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FORUM

Perspectives: The Possible Influence of Assisted Reproductive Technologies on Transgenerational Reproductive Effects of Environmental Endocrine Disruptors

Thomas M. Price,* Susan K. Murphy,† and Edward V. Younglai‡

*Division of Reproductive Endocrinology and †Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Duke University, Durham, North Carolina 27710; and ‡Department of Obstetrics and Gynecology, Reproductive Biology Division, McMaster University Health Sciences Centre, Hamilton, Ontario, Canada L8N 3Z5

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Demasculinization by environmental endocrine-disrupting chemicals (EDCs) is observed in many animal species but less evident in humans. Rodent studies with gestational exposure to either the fungicide vinclozolin or the insecticide methoxychlor demonstrate impaired male fertility with abnormal DNA methylation patterns in spermatozoa. Once established, these epigenetic changes may be permanent and thus paternally passed to subsequent generations. Conclusive evidence of a similar phenomenon in humans has not been established, but several observations bring up the possibility. Some, but not all, studies show an increase in male genital abnormalities after prenatal EDC exposure. Other studies demonstrate sperm abnormalities in males with EDC contact, although it is unclear as to whether this is due to prenatal or postnatal exposure. Although not examined in males with EDC exposure, one study shows gamete DNA methylation abnormalities in males with severe oligospermia. A subsequent study failed to corroborate these findings. The use of assisted reproductive techniques including intracytoplasmic sperm injection has removed natural selection barriers thus enabling reproduction in males that would otherwise be sterile. This review explores the hypothesis that prenatal EDC exposure results in transgenerational male reproductive abnormalities propagated by the use of assisted reproductive technologies.

Key Words: **endocrine-disrupting chemicals; DNA methylation; imprinting; assisted reproductive technologies; intracytoplasmic sperm injection.**

Observations, over several decades, suggest region-specific increases in male genital abnormalities and male-associated infertility. These changes are associated with the increasing presence of pesticides, herbicides, fungicides, and plastic compounds in western society. Such compounds interfere with appropriate sex steroid receptor action, thus being referred to as endocrine-disrupting chemicals (EDCs). Substantial animal data show that prenatal exposure results in male reproductive abnormalities, varying from obvious genital tract anomalies to subtle defects in spermatogenesis. Actions of EDC vary according to the gestational age of the animal, the dose of the agent, and the length of exposure. Multiple molecular mechanisms may be involved in EDC actions, with more recent experiments highlighting changes in gamete DNA methylation at imprinted loci. Of additional concern is animal data demonstrating persistence of imprinting abnormalities through multiple generations leading to transgenerational effects.

Fewer direct observations are available in humans, but epidemiological studies support a relationship between EDC exposure and male reproductive abnormalities. Additionally, initial studies suggest a relationship between sperm abnormalities and changes in imprinted gene loci, bringing into question the possibility that males with significant sperm defects are more likely to pass on imprinted disorders.

Prior to the advent of assisted reproductive technologies (ART), major sperm defects led to sterility, thus eliminating the possibility of transmission of inherited traits. The development of *in vitro* fertilization (IVF) and, subsequently, intracytoplasmic sperm injection (ICSI) eliminated this natural selection, enabling males with dramatic sperm abnormalities to reproduce.

In this paper, we initially review the abundant literature regarding EDC and male reproductive anomalies in animals and basic information regarding imprinting. We then put forth information to support or refute the following hypotheses: (1) EDC exposure during pregnancy adversely affects human male reproduction, (2) abnormal sperm are associated with imprinting abnormalities, and (3) ART enables transgenerational propagation by eliminating the natural selection process.

[1] To whom correspondence should be addressed at 5704 Fayetteville Road, Durham, NC 27713. Fax: (919) 484-0461. E-mail: price067@mc.duke.edu.

