Long-term effects on offspring of exposure of oocytes and embryos to chemical and physical agents

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Central to this review is the knowledge that, in some livestock species, the environment in which fertilization and embryo development occurs influences not only preimplantation embryo development but also the phenotype of resulting offspring. This knowledge is based on in-vitro studies where the induced changes in the embryo can result in an array of developmental abnormalities after transfer including fetal overgrowth. Whilst such findings are of immediate relevance to assisted reproduction in the human, they also raise another equally important but less obvious issue. Can the in-vivo environments in which fertilization and embryo development normally occur be influenced by exogenous factors (either physical or chemical) in such a way that long-term development is adversely affected? In a global environment of increased use of synthetic chemicals and increased production of pollutants, it is an issue of growing relevance. This review examines technical information that is pertinent to these issues together with a brief assessment of some possible molecular mechanisms responsible for aberrant development. The review concludes with an assessment of the clinical significance of the findings.

Key words: assisted reproduction/drugs/embryo/pollutants/toxicants

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Introduction

Both oocytes and preimplantation embryos are exposed to many environmental factors during maturation and development that influence development. However, it cannot be assumed that all factors are beneficial. With oocytes, the nuclear material is arrested in prophase of the first meiotic division and remains in that state from late in fetal development until just before ovulation. In the human, this period can be many years so the probability of DNA being exposed, at some stage, to perturbing factors is reasonably high. Embryos can also be exposed to similar factors. After fertilization, embryos migrate within the oviducts and ultimately enter the uterus in a carefully orchestrated way during which time, in many species, the embryonic genome is activated. Although genomic activation confers a degree of self-determination on the embryo, it is now known that this is a time when the embryo is susceptible to foreign influences (see Figure 1) that can ultimately affect the well-being of resulting offspring.

The susceptibility of both oocytes and preimplantation embryos to perturbing influences raises several important issues. Firstly, do procedures associated with assisted reproductive technology influence the normality of long-term development? This question is particularly relevant in the human given the increasing demands on this technology together with the technical advances that are being made [e.g. intracytoplasmic sperm injection (ICSI), blastocyst transfer]. Secondly, can environmental pollutants (both physical and chemical) or drugs of abuse (e.g. alcohol, caffeine, nicotine etc.) reach levels in the body that are able to induce changes in oocytes and embryos in such a way that long-term consequences result? Studies in the USA on the incidence of congenital defects in humans between 1979 and 1987 indicated that, of 38 defects studied, 29 showed increasing trends in occurrence (Edmonds et al., 1990). The causes are not known but environmental factors may be implicated.
This review examines evidence that both chemical (e.g. culture media, pollutants etc.) and physical agents (e.g. embryo manipulation, exposure to radiation etc.) are able to influence the development of oocytes and preimplantation embryos in such a way that genotypic and/or phenotypic changes are induced in resultant offspring. The review is written in three sections: the first focuses on long-term effects of the exposure of oocytes and embryos to in-vitro conditions (i.e. those associated with assisted reproductive technology); the second examines environmental factors (e.g. chemical pollutants, radiation etc.) that might have similar long-term effects; the third addresses the question of drug intake and subsequent development.

Exposure of embryos and oocytes to chemical agents during assisted reproductive technology

In-vitro embryo technologies invariably involve the procedures of oocyte maturation, fertilization and embryo culture. During these procedures, oocytes and embryos are exposed to different media containing chemicals at concentrations that are not always found in the reproductive tract or, conversely, are not exposed to chemicals that are found in the tract (e.g. various growth factors).

**Figure 1.** Three stages in the maturation and development of the oocyte and preimplantation embryo that are susceptible to genetic and epigenetic modifications. (Panel 1) The oocyte is arrested at prophase of the first meiotic division (up to and including the germinal vesicle stage) for many years before final maturation and fertilization occurs. Both the arrested oocyte and the zygote (fertilized ovum) are susceptible to both physical and chemical agents that are able to induce aneuploidy, polyplody and chromosomal damage. (Panel 2) During early embryo development, epigenetic influences can be experienced that induce aberrant development in the fetus. This is particularly noticeable in some livestock species following in-vitro embryo culture. (Panel 3) It has been postulated (see text) that differentiation of the inner cell mass into ectoderm and endoderm is prone to environmental influence (e.g. embryo culture) with potential adverse effects on offspring.

Culture media

It has been recognized for almost 10 years that the transfer of cultured sheep and cattle embryos can result in the production of abnormal offspring (for recent reviews, see Leese et al., 1998; Young et al., 1998; Boerjan et al., 2000). Abnormalities include increased birth weight (Walker et al., 1992; Thompson et al., 1995), aberrant physiology (Garry et al., 1996) and abnormal organ and skeletal development (Farin and Farin, 1995; Schmidt et al., 1996; Walker et al., 1996; Maxfield et al., 1997; Sinclair et al., 1999). The birth of these offspring is associated with high perinatal mortality (Walker et al., 1992; Ranilla et al., 1998) and placental abnormalities (Hasler et al., 1995; Kruij and den Daas, 1997; Sinclair et al., 1999). Studies indicate that retarded development of the allantochorion might be implicated in fetal loss during late gestation (Peterson and McMillan, 1998a,b). Despite the large increases in birth weights, limited evidence suggests that the mature body weights of oversize lambs and calves do not differ significantly from those of control animals (Wilson et al., 1995; Brown and Radziewic, 1996).

