedures. Searching the literature, the authors found studies linking ART treatment and procedures to a number of adverse obstetric outcomes, imprinting disorders, birth defects, and abnormal birth weight. Furthermore, the present review focusses on the insemination procedure, specifically the epigenetic effect of intracytoplasmic sperm injection (ICSI) upon an embryo's normal development. According to a report, ICSI was the procedure that was most likely to be associated with imprinting errors[3] due to inappropriate methylation of maternal alleles.

METHODS

Articles were identified using multiple formal search methods, which included the searching of key journals and electronic searching of main databases, including the use of free-text, index terms, and authors. Electronic searches of Web of Knowledge, Web of Science, Google Scholar, PubMed, and other databases were conducted. Free-text searches included single and plural keywords, which initially yielded a great number of articles, many unrelated to the review intended, and the articles of interest were highlighted by the authors.

DNA METHYLATION

One of the best-known epigenetic modifications is represented by DNA methylation. Methylation can be defined as the addition of a methyl (CH_3) group, modifying gene function and affecting protein expression. The most widely characterised methylation is the covalent addition of a methyl group to the C-5 position of the cytosine base. Cytosine-phosphate-guanine (CpG) islands are genomic regions containing a high frequency of CG dinucleotides. CpG islands form approximately 70% of promoters in the human genome. DNA methylation generally occurs on the cytosine residues of CpG dinucleotides through the action of several DNA methyltransferase (DNMT).

However, the DNMT process is extremely important for normal embryo development because it plays an essential role in a number of key processes, such as transcriptional repression, suppression of element transposition, imprinting genes, and X chromosome inactivation. On the other hand, methylation defects in humans are involved in various genetic diseases, including Rett syndrome or X-linked mental retardation.[4,5]

Recently, a study by Choux et al.[6] attempted to establish whether reproductive procedures could alter DNA methylation and the transcription of transposable elements and imprinted genes in the placenta and cord blood. The study included 51 IVF/ICSI singleton pregnancies, as well as 58 spontaneously conceived children, and the work focussed on umbilical cord blood and the placenta from which DNA methylation and transcription of three imprinted loci (*H19/IGF2*, *KCNQ1OT1*, and *SNURF* differentially methylated regions [DMR]) and four transposon families (LINE-1, ERVFRD, AluYa5, and ERVW) were assessed by pyrosequencing and quantitative reverse transcription-PCR.[6] The results showed significantly lower methylation levels in the IVF/ICSI group placentas compared with control groups placentas in four of the seven studied markers: *H19/IGF2*, *KCNQ1OT1*, *LINE-1H*, and *ERVFRD-1*. However, there was no difference in the cord blood results.[6]

Another key epigenetic marker that controls gene expression is covalent modification of histone proteins, and noncoding RNA. A histone modification is a covalent post-translational modification (PTM) to histone proteins, which includes methylation, phosphorylation, acetylation, ubiquitylation, and sumoylation. These modifications can induce either gene activation or suppression depending on the nature of the modification and the specific amino acid modified. A non-coding RNA (ncRNA) is a functional RNA molecule that is transcribed from DNA but not translated into proteins. The epigenetic mechanisms are subject to environmental and developmental influences.[2]

IMPRINTING DISORDERS

Imprinting is a chemical process involving the modification of nucleotides. During gametogenesis, the process of genomic imprinting is established. The nucleus of the

zygote has an imprint memory. This memory is retained by the embryo during prenatal and postnatal life. During preimplantation development, imprinting is highly regulated. Information inherited from the previous generation must be erased in primordial germ cells to add new epigenetic information according to whether the primordial germ cell is destined to become an oocyte or a sperm cell.[7] Approximately 40 genes are known to be imprinted in humans and imprint abnormality is understood to result in 10 syndromes.