ANIMAL STUDIES OF EDC

Vinclozolin

The majority of data regarding the effect of EDC and male reproduction is derived from rodent studies with compounds of vinclozolin (VCZ), methoxychlor (MXC), and/or metabolites of these chemicals. Studies often vary according to the dose, route, and time of administration. Complicating such studies is the fact that EDC have complex dose-response relationships and often act through multiple signaling pathways (Henley and Korach, 2006). VCZ (3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione) is a fungicide used on turf grass and on vine plants such as grapes and strawberries. It is metabolized to two hydrolytic degradation products which have higher binding affinities for the androgen receptor (AR) than VCZ itself (Kelce *et al.*, 1994). *In vitro* studies have shown binding to the AR with inhibition of androgen-induced transcription (Kavlock and Cummings, 2005; Kelce and Wilson, 1997; Shimamura *et al.*, 2002). The major effects of VCZ result from prenatal exposure.

Peripubertal exposure of Long Evans rats to VCZ by gavage (0, 10, 30, or 100 mg/kg daily from day 22 of life) delayed pubertal maturation and retarded sex accessory gland and epididymal growth at 30 and 100 mg/kg (Monosson *et al.*, 1999). At higher doses (100, 200, and 400 mg/kg), given between days 35 and 44, VCZ decreased sex accessory gland weights, caused hypertrophy of Leydig cells, and sloughed cells in the epididymis of Sprague-Dawley rats immediately after cessation of treatment (Yu *et al.*, 2004). When animals were allowed to mature, epididymal sperm counts were reduced.

In utero and neonatal exposures to VCZ have more detrimental effects on the developing male. Exposure of pregnant rats to 100 mg/kg/day VCZ on days 8–14 of pregnancy led to higher numbers of apoptotic germ cells on postnatal day 20, but testis weight was not affected (Uzumcu *et al.*, 2004). Sperm motility was also impaired on day 60. Exposure of pregnant mice to 100 mg/kg/day of VCZ on days 10–18 caused a significant decrease in anogenital distance (AGD) of F1 neonates and reduced spermatogenesis in F1 males at 42 days of age (Shimamura *et al.*, 2002). Perinatal exposure of Long Evans rats to low doses of VCZ (1.5–12 mg/kg/day) from day 14 of pregnancy to postnatal day 3 did not produce any obvious structural changes or weight reductions in androgen-sensitive reproductive organs of male offspring, but a significant reduction of erections was observed at all dose levels (Colbert *et al.*, 2005). However, at a dose of 200 mg/kg/day during the same period of exposure, the male offspring of both pregnant Long Evans and Wistar rats had major adverse effects in their sex-specific organs (Hellwig *et al.*, 2000). The no-observed-adverse-effect levels were 12 and 6 mg/kg body weight in Wistar and Long Evans rats, respectively. When pregnant rabbits were treated with VCZ, 7.2 or 72 mg/kg/day, from day 15 of pregnancy through 4 weeks postnatally, the male offspring older than 12 weeks had twice as many morphologically abnormal sperm in their semen (Veeramachaneni *et al.*, 2006). Interestingly, administration of 200 mg/kg/day VCZ on days 12–19 of pregnancy caused no alteration in the pattern or expression level of AR on pregnancy day 19, neither was there any gene expression alteration as assessed by microarray analysis (Mu *et al.*, 2006). These studies illustrate the diversity of effects depending on species, dose, and timing of ED administration.

Two-generation studies with VCZ reveal that 40–200 ppm given in the diet to pregnant rats could result in changes in the AGD, sex organ weights, sexual maturation, and blood hormones in both F1 and F2 male offspring (Matsuura *et al.*, 2005; Yamasaki *et al.*, 2005). The effects of VCZ are permanent and are most pronounced when administered transiently during the period of gonadal sex differentiation (Anway and Skinner, 2006; Anway *et al.*, 2005; Skinner and Anway, 2005). The mechanism appears to be epigenetic and involves altered DNA methylation and permanent reprogramming of the male germ line leading to spermatogenic cell apoptosis and subfertility. The phenotypic changes can extend to the F4 generation with high penetrance. These findings suggest that an environmental exposure during pregnancy could lead to permanent epigenetic changes in male offspring that persists through multiple generations.