On the other hand, the in-vitro culture of human embryos can be associated with lower birth weights although the decreases can be minimal (Beral and Doyle, 1990; Doyle et al., 1992; Tan et al., 1992; McFaul et al., 1993; Wang et al., 1994; D’Souza et al., 1997; Ménézo et al., 1999). D’Souza and colleagues (1997) reported a mean birth weight for singletons following IVF of 3.016 kg compared with 3.400 kg for control babies. This study also reported a significant reduction in the mean gestation length for multiple births (35.2 weeks versus 39.5 weeks) while more premature births of singletons following IVF have also been observed (Gissler et al., 1995; Tanbo et al., 1995). Although some studies have reported a higher incidence of birth abnormalities following IVF compared with natural pregnancies (Rizk et al., 1991; Silver et al., 1999; Ménézo et al., 2000), the physical and mental health of IVF offspring is not generally impaired (Morin et al., 1994; D’Souza et al., 1999). The birth of these offspring is compromised development in-vitro embryonic environment 565

**Panel 1 - Oocyte arrest and maturation**

- Primary oocyte
- GV stage
- Secondary oocyte
- Zygote (2PN)

**Panel 2 - Embryo development and hatching**

- 2 - Cell
- 8 - 16 Cell
- Blastocyst
- Hatching blastocyst

**Panel 3 - Hatched blastocyst and cell differentiation**

- Inner cell mass
- Ectoderm
- Endoderm
- Trophoderm

Long-term effects of in-vitro embryonic environment
Progesterone concentration

High peripheral progesterone concentration is implicated in enhanced fetal growth. Evidence implicating progesterone in the regulation of fetal development after embryo transfer comes from studies where animals were either supplemented with progesterone shortly after ovulation (Garrett et al., 1988; Kleemann et al., 1994) or where asynchrony was induced in embryo recipients (Wilmut and Sales, 1981). Both conditions induced fetal overgrowth following embryo transfer. There is the potential in assisted reproductive technology programmes for progesterone concentrations to play a more significant role in development than normal by virtue of there being multiple corpora lutea and because of the potential for asynchronous embryo transfer to occur.

Gonadotrophins

Animal studies indicate that high doses of gonadotrophin can induce higher than normal levels of aneuploidy (Hansmann et al., 1980; Hansmann and Jenderny, 1983; Mailhes et al., 1994). Mailhes et al. (1994) suggested that gonadotrophin treatment might increase the time needed for oocytes to complete meiosis. Such a change could predispose the oocyte to abnormal chromosome segregation (Eichenlaub-Ritter and Boll, 1989; Hansmann and Pabst, 1992; Mailhes et al., 1994). Several studies have reported abnormal fetal development in the mouse following gonadotrophin treatment (Ertzeid and Storeng, 1992; Ertzeid et al., 1993) including congenital defects (Sakai and Endo, 1987) and forelimb and central nervous system defects (Elbing, 1975). These abnormalities might not only result from aneuploidy but also from gonadotrophin-induced changes in the maternal environment. Aneuploidy in human oocytes obtained after gonadotrophin treatment is relatively high (e.g. Wramsby and Liedholm, 1984; Bongo et al., 1988; Djalali et al., 1988). However, this high incidence may be inherent and unrelated to the treatment protocol (Gras et al., 1992).

Anaesthetics

The development of mouse embryos both in vitro and in vivo can be adversely affected by exposure to various anaesthetics including nitrous/nitric oxide (Warren et al., 1990; Barrosa et al., 1998), propofol (Alsalihi et al., 1997; Tatone et al., 1998) and lidocaine (Del Valle and Orihuela, 1996). Chronic exposure to halothane can reduce fetal weight (Wharton et al., 1978). However, no studies have examined the link between acute exposure of oocytes/embryos and the normality of subsequent development.

Exposure of embryos and oocytes to physical agents during assisted reproductive technology

Physical agents include relevant parameters of the culture system (e.g. pH, osmolarity, oxygen tension) as well as various manipulation procedures associated with assisted reproductive technology (e.g. cryopreservation, embryo biopsy for genetic diagnosis and embryo splitting). Although the physical features of the culture system can have substantial influences on embryo growth and survival, there appears to be no evidence indicating that these factors can induce adverse long-term effects. However, it is possible that changes in pH are involved in the way in which the in-vitro environment is able to induce fetal overgrowth following embryo culture (e.g. effects due to ammonium accumulation). On the other hand, embryo manipulation procedures can cause damage of both a physical and chemical nature, the latter resulting from the composition of medium in which the manipulation procedures occur (e.g. cryopreservation medium).

Cryopreservation of embryos and oocytes

In the human, there is both a trend towards the freezing of blastocysts (Menezo and Veigi, 1997) and a requirement for the establishment of banks of frozen ova. The question of whether assisted reproductive technology can influence normality of development is never more relevant than as it applies to the use of frozen blastocysts and oocytes. This important topic is covered elsewhere in this symposium.