One of the greatest concerns regarding all ART treatments and procedures is an apparently higher percentage of imprinting disorders, such as Beckwith–Wiedemann syndrome (BWS: ONIM 130650) and Angelman syndrome (AS: ONIM 105830), seen in ART babies compared with the general population. Several studies were conducted to evaluate the correlation between the most common imprinting disorders and offspring born after an infertility treatment.[8-10] Besides BWS and AS, other imprinting disorders, including Prader–Willi syndrome (PWS: OMIM 176270) and Silver–Russell syndrome (SRS: OMIM 180860), showed either poor or non-existent association with ART.[11]

BWS is a congenital disorder that involves overgrowth and neoplasia.[12] It has recently gained considerable interest because the molecular cause in most cases is epigenetic, rather than genetic. BWS has been shown to involve loss of imprinting of a group of imprinted genes on 11p15. Approximately 15% of patients with BWS have aberrant methylation and imprinting of *H19* and almost half of patients with BWS have aberrant methylation and imprinting of *LIT1.*[12] The first evidence that suggested that ART is associated with BWS was published by DeBaun et al.[12] The study presented seven cases of children born after ART, all of whom were diagnosed with BWS. ICSI was performed in five cases, and in the other two cases conventional IVF was used. Molecular studies of six of the children indicate that five of the six had specific epigenetic alterations associated with BWS, four at *LIT1* and one at both *LIT1* and *H19.*[12]

In the following years, other authors, among them Rossignol et al.,[13] supported the same conclusion. On the other hand, a Danish National IVF Cohort Study followed 442,349 singleton non-IVF children and 6,052 IVF children[14] and concluded that no imprinting disorder was found in the IVF cohort. An Irish study published in 2007 supported that there is a small risk of imprinting diseases among ART conceived children.[15]

A recent study published in 2016 by Tenorio et al.[16] followed and assessed 187 children with BWS, conceived naturally or following ART. The researchers concluded that there was a link between molecular aetiology of the disease and the type of conception, and that the odds ratio for BWS in children conceived by ART is 7-fold higher than babies conceived naturally. In addition, the hypomethylation of KCNQ1OT1:TSS-DMR was present in the ART group, while it was observed in approximately 50% of cases in the spontaneous conception group.

AS affects approximately 1 in 16,000 children, and it is characterised by severe intellectual disability, speech impairment, ataxia, a happy demeanour, seizures, and microcephaly.[17] Approximately 3% of patients with AS have an imprinting defect, evidenced by a paternal-only pattern of methylation. AS was first related to ART in general, and to ICSI in particular, in 2002 in a study conducted by Cox et al.[3] reporting two cases of ICSI treatment followed by AS diagnosis. ART has been implicated in AS by reports of five ART-conceived patients with epimutation-AS.[3,8,18] Of these, four were conceived using ICSI[3] and one using ovarian hyperstimulation alone.[8] However, the literature data are inconclusive and need further investigation. A Danish survey from 2005 concluded that in 25,000 offspring born after IVF no cases of AS were found.[14] In the same year, another German study suggested, for the first time, a possible link between subfertility, by itself, and the increased number of AS diagnosis among children born after infertility treatment.[8] In their study, Ludwig et al.[8] found no difference regarding the relative risk of an imprinting disorder between the infertile couples who were treated by ICSI or hormone therapy and the subfertile couples who did not undergo a fertility treatment. Furthermore, another study by Doornbos et al.[19] established that the major risk factors for infertility treatment related to AS were long-term infertility and ovulation induction.[19]

PWS affects approximately 1 in 17,500 children and is characterised by neonatal hypotonia, childhood onset obesity, cognitive impairment, distinctive behavioural characteristics, hypogonadism, and a characteristic facial appearance.[20] There is no single gene responsible for PWS, but most aspects of the PWS phenotype result from the absence of paternal expression of a cluster of non-coding RNA known as 'HBII-85'.[21]

SRS is a disorder of decreased growth that is estimated to affect 1 in 100,000 children. SRS is characterised by intrauterine and post-natal growth retardation plus variable additional features, including fifth finger clinodactyly, limb length asymmetry, a typical facial phenotype, and variable learning disabilities.[22] SRS differs from other imprinting syndromes in that three distinct imprinted loci on two different chromosomes have so far been implicated.[22,23] There is currently little evidence linking SRS with ART. To date, there have been five patients reported with SRS and who were conceived using IVF or ICSI[23] and molecular data are available for only two. One ICSI-conceived girl with an SRS-like phenotype was found to have hypomethylation at the paternal allele.[23]