Methoxychlor

MXC (2,2-bis(4-methoxyphenyl)-1,1,1-trichloro-ethane) is an insecticide developed to replace dichlorodiphenyltrichloroethane. Its metabolite, 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane, is also active in inhibiting testosterone formation by cultured Leydig cells from neonatal rats (Akingbemi *et al.*, 2000; Murono and Derk, 2005). Effects of exposure to MXC in adult rodents are not known. However, in young adult male rats, age 54–60 days, MXC administration at 200 mg/kg body weight reduced serum testosterone levels and *ex vivo* Leydig cell testosterone formation (Murono *et al.*, 2006).

Transient exposure to MXC during sexual differentiation *in utero* can lead to disruption of adult male function in Sprague Dawley rats but fertility is not reduced (Cupp and Skinner, 2001; Cupp *et al.*, 2003). Preimplantation exposure of male rats to MXC can lead to reduced testosterone levels at 3 and 6 months of age, but only the seminal vesicles were heavier at 6 months (Amstislavsky *et al.*, 2006). *In utero* exposure to high doses of MXC also alters mammary gland development in male rats (Wang *et al.*, 2006; You *et al.*, 2002). When rats were fed MXC from day 15 of pregnancy to day 10 after parturition, luteinizing hormone and follicle stimulating hormone levels were decreased but not testosterone levels or lordosis behavior in male offspring (Suzuki *et al.*, 2004).

Transgenerational effects similar to those observed for VCZ have been associated with prenatal exposure to MXC as well (Anway and Skinner, 2006).

GENOMIC IMPRINTING

Genomic imprinting refers to a mechanism of gene regulation whereby one of the two copies of a gene within a cell is transcriptionally silenced. This silencing is a result of epigenetic modifications that are established differentially in sperm and oocytes during gametogenesis, are preserved during genome-wide epigenetic remodeling that occurs after fertilization, and are maintained in somatic tissues throughout the life span of the individual. There are approximately 50 imprinted genes identified thus far in humans (Morison *et al.*, 2001) with up to several hundred estimated to exist (Morison *et al.*, 2005). The epigenetic mediators of imprinted expression include histone modifications and DNA methylation, neither of which changes the DNA sequence. Although both modifications are involved in imprint regulation in somatic tissues, how histone modifications can be transmitted through the male germ line is not clear. During spermatogenesis, the histones are removed from the genome and are replaced temporarily by protamines. It is difficult to envision how conveyance of imprint regulatory information at the histone level would occur during the course of histone extraction, protamine substitution, and histone replacement. Intriguingly, a recent study demonstrated that this might be possible through retention of at least some modified histones during this process (van der Heijden *et al.*, 2006).

The most obvious consequences of imprinting are the loss of protection from recessive mutations that functional diploidy confers and the epigenetic vulnerability to alterations in expression. Disruption of the mechanisms regulating imprinted expression, through a single epigenetic (or genetic) event, can lead to activation of the normally silent allele, or conversely, repression of the normally active allele. Such loss of imprinting (LOI) is observed in many human malignancies and in neurodevelopmental disorders, connecting epigenetic alterations at imprinted regions to phenotypic consequence. It is unclear why imprinting evolved given the risks associated with monoallelic expression, but the reasons may involve differential influence of the female and male parental genomes that compete in the control of placentation and growth of the offspring.

There are several critical periods of development in which imprint marks may be particularly vulnerable to disruption that can lead to lifelong and potentially multigenerational changes in gene expression. The first is during embryogenesis when global methylation patterns and imprinting marks are erased as primordial germ cells migrate along the genital ridge (Hajkova *et al.*, 2002; Yamazaki *et al.*, 2003). Another vulnerable period is during the reestablishment of gametic imprint marks to reflect the sex of the individual in which the gametes now reside. This may begin prenatally and be completed during gamete maturation after puberty, with the exact timing of imprint establishment varying by gene. Hence, postpubertal environmental influences might also alter the establishment of imprint marks; transmission of altered imprint marks disrupts the normal methylation erasure and reestablishment in subsequent generations.