Embryo biopsy

Preimplantation genetic diagnosis in human embryos is a relatively new procedure, and, of the small number of babies so far born, there is no evidence of any adverse long-term effect (Ménézo et al., 2000). Interestingly, the freezing of biopsied human embryos is associated with substantially reduced viability (Magli et al., 1999) but there is no evidence of any adverse longer-term consequence.

Embryo splitting

Studies in mice indicate that the halving of 2-cell embryos has no lasting effect on body size or postnatal growth rate (Papaioannou et al., 1989; Biggers and Papaioannou, 1991). These studies and others (Lewis and Rossant, 1982; Rands, 1986) indicate that compensatory growth of the embryo occurs after embryo splitting. However, splitting of 2-cell embryos might be at the expense of a decreased proportion of inner cell mass (ICM) cells in the blastocyst (Rands, 1985) and subsequent development (for review, see Tarín and Handyside, 1993). There appears to be no evidence of abnormal development of offspring following the transfer of livestock embryos split at different stages of development (e.g. Ozil et al., 1982; Williams et al., 1984; Takeda et al., 1986; Kippax et al., 1991).

Manipulation of oocytes

The susceptibility of the oocyte to manipulation, other than freezing, is not well understood. One common form of oocyte manipulation is ICSI. ICSI is the subject of a future symposium article and it is sufficient to indicate that the rates of congenital abnormalities, organ malformations and chromosomal abnormalities are increased compared with spontaneous pregnancies (Ménézo et al., 2000). Another common form of manipulation in livestock ova is enucleation of the metaphase chromosomes in nuclear transfer programmes. The production of clones by the transfer of either somatic cells or blastomeres to these ova is associated with many of the problems associated with in-vitro culture of livestock embryos (Willadsen et al., 1991; Wilmut et al., 1997). It is not known if the underlying molecular mechanisms are similar. However, enucleation damages the cytoskeleton as well as removing mitochondria and other
organelles, whereas in-vitro culture induces mitochondrial degeneration (Dorland et al., 1994; Shamsuddin and Rodriguez-Martinez, 1994) and, presumably, adversely affects the function of other organelles.

Exposure of embryos and oocytes to environmental pollutants

Exposure to environmental pollutants is known to have many adverse influences on female reproduction (for review, see Sharara et al., 1998). However, much of the information has been collected under uncontrolled conditions and/or where individuals have been exposed to toxicants for prolonged or unspecified periods. Many pollutants are able to affect chromosome segregation, resulting in aneuploidy with the first meiotic division in female germ cells being particularly susceptible (for review, see Mailhes, 1995). It was generally believed that fetal defects associated with exposure to pollutants were inducible only when the conceptus was exposed during the period of organogenesis. Studies during the 1980s involving the exposure of subjects to various toxicants demonstrated the importance of exposure at specific stages of oocyte maturation, fertilization and embryo development (for review, see Rutledge et al., 1992). There are many different toxicants and, for the sake of brevity, they are divided into the following categories.

Oestrogenic substances

There is concern that exposure to environmental oestrogens might be having widespread adverse effects on the reproductive health of humans and wildlife. Relevant oestrogens include compounds such as polychlorinated biphenyls (PCB, once used widely in capacitors, transformers and lubricants), organochlorine pesticides (e.g. chlorophenothane (DDT)), phthalates (used in beverage containers, automobile parts and medical supplies), phyto-oestrogens and some industrial wastes as well as synthetic oestrogens (e.g. diethylstilboestrol). Specific studies indicate that prolonged exposure to some of these substances can lead to reduced fertility, abnormal sexual development, anovulation, reduced gestational age and low birth weights (Taylor et al., 1984; Heindel et al., 1989; Taylor et al., 1989; Colborn et al., 1993; Battershill, 1994; Davis et al., 1994; Cummings, 1997; Golden et al., 1998; Goldberg and Falcone, 1999).

Very few studies have examined long-term development following acute exposure of either oocytes or embryos to environmental oestrogens even though the transcripts for the oestrogen receptor are present in the preimplantation embryo, at least in the mouse (Hou and Gorski, 1993; Hou et al., 1996). The exposure of mouse embryos, including zygotes, to organochlorine pesticides significantly reduced rates of blastocyst formation in association with reduced cell numbers and increased rates of apoptosis (Kholkute et al., 1994a; Alm et al., 1996; Greenlee et al., 1999). The adverse effects on IVF rates of exposing mouse ova to PCB was shown not to be due to the oestrogenicity of the substance, thus implicating other inhibitory mechanisms (Kholkute et al., 1994a). Oestradiol may also exert indirect influences including a delay in the initiation of oocyte meiotic maturation (Racowsky, 1991, 1993a,b) which might predispose the oocyte to abnormal chromosome segregation. It has been suggested that oestrogens might induce this effect by disturbing various metabolic pathways (Masumbuko et al., 1992).