Three publications have indicated the existence of a novel imprinting syndrome resulting from maternal hypomethylation at multiple loci.[13,23,24] Results indicate that the maternal hypomethylation syndrome can be associated with, but is not limited to, ART conceptions.[7]

In a more recent meta-analysis, published in 2018, Cortessis et al.[25] reviewed 23 studies from the literature concentrating on the correlation between ART and imprinting diseases incidence and concluded that there was a positive association among them.

In conclusion, evidence of imprinting syndromes resulting from epimutations in ART-assisted pregnancies is so far confined to three syndromes: BWS, AS, and the maternal hypomethylation syndrome. It is notable that for all three syndromes the observed epigenetic defect is hypomethylation on the maternal allele.

IN VITRO FERTILISATION VERSUS INTRACYTOPLASMIC SPERM INJECTION

ICSI is a procedure widely used for achieving fertilisation of oocytes. The procedure was first described by Palermo et al.[26] in 1992 and represents a major advance in infertility treatment for couples for whom classical IVF is not an option due to low sperm count. The technique involves the injection of a single sperm cell into the oocyte.[26] There have been many studies to date concerning the safety of this procedure. The present paper reviews the data available in the literature concerning the link between ICSI and epigenetic modifications.

Over the years, a lot of theories regarding IVF or ICSI-born children have been suggested but the debate continues with more studies performed and interest in epigenetic activity increasing.

In a retrospective cohort study of children born between 2002 and 2008, Whitelaw et al.[27] measured the DNA methylation in paternally expressed gene 3 (*PEG3*), insulin-like growth factor II (*IGF2*), *SNRPN*, long interspersed nuclear element I (*LINE I*), and the insulin gene (*INS*) in buccal cell DNA obtained from children born following IVF (n=49) and ICSI (n=20) procedures and then compared them to the spontaneously conceived children (n=86). The characteristics of the spontaneously conceived group were matched as closely as possible to the ART group and there were no significant differences in any of the subject characteristics. The results showed no significant differences between the children born using ART and the control group for three of the four genes studied and for the repeat element. The only significant difference was related to the *SNRPN* methylation, which was significantly higher in the ICSI group compared to the spontaneous conceived group. The difference remained important in a comparison between ICSI and standard IVF, and between ICSI and combined IVF and control groups. Additionally, higher levels of *SNRPN* methylation was associated with a longer infertility duration.

Another study, conducted by Rancourt et al,.[28] aimed to establish if there is a connection between the method of conception and an

increased risk of rare childhood disorders. The study collected data and biospecimens (placenta and umbilical cord samples) from three groups of women, 61 of whom conceived spontaneously, 59 of whom conceived by IVF, and 27 of whom conceived by ovulation induction. The population studied was restricted to non-Hispanic, white individuals, because of the evidence that methylation may vary with race,[5] and to singleton births resulting from one implanted placenta. To analyse the role of the conception method in the epigenetic activity, six DMR were examined by bisulfite pyrosequencing in both the cord blood (embryonic) and the placenta (non-embryonic). The authors observed that the methylation levels of *GRB10*, *MEST*, *H19*, *SNRPN*, and *KCNQ1,* as well as *IGF2*DMR0, were not disrupted by the fertility treatments. However, the methylation levels for *H19* were marginally lower in placentas from children conceived by fertility treatment. Although the study did not consider the different ART procedures in detail, the conclusions are reassuring for infertile couples.[28]

A study reported by Tierling et al.[29] concluded that there was no significant difference between the epigenetic effects of IVF and ICSI, but the researchers followed a more heterogeneous population, including twin pregnancies.