The time between fertilization and implantation represents another period when the genomes are epigenetically vulnerable. Just after fertilization in the zygote, the male pronucleus undergoes chromatin decondensation and is rapidly demethylated. This is followed by a more gradual demethylation of the female pronucleus (Santos *et al.*, 2002). Remarkably, the gametic imprint marks are normally resistant to this wave of genome demethylation (Mayer *et al.*, 2000; Oswald *et al.*, 2000). After erasure, the methylation profile in the early embryo is restored by *de novo* methylation, a process that continues through peri-implantation (Reik *et al.*, 2001). Differentiation brings about additional tissue-specific changes in the epigenome (Ehrlich, 2003) and the rapid cell division that occurs during prenatal development necessitates that these profiles be faithfully recapitulated through each round of DNA replication. Each one of these epigenetic milestones is a potential target for the action of EDC at imprinted or other loci. Changes in the epigenome are clonally heritable. The scope of epigenetic alterations then depends on the point at which epigenetic alterations occur. If they occur prior to or during germ line specification, the resulting epigenetic changes will be passed on, potentially, to multiple generations, as has been observed in rodents exposed during gestation to VCZ and MXC (Anway *et al.*, 2005).

Common Assays for Imprinting

Imprinted genes are characterized by mRNA expression primarily from one of the two inherited alleles. As such, demonstration of imprinted expression is dependent on the ability to distinguish between transcripts produced from each parental allele. This is accomplished by analyzing cDNAs from heterozygous individuals in the tissue of interest for the presence of genetic polymorphisms. Since most genes are expressed biallelically, nucleotide sequencing of cDNAs, for example, will reveal the presence of both allelic variants at polymorphic sites within the region sequenced. For imprinted genes, sequencing of cDNA will reveal predominance of only one of the two parental alleles in normal circumstances. Under abnormal circumstances, such as in cancer and other diseases and disorders, imprinted genes can lose their imprinted pattern of expression, such that the normally silenced allele becomes active (and is detectable by nucleotide sequencing) or conversely that the normally active allele becomes silenced.

The study of epigenetic characteristics associated with imprinted domains has shown that histone modifications and/or DNA methylation go hand in hand with imprinting status (Cui *et al.*, 2003; Hu *et al.*, 2000; Vu *et al.*, 2006). DNA methylation has been most intensively studied as nearly all imprinted genes are directly or indirectly associated with genomic regions rich in CG dinucleotides that are methylated differentially according to the parental origin of the particular allele. Analyses of such differentially methylated regions (DMRs) are performed using a number of methods.

Traditional methodology uses isoschizomeric restriction enzymes (e.g., *Msp*I and *Hpa*II) that recognize the same CG-containing recognition sequence differentially based on methylation status, followed by Southern blotting to detect the presence of the fragments of interest. Although robust, this method is limited by the requirement for large amounts of genomic DNA and in its ability to assess methylation of the CG dinucleotides only within the restriction site used for analysis. Most current techniques utilize genomic DNA that has been treated with sodium bisulfite. Sodium bisulfite mutagenesis causes all unmethylated cytosines to become uracils through deamination; all methylated cytosines are resistant to this deamination. Because uracil pairs with adenine, sodium bisulfite–treated genomic DNA can be used as a template for PCR. The amplicons thus produced will therefore contain thymines in place of unmethylated cytosines, including all cytosines that were not in CG context, and cytosines at all positions that were methylated in the original DNA sequence.

This has led to the widespread use of PCR-based methods to analyze DNA methylation (Wong, 2006). Methylation-specific PCR (MS-PCR) is a commonly used technique employing two independent primer sets that are designed to specifically amplify either methylated or unmethylated versions of the same bisulfite-modified DNA sequence by annealing to potentially methylated cytosines. Although rapid and relatively inexpensive, MS-PCR provides qualitative rather than quantitative information about methylation. Quantitative methylation data can be obtained using other techniques such as combined bisulfite restriction analysis (Eads and Laird, 2002), pyrosequencing (Colella *et al.*, 2003), real-time MS-PCR (e.g., MethyLight) (Eads *et al.*, 2000), or by bisulfite sequencing (Frommer *et al.*, 1992).