Heavy metals

Prolonged exposure of adults or prenatal exposure of fetuses to lead, mercury, cadmium, uranium and manganese have all been linked to adverse reproductive outcomes in several species (for reviews, see Andrews et al., 1994; Anntilla and Sallmen, 1995; Ratcliffe et al., 1996; Sharara et al., 1998). These outcomes include spontaneous abortion, miscarriage, fetal death and premature parturition. Brief in-vitro exposure of preimplantation mouse embryos to heavy metals is detrimental to subsequent development. Metals known to adversely affect in-vitro development include lead (Jacquet et al., 1976; Wide, 1978), lithium (Fernandez and Izquierdo, 1983), cadmium (Storeng and Jonsen, 1980; Watanabe and Endo, 1982; Yu et al., 1985; De et al., 1993) and nickel (Storeng and Jonsen, 1980). In a separate study, both essential (Cu, Mn, Fe and Zn) and non-essential metals (Cr, Hg, Pb, V, Al, Ag, Cd and As) were found to be embryotoxic at relatively low concentrations (Hanna et al., 1997). There is little evidence linking acute exposure of either oocytes or embryos to long-term effects in offspring although Wide (1983) reported a significant increase in the incidence of exencephaly following exposure of mouse blastocysts to lead.

Solvents

Solvents are used widely in industry and include substances such as perchloroethylene, toluene, xylene and styrene. Prolonged exposure to these substances can result in increased perinatal death, reduced fetal weight and infertility (Ungvary and Tatrai, 1980; Harkonen et al., 1984; Lindbohm et al., 1990; Donald et al., 1991). Exposure of mice to n-hexane for 4–6 weeks resulted in a significant reduction in the number of growing oocytes (Siracusa et al., 1992) and prolonged exposure of women in the leather industry was associated with congenital malformations and/or stillbirths (Clarke and Mason, 1985, 1986; McDonald and McDonald, 1986).

Studies that examined the effects of acute exposure of the preimplantation mouse embryo to the solvent methylnitrosourea (either in vitro or in vivo) indicated that exposure was related to an increase in the incidence of abnormal fetuses (cleft palate, exencephaly and malformed vertebrae) (Bosser and Iannaccone, 1985; Speilmann et al., 1989; Nagao et al., 1991). In-vitro exposure of blastocysts to this solvent resulted in a 3-fold increase in mortality rate during the first year of life (Iannaccone, 1984). The exposure of female rats to toluene vapour from 14 days before until 7 days after mating also increased fetal mortality (Ono et al., 1996).

Other industrial chemicals

Other relevant industrial chemicals include ethylene oxide (a sterilizing agent), 4-vinylcyclohexene (produced from the manufacture of synthetic rubber and insecticides), other PCB and dioxin-derivatives (produced from the bleaching of paper and the manufacture of some pesticides). Prolonged exposure to these chemicals can cause reproductive malfunctions in various species including oocyte degeneration in primordial and primary follicles (Smith et al., 1990; Hooser et al., 1991; Douds et al., 1992), prenatal death, infertility and fetal growth retardation (Colborn et

Ethylene oxide binds covalently to DNA and has been shown in several species to induce point mutations (for review, see Landrigan et al., 1984). Acute exposure is toxic to bovine embryos (Schiewe et al., 1985; Holyoak et al., 1996) and induces aneuploidy in mice in association with high rates of fetal abnormality and death following exposure near the time of fertilization (Generoso et al., 1987; Rutledge and Generoso, 1989; Katoh et al., 1989). Other mutagens (ethyl methanesulphonate, ethyl nitrosourea and triethylene melamine) are known to produce similar effects following exposure of zygotes (Generoso et al., 1988). Dioxins have been detected in human follicular fluid and the in-vitro exposure of mouse embryos to tetrachlorodibenzo-p-dioxin significantly influenced embryo development including an increase in the mean number of cells in blastocysts (Tsutsumi et al., 1998) and accelerated differentiation in the embryo (Blankenship et al., 1993). Exposure of mouse oocytes to PCB in vitro adversely affected fertilization rates and increased the incidence of degenerative ova and abnormal embryos (Kholkute et al., 1994b). Similarly, acute exposure of embryos of several species to PCB in vitro resulted in a reduction in cell proliferation and an increased rate of degeneration compared with control embryos (Lindenau and Fischer, 1996; Kuchenhoff et al., 1999).

**Pesticides, herbicides and insecticides**

Prolonged exposure to organochlorine pesticides (e.g. DDT, methoxychlor and chlordecone), organophosphate pesticides (parathion, malathion and diazinon), herbicides and insecticides is associated with reproductive abnormalities in several species (for review, see Sharara et al., 1998). These abnormalities are likely to result, at least in part, from the oestrogenic nature of some of these chemicals (see earlier). In the human, pollutants have been detected in follicular fluid (Trapp et al., 1984) and epidemiological studies indicate an association between occupational exposure and fetal death, abortion and congenital defects (Nurminen, 1995; Pastore et al., 1997; Arbuckle and Sever, 1998; Shaw et al., 1999). The organochlorines are known to adversely affect bovine oocyte maturation in vitro (Alm et al., 1998), embryo transport through the reproductive tract (Cummings and Perreault, 1990) and embryo development in association with reduced rates of implantation (Cummings and Gray, 1989; Sircar and Lahiri, 1989; Seiler et al., 1994; Hall et al., 1997). Furthermore, treatment of hamsters with the formamidine pesticide chloridimeform just prior to the preovulatory LH surge delayed the ultimate timing of the surge and impeded the time of ovulation (Goldman et al., 1993). Despite these effects, there appears to be nothing known of the relationship between acute exposure of embryos and oocytes to these chemicals and long-term development.