Sutcliffe et al.[9] suggested that there is a strong link between different types of ART (IVF and ICSI) and imprinting syndromes. However, due to the fact that this study was underpowered and suffered from methodological difficulties, the research lacked reliable data on this subject. The incidence of imprinting disorders overall is small (<1:12,000 births).[30]

Kobayashi et al.[31] showed that 14% of infertile men had an abnormal methylation imprints in their sperm. The outcome of ART with sperm shown to have an abnormal DNA methylation pattern was generally poor. Their data suggested that sperm from infertile patients, especially those with oligozoospermia, may carry a higher risk of transmitting incorrect primary imprints to their offspring, highlighting the need for more research into ART.

Additionally, a study by Marques et al.[32] showed that 30% of men with severe oligozoospermia had an altered *H19* methylation profile. The data suggested an association between abnormal genomic imprinting and hypospermatogenesis, and that spermatozoa from oligozoospermic patients carry an increased risk of transmitting imprinting errors. Ørstavik et al.[18] concluded that ICSI can lead to an increased risk of imprinting defects. Cox et al.[3] concluded that there are some indications that ICSI might interfere with the establishment of the maternal imprint in the oocyte or pre-embryo and increase the risk of imprinting defects. Estill et al.[33] concluded that ICSI culture conditions and parental infertility itself have a lasting impact on a child's epigenome.

There are studies published asserting that the epigenome of ART children remains essentially unchanged.[34-36] Santos et al.[37] concluded, following research on 76 ICSI embryos, that this insemination procedure does not lead to an increased incidence of epigenetic errors.[37] The study showed that DNA methylation pattern was consistent with the normal one, up to the blastocyst stage. Santos et al.[37] found no evidence that blastocysts obtained from injected oocytes were more severely affected than those obtained from conventional insemination. Ghosh et al.[38] compared methylation of CCGG sites in the placentas between ICSI and IVF and no significant differences were observed. The study published by El Hajj et al.[39] on DNA methylation signatures in the cord blood of ICSI children raised concerns that ART-induced epigenetic changes may be transmitted to the offspring, conferring a higher risk of imprinting and other disorders. To study the possible impact of ICSI on the epigenome of the exposed offspring and to identify susceptible loci, El Hajj et al.[39] compared the cord blood methylomes of healthy ICSI newborns versus naturally conceived newborns, using 450,000 methylation arrays. The observed methylation patterns in both the ICSI and the control group were within the normal range of methylation variation.

CONCLUSIONS

The continuous development of ART procedures and treatment has led to more and more ART-conceived children; however, with this rise has come growing concern about the safety of these techniques. When ICSI was first introduced it was intended to help oligozoospermic males to

conceive, but nowadays this technique is used worldwide in many cases that do not involve male infertility. Therefore, it has become very important to establish the possible side effects on the embryos and future children conceived by IVF-ICSI. Many scientists have concentrated on retrospective and prospective follow-up studies of babies born after an infertility treatment to determine the possible alterations of the embryo genome and the risk of rare malformation or genetic diseases.

Molecular analysis permits the identification of various gene defects due to epigenetic abnormalities, that may lead to imprinting disorders, such as BWS, AS, and PWS, or to defects of methylation-related syndromes, like Rett syndrome. Some authors have associated these disorders with infertility procedures and treatment, reporting a significantly increased prevalence in ART-conceived offspring. Meanwhile, other studies showed no difference between the general population and infertile couples or linked the genetic disorders to long-term infertility and ovulation induction, rather than to ART treatment.

Based on the available literature data, it is difficult to conclude that there is a strong correlation between ICSI and these epigenetic syndromes. Furthermore, all of the parameters involved, including infertility duration, ovulation induction, oocyte retrieval, fertilisation, and various lab variables, need to be taken into account.

At the same time, epigenetic abnormalities can be found in a large and heterogeneous variety of genes leading to the need for more exact studies. Additionally, there is a lack of long-term follow-up studies due to the fact that the phenotypes associated with epigenetic disorders are sometimes difficult to establish early in life or may be very subtle, for example predisposing neoplasia.

In conclusion, the available data suggest an association between ART overall and the incidence of three imprinting disorders: BWS, AS, and maternal hypomethylation syndrome. ICSI may be a technique that can lead to a higher incidence of imprinting disorders, but additional data is necessary to evaluate the factors involved and to determine the safety of every single ART step.

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