Bisulfite sequencing is performed using primers for PCR that anneal to regions devoid of CGs on bisulfite-converted DNA to generate amplicons used for nucleotide sequencing. This method offers single-nucleotide resolution of the methylation status of individual CGs, which can be quantified with imaging software. For imprinting analysis, amplicons derived from such a reaction are often cloned into plasmids, and the individual alleles thus represented can be sequenced. If an allele-distinguishing polymorphism is present within the cloned region, then methylation patterns specific to each allele can be determined. Variations on these basic methods have also been used that are beyond the scope of this review. High-throughput methods for methylation analysis are beginning to be developed, and this technology will be a requirement for improving our understanding of the normal epigenome as well as the genome-wide epigenetic consequences of exposure to endocrine-disrupting agents.

Abnormal Imprinting in Animals Exposed to VCZ and MXC

As mentioned above, a recent study demonstrated that transient exposure of pregnant rats to VCZ or MXC caused impaired fertility in male offspring (Anway *et al.*, 2005). The timing of the exposure (between E8 and E15) was concomitant with the time that methylation marks are reprogrammed within the germ cells in the developing offspring. The fertility defect was transmitted to over 90% of all subsequent male offspring through at least the F_4 generation. The high frequency of this phenotype and the specificity of exposure timing indicates that it is likely not attributable to a genetic mutation but more likely associated with a transmissible epigenetic alteration in the germ line. Although the authors did not observe altered methylation of maternally expressed *H19* in the affected animals, they did find that a number of genomic loci that they termed ''imprinted-like genes/DNA sequences'' exhibited methylation differences in testes and sperm between affected animals and controls (Anway *et al.*, 2005; Chang *et al.*, 2006). Many of these loci were shown to exhibit the same patterns of altered methylation in sperm of male rats in subsequent generations. Analysis of the adult rats from the F_1 to F_4 generation revealed abnormalities in the kidney, prostate, testis, breast and skin; immune abnormalities; hypercholesterolemia; and tumor formation, some malignant. Transgenerational inheritance of the adult-onset phenotypes was more frequently observed through the male germ line, but the female germ line may also have a contributing role (Anway *et al.*, 2006). Together, these observations indicate that the shift in methylation induced by the EDC exposure is heritable and associated with frequent occurrence of later onset diseases. It is unclear at this point whether the genomic loci with abnormal methylation resulting from EDC exposure have themselves been induced to become imprinted or whether the methylation changes are occurring at loci that are vulnerable to such effects without imposing imprinted expression. In developing oocytes and sperm, methylation marks are established differentially not only at regions that regulate genomic imprinting but also at certain repetitive elements and perhaps other regions that do not exhibit imprinted expression (Howlett and Reik, 1991; Sanford *et al.*, 1987). Such loci may be targets of EDC exposure. Further studies are needed to determine whether the genes showing transmissible methylation changes as a result of prenatal VCZ and MXC exposure exhibit imprinted (i.e., monoallelic, parent-of-origin dependent) expression and also whether known imprinted genes are affected.

In the above sections, we have provided strong evidence in animal studies that EDC exposure during gestation results in abnormal male reproduction, varying from genital anomalies to only spermatozoa defects. One mechanism includes abnormal DNA methylation with persistence in spermatozoa through several generations. We now explore what similar evidence is available in humans and the potential impact of ART.

EDC AND HUMAN REPRODUCTION

Evidence of an EDC effect on male reproduction is not as clear; reports consist of both positive and negative results.

Additionally, most studies evaluated broad chemical categories such as pesticides and were unable to focus upon a single compound. A study of semen parameters in regions of the United States found significantly lower sperm concentrations and motility in Columbia, MO, an agricultural dominant region, compared to more urban locations of New York, NY, Minneapolis, MN, and Los Angeles, CA. A comparison of men in Columbia with low sperm concentrations to those with normal parameters showed increased urinary concentrations of pesticides in the former, even though only 12% reported any known exposure (Swan, 2006). In a study of Mexican agricultural workers, sperm aneuploidy rates correlated with urinary organophosphate pesticide levels. Seventy-five percent had abnormally high sperm DNA fragmentation indices compared to 12% of urban, nonexposed, controls (Recio *et al.*, 2001; Sanchez-Pena *et al.*, 2004). Likewise, exposure to the common pesticide, carbaryl, resulted in increased sperm aneuploidy, DNA fragmentation, and abnormal gross morphology in pesticide factory workers in China (Xia *et al.*, 2005).