**Microtubule poisons**

The efficacy of some drugs used in medicine as well as some fungicides relies on the ability of the active chemical to inhibit spindle formation. Many spindle inhibitors have a trimethoxybenzene ring in their molecules and so trimethoxybenzoic compounds (and their analogues) may potentially induce non-disjunction in oocytes (Tateno et al., 1995). These authors found that the acute exposure of Chinese hamster oocytes to substances such as reserpine (an antihypertensive), podophyllotoxin (an antiviral) and vinblastine sulphate (an antineoplastic) resulted in morphologically abnormal oocytes, with podophyllotoxin and vinblastine sulphate inducing high levels of aneuploidy. High levels of aneuploidy have also been reported following the acute exposure of mice to vinblastine (Russo and Pacchierotti, 1988). Other drugs that are known to induce aneuploidy in association with various reproductive disorders include colchicine (Hummler and Hansmann, 1985; Tease and Fisher, 1986; Mailhes and Yuan, 1987; Mailhes et al., 1988, 1990), the fungicide carbendazim (Hummler and Hansmann, 1988; Perreault et al., 1992; Zuelke and Perreault, 1995; Jeffay et al., 1996; Can and Albertini, 1997), griseofulvin (Tiveron et al., 1992; Mailhes et al., 1993; Marchetti and Mailhes, 1995), benomyl (Mailhes and Aardema, 1992) and etoposide (Mailhes et al., 1994).

**Exposure of embryos and oocytes to physical agents**

Various forms of radiation including ionizing radiation (e.g. X-rays, ultrasound and low level electromagnetic fields including microwaves) can potentially affect the development of embryos and oocytes. In addition to their potential primary effects, both microwaves and ultrasound are able to induce local hyperthermia in subjects, and ionizing radiation has the specificity to damage DNA.

**Ultrasound**

Studies in animals and humans indicate that the diagnostic use of ultrasonography during pregnancy is without risk when used at recommended intensity levels and in the absence of any thermal effect (for reviews, see Brent et al., 1991; Jensch and Brent, 1999). An important question is whether the use of ultrasound for oocyte recovery in human IVF programmes exposes the oocyte to risk during the final stages of meiosis. In the study of Mahadevan et al. (1987), the exposure of human oocytes to ultrasound, either during meiosis or after its completion, did not significantly influence the developmental ability of resultant embryos. Furthermore, Heyner et al. (1990) found that the exposure of mice to diagnostic levels of ultrasound did not affect the number of embryos produced nor the ability of embryos to incorporate labelled precursors into DNA and RNA. However, directly exposing mouse blastocysts to ultrasound in vitro for 1 or 5 min increased the rates of resorption and stillborn (Iannaccone et al., 1991). Hande and Uma Devi (1992) also reported that the exposure of preimplantation mouse embryos to ultrasound increased the level of prenatal mortality although a comparable finding was not obtained in a subsequent study (Hande and Uma Devi, 1993).

**Electromagnetic fields (EMF)**

Electromagnetic radiation is widely encountered in daily living: it is being emitted from high voltage power lines, electrically heated apparatus, video display terminals, microwave ovens, cellular phones and other appliances. The effects of EMF on reproduction have been widely researched and thoroughly reviewed in recent
years (for reviews, see Chernoff et al., 1992; Robert, 1996; Brent, 1999; Robert, 1999). All reviewers reach a similar conclusion – that despite some contrary findings, there is no consistent evidence to indicate that EMF, of the nature commonly experienced, are able to significantly influence any stage of the reproductive process.

**Ionizing radiation**

Exposure to ionizing radiation may take the form of accidental exposure, low level exposure in diagnostic laboratories or exposure during radiation therapy. It has been known for many years that the exposure of preimplantation mouse embryos to relatively low doses of X-rays can lead to an increased incidence of malformed fetuses (Rugh and Grupp, 1959; Rugh et al., 1969). This teratogenic risk is both dose and strain dependent (Pampfer and Streffer, 1988; Jacquet et al., 1995; Streffer and Muller, 1996). Abnormalities include embryonic and fetal mortality, exencephaly, cleft palate, gastrochisis, anophthalmia and dwarfism (Jacquet et al., 1995; Streffer and Muller, 1996; Gu et al., 1997).

In the mouse, the zygote appears to be the embryo stage most sensitive to the effects of radiation (Russell and Montgomery, 1966; Jacquet et al., 1983; Gu et al., 1997). Studies of chromosomes (Pampfer and Streffer, 1989) and protein levels (Hillenbrandt and Streffer, 1994) indicate that the induced damage is mediated through genetic change. Abnormal fetuses can also result from exposing maturing mouse oocytes to X-rays (Oakberg, 1979; Kirk and Lyon, 1982; Russell and Russell, 1992; Muller and Schottten, 1995).

The extent to which such findings can be extrapolated to the human is not known, given the likely differences in response between species (Jacquet et al., 1995). Most of the above studies used radiation doses below those expected in diagnostic laboratories but comparable with those that can be experienced during radiation therapy.

**Exposure of embryos and oocytes to drugs of abuse**

The most commonly used drugs of abuse (alcohol, caffeine, nicotine, marijuana and cocaine) are able to modify signalling of neurotransmitter systems and intracellular messengers and consequently have the potential to induce reproductive abnormalities. Caffeine, cocaine and nicotine are central nervous system stimulants, whereas alcohol and marijuana are depressants (for review, see Smith and Asch, 1987). One major problem in assessing the adverse effects of the intake of any of these drugs is the inability to separate the effects of chronic intake from the effects due to short-term intake. A second problem, particularly in epidemiological and retrospective studies, is the likely concurrent use by individuals of several drugs.

**Alcohol**

Alcohol is a known teratogen that causes a variety of developmental abnormalities in the fetus following prolonged maternal consumption. Abnormalities include growth retardation, craniofacial anomalies and neurological disorders (collectively known as the fetal alcohol syndrome; Jones et al., 1973; Ouellette et al., 1977; Russell, 1977). In the studies of Kaufman (1983) and Kaufman and Bain (1984), mouse oocytes exposed in vivo to ethanol showed a high incidence of aneuploidy. These authors suggested that ethanol induced these effects by acting on the meiotic spindle, thus causing aberrant chromosome segregation. Subsequent in-vivo and in-vitro studies in several species indicate that acute ethanol exposure can adversely affect fertilization rates and embryo development (Pennington et al., 1984; Leach et al., 1993; Cebral et al., 1999), increase the incidence of cytoplasmic fragmentation and spontaneous activation of oocytes (Cebral et al., 1999) and decrease fetal survival (Washington et al., 1985; Wiebold and Becker, 1987). Some of these effects can be induced in rats with a single dose of ethanol administered in the mating period (Pennington et al., 1984). Such observations raise the question of what effect a single episode of alcohol ingestion in the human (e.g. in the peri-ovular period) has on the normality of embryo and fetal development. Furthermore, alcoholic women who abstained during pregnancy produced infants of lower birth weight than did non-alcoholic women (Little et al., 1980), indicating a possible ‘carry over’ effect of chronic alcohol exposure.

**Caffeine**

Numerous epidemiological studies in the human indicate an association between prolonged caffeine intake and intrauterine growth retardation, fetal loss and infertility (e.g. Martin and Bracken, 1987; Wilcox et al., 1988; Fortier et al., 1993; Infante-Rivard et al., 1993) as well as delays in conception (Hatch and Bracken, 1993). Studies show that caffeine can directly affect meiotic maturation in the hamster (Prather and Racowsky, 1992) and preimplantation development in laboratory animals (Vogel and Spielmann, 1987; Loupis et al., 1996). Evidence in the rat also indicates that maternal consumption, although not affecting preimplantation embryo development (Jacobs et al., 1999), can result in reduced growth rates in both the neonatal and prepubertal periods (Pollard et al., 1999). However, there appears to be no evidence that links acute exposure of either oocytes or preimplantation embryos to caffeine and the normality of subsequent development.

**Nicotine**

Prenatal nicotine exposure is known to reduce birth weights in humans (Lowe, 1959; Underwood et al., 1967) and to influence postnatal growth rate, development and sensory motor reflexes in laboratory animals (Daeninck et al., 1991; Ajarem and Ahmad, 1998). The in-vitro exposure of oocytes and embryos of these animals to high concentrations of nicotine (e.g. 1–5 mmol/l) delayed or inhibited subsequent development (Balling and Beier, 1985; Baldwin and Racowsky, 1987; Racowsky et al., 1989). Similar responses have not been obtained at concentrations normally found in the circulation of smokers (0.1–1.0 μmol/l; Langone et al., 1973). However, there is evidence that links cigarette smoking in humans with aberrant meiotic maturation in oocytes (Zenzes et al., 1995).

**Cocaine**

Epidemiological studies in the human indicate that prolonged cocaine use during pregnancy increases the risk of low birth
weight and other developmental abnormalities (Bingol et al., 1987; Chasoff et al., 1989; Little et al., 1989; Handler et al., 1991; Plessinger and Woods, 1998; Smeriglio and Wilcox, 1999). These conclusions are supported by similar observations in laboratory animals (Mackler et al., 1996). Cocaine is able to block in-vitro embryo development in the mouse, particularly during the early stages (Kaufman and Armani, 1992). Similar effects have been found in laboratory animals following in-vivo exposure (Atlas and Wallach, 1991; El-Bizri et al., 1991).

**Marijuana**

Regular use of marijuana is associated with an increased risk of low birth weight (Hatch and Bracken, 1986), anovulation in rhesus monkeys (Asch et al., 1981) and shorter menstrual cycles in women (Bauman, 1980). In-vitro studies with mouse embryos indicate that exposure to low levels of cannabinoids can result in abnormal development (Paria et al., 1995; Yang et al., 1996). Similar studies with blastocysts showed an accelerated differentiation of the trophectoderm compared with embryos not exposed to cannabinoids. However, as with other drugs of abuse, information is lacking on long-term effects of brief exposure of either oocytes or embryos.