Male reproductive changes from prenatal exposures include genital abnormalities of hypospadias and cryptorchidism. A role of environmental factors is supported by an increased incidence of hypospadias in agricultural workers applying pesticides (Garry *et al.*, 1996). The highest incidence was found in conceptions occurring in the Spring, the time of greatest pesticide application. Other evidence implicating an environmental factor includes a dose-dependent increase in hypospadias after an industrial accident releasing dioxin in Italy (Mastroiacovo *et al.*, 1988) and increased cases in subjects living near a hazardous waste landfill in Europe (Dolk *et al.*, 1998).

Contrasting studies are also available. In a case-controlled study, no differences in sperm aneuploidy rates were present in 20 pesticide appliers in Minnesota compared to 20 controls. This study did not verify exposure with any type of biochemical testing (Smith *et al.*, 2004). Concentrations of MXC in water near pesticide dump sites in Poland approached 0.31 μg/l, but no abnormal reproductive function was found in men living nearby (Buczynska and Szadkowska-Stanczyk, 2005). In a study of 30 Danish farmers, sperm samples analyzed for abnormalities of chromosomes 1 and 7 by fluorescent in situ hybridization showed no difference prior to and after a season of fungicide usage (Härkönen *et al.*, 1999).

The above observations support a role for pesticides in male reproductive abnormalities after prenatal exposure, but an effect in subsequent generations has not been studied. A possible transgenerational effect is seen with the synthetic estrogen, diethylstilbestrol (DES). Several studies (Brouwers *et al.*, 2006), but not all (Plamer *et al.*, 2005), show an increased incidence of hypospadias in males born of women exposed to DES *in utero*. Rodent studies show transmission of transgenerational DES effects through the maternal germ line (Newbold *et al.*, 2006) as opposed to observations for VCZ and MXC.

Abnormal Imprinting in Impaired Spermatogenesis

To our knowledge, there have not been any studies evaluating changes in spermatozoa DNA methylation in males exposed to EDC. Recent studies have examined the methylation status of the imprint center DMR located near the maternally expressed *H19* in spermatocytes and spermatogonia from men with abnormal sperm that underwent ICSI. This DMR is methylated during spermatogenesis and carries this paternal methylation mark throughout life (Davis *et al.*, 2000). No differences in the methylation status were observed for the *H19* imprint center in two studies (Hartmann *et al.*, 2006; Manning *et al.*, 2001), while significant hypomethylation was observed in another study of oligospermic samples (Marques *et al.*, 2004). Analysis of the maternally methylated *MEST* locus for methylation changes in the oligospermic samples showed that the maternal methylation imprint mark had been completely erased, at least in the region analyzed (Marques *et al.*, 2004). Although these results suggest that the maternal methylation marks associated with *MEST* are appropriately erased in men with oligospermia, other imprinted genes with maternal methylation imprints will need to be similarly analyzed.

There are only two other imprinted regions known to carry paternal germ line methylation imprints: *Rasgrf1* (Yoon *et al.*, 2002) and *Dlk1/Gtl2* (Takada *et al.*, 2002), whose germ line methylation status has been thus far determined only in mice. These paternal imprint marks need also to be investigated for potential alterations in methylation resulting from EDC exposure, and it will be important to determine whether these genes have a role in fertility. Intriguingly, paternally expressed *DLK1*, which encodes an EGF-like homeotic protein, was recently identified as one of the genes most differentially expressed when comparing normal sperm to that from men with impaired fertility (Rockett *et al.*, 2004).

Another possible mediator of abnormal paternal imprint establishment is the CTCF-like (CTCFL) protein, which is reportedly involved in targeting methylation imprints to appropriate loci during spermatogenesis (Loukinov *et al.*, 2002). *CTCFL* is a paralog of the zinc finger gene, *CTCF*. CTCF binding to genomic DNA induces changes in chromatin structure leading to the creation of insulator sequences that function to physically separate *cis*-acting elements, for example, promoters and enhancers, from one another. In the imprinted *IGF2/H19* domain, CTCF binds to the unmethylated maternal allele at the DMR upstream of *H19* and creates an insulator element that blocks access of downstream enhancers to the upstream *IGF2* promoter. The paternal allele is methylated at this DMR, which prevents CTCF binding and allows the enhancers to activate transcription of *IGF2*. The binding specificity of CTCFL is identical to that of CTCF, but CTCFL is only expressed during discrete stages of spermatogenesis. This is thought to allow for CTCFL to aid in establishment of methylation at imprinted loci (Loukinov

et al., 2002). *CTCFL* is itself epigenetically regulated (Hoffmann *et al.*, in press), with dense promoter methylation correlating with transcriptional repression in normal adult testis and other tissue types. Adult spermatozoa, however, are completely unmethylated, supporting the idea that CTCFL expression may be involved with remethylation of the genome prior to sperm maturation. It is plausible that CTCFL may be vulnerable to promoter methylation and silencing during spermatogenesis as a result of EDC exposure. If so, the establishment of imprints at paternally methylated loci may be impaired. Although the studies to date differ with respect to the methylation status of the paternal imprint at *IGF2/H19* in sperm from men with impaired spermatogenesis, other paternal methylation imprints also need to be examined, as well as the promoter region of CTCFL itself to determine if these sequences are epigenetically targeted as a result of EDC exposure.

IVF, ICSI, AND MALE REPRODUCTION

The advent of IVF with ICSI dramatically changed therapy for male infertility. Men with sperm abnormalities consistent with sterility are now able to reproduce by the injection of a single sperm into an oocyte. This procedure thus provides an excellent model to determine possible transmission of traits linked to abnormal semen parameters. There are no studies evaluating IVF/ICSI outcomes in males exposed to EDC. There is limited evidence that males with abnormal sperm may have higher male reproductive defects in their offspring. In most cases, there is not any difference in male reproductive abnormalities between IVF and ICSI, except for the possibility of hypospadias. Some studies (Ericson and Kallen, 2001; Kallen *et al.*, 2005; Wennerholm *et al.*, 2000), but not all (Bonduelle *et al.*, 2002), have shown an increase in hypospadias with ICSI compared to IVF conceptions. A contributing factor to this discrepancy may be the evolving use of ICSI. When first put into clinical care, the process of ICSI was solely used for males with severe sperm abnormalities including low concentration, low motility, or abnormal morphology. With time, the use has broadened to conditions not involving sperm problems. The observation of increased hypospadias with ICSI has been attributed to paternal transmission of abnormal male genes. The possibility of DNA methylation changes in the paternal germ line has not been evaluated. Additionally, other indicators of male genital development such as AGD have not been evaluated in the offspring of ICSI conceptions, and these children are not yet old enough to determine semen parameters.

IMPRINTING DEFECTS AND IVF

IVF may contribute to imprinting disorders *via* two mechanisms. First, the IVF process may lead to imprinting changes from exposure of the oocyte or developing embryo to exogenous factors in culture media. Second, IVF may indirectly contribute by allowing propagation using abnormal sperm containing imprinting defects. These processes could potentially lead to transgenerational effects.

Some evidence has accumulated that *in vitro* culture of embryos may lead to imprinting abnormalities. First observed in animals, *in vitro* culture of sheep embryos leads to a ''large offspring syndrome.'' This is associated with aberrant methylation of the insulin-like growth factor 2 receptor (Igf2r) (Young *et al.*, 2001) which is imprinted in sheep (Young *et al.*, 2003) but not in humans (Killian *et al.*, 2001). Likewise, alterations in embryo culture media may lead to epigenetic changes in rodents. Alterations in fetal calf serum supplementation of mouse embryo media led to smaller fetuses associated with abnormal expression and methylation of *IGF2/H19* (Khosla *et al.*, 2001; Li *et al.*, 2005; Lucifero *et al.*, 2004).