**Are fetal oocytes sensitive to chemical and physical agents?**

Many studies, some of which are cited above, clearly indicate that organogenesis in the fetus can be disturbed when the fetus is exposed to either chemical or physical agents. An important and separate issue is whether fetal oocytes might also be influenced by these agents and whether there are long-term consequences in progeny that result from these oocytes.

It is now recognized that exposure of pregnant mothers (F₀) to a variety of agents is able to induce phenotypic changes not only in the first generation (F₁) but also in the second generation (F₂). Examples of second generation effects, transmitted through the female, include the ingestion of high levels of caffeine by rats (Pollard et al., 1987), the exposure of rats to ether vapour (Carney et al., 1999), the exposure of rats and hamsters to dioxin (Hurst et al., 1998; Wolf et al., 1999), the exposure of mice to dimethylbenzanthracene (DMBA), methyltritosurea (MNU) and ethyltritosurea (ENU) (Tomatis, 1979) and the exposure of mouse zygotes to X-rays (Pils et al., 1999). Undernutrition in the human can also induce second generation effects (Lumley, 1992) but there appears to be no evidence that diethylstilboestrol exposure has such an effect (Giusti et al., 1995; Mittendorf, 1995).

It is not known if these transgenerational effects are genetic or epigenetic in origin (John and Surani, 1999). However, the existence of such a phenomenon demonstrates the need to examine the F₂ generation when studying potential toxicants and their effects on the normality of development in the F₁ generation.

**What factors exist in in-vitro systems to induce aberrant development?**

As reviewed earlier, the in-vitro culture of embryos, particularly of livestock species, can result in marked phenotypic changes to offspring. Little is known at the cellular level on how these changes are induced.

**Clues from embryo culture studies**

The lumen of the oviduct provides a unique environment for fertilization and early cleavage, and the mimicking of its physical and chemical properties in vitro is difficult. It is therefore not surprising that the in-vitro culture of embryos of various species is associated with an array of developmental abnormalities including mitochondrial degeneration, cytoplasmic fragmentation, high lipid content in cytoplasm, disturbed cell lineage differentiation and abnormal metabolism and gene expression (for review, see Walker et al., 1998). However, none of these abnormalities have been definitively linked with the long-term effects described earlier. In livestock, enhanced fetal growth and associated problems are most common when (i) the culture period includes the time of genomic activation (Walker et al., 1992) and (ii) when the culture medium contains serum (Thompson et al., 1995). It is not known if the causative factor(s) are native to serum or are a product of serum decay or embryo metabolism. However, aberrant development can occur following the transfer of embryos cultured in medium without serum (McEvoy et al., 1999; Hartwich et al., 2000) thus implicating other chemical and/or physical agents. One factor that is implicated is ammonium ion accumulation in culture media, which, at relatively high concentrations, can cause fetal retardation and exencephaly in the mouse (Lane and Gardner, 1994) and increased fetal weight in the sheep (Sinclair et al., 1998; Hartwich et al., 2000).

**Possible underlying molecular mechanisms**

The molecular mechanisms underlying these epigenetic changes following embryo culture are not known. One essential requisite is that the changes induced during the culture period are manifested during fetal growth and development. One hypothesis is that the function of imprinted genes, several of which are associated with fetal growth, is perturbed during embryo culture (for review, see Young and Fairburn, 2000). As indicated by these authors, putative imprinting mechanisms such as DNA methylation can be transmitted through cycles of cell division such that changes induced in the embryo can influence fetal development. Furthermore, there is increasing evidence that epigenetic phenotypes can be maternally inherited (Wolff et al., 1998; Morgan et al., 1999). It is interesting to note that the administration of alcohol to pregnant mice in relative doses sufficient to induce the fetal alcohol syndrome in humans can induce hypomethylation of fetal DNA (Garro et al., 1991). It is possible that aberrant fetal development after in-vitro embryo culture and after acute alcohol exposure share similar cellular mechanisms.

It has also been postulated that fetal abnormalities following embryo culture might result from a disturbance in the time of expression of the Hox genes (Gellon and McGinnis, 1998). Expression, which is required for normal development, is dependent on the presence of embryonic ectoderm and endoderm (see Figure 1). Boerjan et al. (2000) postulated that the differentiation of ICM cells into ectoderm and endoderm is affected by in-vitro culture in such a way that fetal development is
compromised. It is known that cell lineage differentiation in the cultured blastocyst (i.e. before the formation of the endoderm and ectoderm) can be disturbed, resulting in a disproportionate decrease in the number of ICM cells compared with trophectoderm cells (Marquant-Le Guenne et al., 1989; Iwasaki et al., 1990; Erbach et al., 1994; Du et al., 1996). Subsequent impaired formation of the endoderm and ectoderm might be a continuation of the same process or be a consequence of it.

Clinical significance

**Adverse consequences of assisted reproductive technology**

The studies reviewed above indicate that some developmental abnormalities in the adult can have their origins in events that happen during the preimplantation period of development. This conclusion, which has also been reached by other authors (Seamark and Robinson, 1995; Young et al., 1998; Boerjan et al., 2000), is an extension of the hypothesis that the fetus develops in response to various environmental stimuli (Barker, 1993; Barker and Clark, 1997). Fortunately, the extremes in aberrant development that can occur following the culture of livestock embryos have not been observed in human assisted reproductive technology programmes. As indicated by Young et al. (1998), there are several inherent differences in embryo development between species (e.g. the timing of gene expression and differences in gene imprinting) which makes extrapolation between species difficult. The relatively small effects observed in the human might result from the fact that conventional IVF involves the transfer of very early stage embryos, thereby minimizing exposure of embryos to epigenetic influences at critically important times. Alternatively, the in-vitro environments used in human assisted reproductive technology might simply be free of such influences. However, several important points need to be reiterated from the livestock studies. Firstly, the physical abnormalities observed following the in-vitro procedures are not consistently obtained and the magnitude of each trait can vary considerably between individuals. There are probably biochemical and physiological changes that are not so easily detected. Secondly, the aberrations are most often associated with prolonged periods of culture (e.g. from the zygote to the blastocyst stage) whereas procedures such as in-vitro maturation and fertilization appear to have minimal influence. Thirdly, the abnormalities are most common when serum is used in the medium although enhanced fetal growth can result from the culture of embryos in serum-free systems, albeit at a reduced frequency. Given these observations, the trend in human assisted reproductive technology towards the use of serum-free systems is desirable but the trend towards the culture and transfer of blastocysts (Gardner and Lane, 1997; Jones et al., 1998) should proceed with caution. Human in-vitro technology has made substantial progress in the last two decades and this progress is likely to continue as advances are made in improving the quality of the in-vitro environment.

**Adverse consequences of exposure to environmental agents**

Generally, the exposure of oocytes and embryos in situ to pollutants and drugs is a poorly researched area even though such exposure is likely to have a serious impact on human reproduction. Studies reviewed indicate that brief in-vitro exposure of oocytes and/or embryos of laboratory species to agents such as lead, MNU, toluene vapour, alcohol, caffeine, ethylene oxide, microtubule poisons and ionizing radiation can all adversely affect the normality of fetal development. However, the significance of these results and their extrapolation to the human is difficult. The approach of using in-vitro embryo culture to assess the effects of exposure to any toxicant would only be meaningful if the concentration of the toxicant in the reproductive tract is known and where there is some knowledge of the pharmacokinetics of the substance. Given increases in global pollution together with the widespread use of drugs in society, it is important that a concerted effort is made towards the gathering of appropriate basic information. Until such information is available, the clinical assessment of problems arising from such exposure is likely to be very difficult.

**The dilemma of the recessive mutation**

The long-term consequences outlined above may result from either genetic or epigenetic changes to the genome. Exposure to pollutants is generally associated with an increased incidence of aneuploidy whereas assisted reproductive technology procedures are more likely to induce epigenetic changes, although these consequences are not mutually exclusive. Dominant mutations, with adverse effect, are usually evident in higher than normal rates of abortion, birth defects and death of offspring. However, it is also possible for recessive mutations to occur. The presence of these mutations will remain undetected until progeny that result from affected oocytes or embryos produce offspring from within their own population. Even then, these individuals will only be detected if homozygosity is associated with an identifiable phenotypic character. The probability of sufficient numbers of homozygous individuals being produced to facilitate detection in epidemiological studies is remote. Similarly, their clinical detection might also be difficult depending on the nature of the recessive mutation. The possible induction of recessive mutations, either through assisted reproductive technology procedures, drug abuse or exposure to environmental pollutants, is an ongoing dilemma.

**Conclusions**

It is concluded that the exposure of embryos to chemical influences during human assisted reproductive technology can have small but inconsistent effects on the development of offspring, despite the occurrence in livestock species of much larger adverse consequences. With the exception of cryopreservation (see elsewhere in this symposium) and ICSI, the physical manipulation of ova and embryos appears to be without any long-term adverse effect. This review also indicates an association between the exposure of oocytes and embryos to environmental pollutants and the normality of long-term development. Development can be adversely affected if exposure occurs at precise times during the periods of oocyte maturation, fertilization and early embryo development. However, extrapolation of these findings (obtained from laboratory animals) to the human is difficult. Less informa-
tion is available on exposure to drugs of abuse although there is sufficient evidence to indicate that intake can be associated with retarded embryo development. Whereas exposure can increase the incidence of aneuploidy it is not known if exposure can also elicit epigenetic change. Chronic intake of alcohol can lead to hypomethylation of fetal DNA, and it might be that methylation during preimplantation development is also affected. Is female fertility in the human jeopardized by exposure to physical and chemical agents in the environment? Information summarized in this review indicates that this is a possibility. Many studies are needed to validate the possibility that exposure, particularly short-term exposure, is having adverse long-term effects on offspring. The paradox is that couples that are infertile because of exposure to environmental factors might ultimately rely on assisted reproductive technology for the production of offspring. It is important that progress continues to be made on improving the quality of in-vitro environments used in this technology. It is equally important that the natural environment for fertilization and early development is free from adverse environmental influences.

References


Long-term effects of *in-vitro* embryonic environment


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