For obvious reasons, similar studies in human embryos have not been performed, but clinical data on subsequent IVF babies has been reported. DNA methylation status of the imprinted gene region in humans, located at chromosome 15q11–q13, showed no abnormalities in 92 random children conceived *via* ICSI. Yet, an increased incidence of rare imprinting-related diseases, including Beckwith-Wiedemann syndrome (BWS) and Angelman syndrome (AS), in IVF offspring has suggested an association. Data thus far has suggested a sixfold increase in the incidence of BWS in couples conceiving with IVF/ICSI (DeBaun *et al.*, 2003). This estimate must be cautiously considered given the rarity of this disease at one in 14,500 live births (Sutcliffe, 2000). BWS involves LOI of genes encoding for *H19* and *LIT1* located on chromosome 11p15.5. *H19* encodes a nontranslated maternally expressed RNA, which appears to inhibit expression of insulin-like growth factor 2 (Wilkin *et al.*, 2000). Approximately 15% of BWS cases have maternal aberrant methylation of *H19*, thus leading to *IGF2* expression from both maternal and paternal genomes (DeBaun *et al.*, 2003). *LIT1* is a paternally expressed antisense RNA that leads to decreased expression of the cell cycle inhibitor, $p57^{KIP2}$. In approximately 50% of cases of BWS, maternal LOI of the *LIT1* gene with hypomethylation leads to increased expression of LIT1 with resultant decrease in $p57^{KIP2}$ expression (Arima *et al.*, 2005). In a previous study of 14 children with BWS born after IVF/ICSI, 13 had LOI of the *LIT1* gene and 1 had LOI of both *H19* and *LIT1* genes (Allen and Reardon, 2005; DeBaun *et al.*, 2003).

AS results from loss of function of the enzyme ubiquitin protein ligase E3A (UBE3A) in the brain. The *UBE3A* gene (chromosome 15q11–q13) is normally maternally expressed in the brain with methylation of the paternal copy. LOI of the maternal gene due to aberrant hypermethylation results in loss of enyzme production. On average, only 5% of AS are caused by imprinting disorders, while most are due to deletions, point mutations, or uniparental disomy. Of the three studied cases of AS from ICSI, all have been associated with LOI (Allen and Reardon, 2005).

Interestingly, the cases of imprinting diseases associated with IVF/ICSI have thus far all been associated with maternal LOI, suggesting a defect in the oocyte or a defect occurring during the time of fertilization and early embryo culture. This may be due to differential timing of completion of imprinting in oocytes versus sperm. Imprinting appears to be complete when the spermatozoan becomes haploid, whereas in the oocyte, imprinting may continue up to the time of ovulation (Davis *et al.*, 2000; Kerjean *et al.*, 2000; Thompson and Williams, 2005).

The possibility that IVF/ICSI will propagate imprinting disorders associated with abnormal spermatogenesis has not been investigated. As discussed previously, males with oligospermia may have increased LOI of the *H19* gene. Could this defect lead to growth consequences in the fetus and could this defect become transgenerational in subsequent male offspring?

CONCLUSION

This review explores interactions between EDCs, abnormal spermatogenesis, imprinting, and advanced reproductive technologies. Animal studies are clear that prenatal exposure to EDC such as VCZ and MXC results in abnormal DNA methylation patterns of spermatozoa that may persist for generations.

Human observations are less concise and may be summarized as follows. Not without controversy, there is epidemiological evidence that exposure to pesticides/fungicides during pregnancy may result in male reproductive abnormalities. Some, but not all studies show abnormal methylation in oligospermic semen samples, but this has not been evaluated in males exposed to EDC. Some studies of ICSI using males with significant oligospermia show an increased risk of hypospadias. Other markers such as anogenital distance have not been evaluated, and the ICSI population has yet to reach the necessary age for analysis of spermatozoa. The question remains as to whether these observations can be tied together in a common theme or whether they are simply true—true but unrelated. The common theme hypothesized is that an original prenatal exposure to EDC results in abnormal DNA methylation in sperm associated with abnormalities in concentration, motility, and/or morphology. This epigenetic abnormality is then propagated through the use of IVF/ICSI.

Additional studies will be necessary to test such a hypothesis. These include the evaluation of DNA methylation in the sperm of males with a known family history of EDC exposure and the persistence of abnormal DNA methylation patterns in the sperm of male offspring from a father with a known defect. Although not specific for imprinting disorders, additional studies need to evaluate the anogenital distance in offspring from males with severe sperm defects using ICSI and semen parameters when the offspring reach the appropriate age.

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