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MINI-REVIEW ***Developments in Reproductive Biology and Medicine***

Developmental plasticity and its relevance to assisted human reproduction

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ABSTRACT: The advent of assisted reproduction has allowed the conception of millions of individuals who otherwise would not have existed. Although most ART children are born healthy, there is increasing awareness of the plasticity of the human embryo causing concerns about potential long-term consequences of ART for the growth, development and health of this growing population of individuals. Evidence from studies in animals and humans suggest that physiology and metabolism may be permanently affected by ART. It suggests that ART children may be at increased risk of later cardiometabolic diseases. Part of this increased susceptibility to cardiometabolic diseases seems to be due to parental predisposition, while part of the increased susceptibility seems to be due to the ART procedure itself. Due to the fast development of new techniques in ART, it is unclear whether newer techniques are associated with similar risks. There is evidence to suggest that the newer techniques are safer, but the rapid developments in reproductive medicine and ever increasing indications for ART make it difficult to draw conclusions. Until more is known about the effectiveness and safety of ART for the broader indications in which ART is currently used, caution in using ART is mandatory.

Further progress could be made if long-term follow-up studies were included in the development of new ART techniques. Harmonization of measurements in human and animal studies of ART would allow faster scientific progress and less scientific waste. Also, including more details of the ART procedures in ART registries and allowing follow up of ART children through linking registries with already collected data from perinatal registries, child health clinics and schools would help to provide a better understanding of the growth, development and health of the growing population of ART children. Ultimately, these studies will provide the much needed information on how to provide ART children with the best possible start in life.

Key words: developmental plasticity / developmental origins of health and disease / assisted reproduction / early embryo environment / child follow up / long-term health

Introduction: the developmental origins of health and disease

The process of human reproduction is fascinating. It is the wonder of life. When oocyte and sperm fuse, a new individual comes into being. A new life begins. With the beginning of a new life, a developmental trajectory is initiated, which will ultimately determine how we become who we are.

Every human being has started life as a single fertilized oocyte. The newly formed genetic code provides the blueprint for the developmental trajectory of the new individual. The DNA, the epigenetic information on the DNA and signals from the direct environment, all work together to orchestrate the development of the single fertilized oocyte into a new individual. The phenotype of the embryo, i.e. its morphology, physiology and metabolism as well as its developmental potential, is shaped by the interplay between its genotype and its early environment. In responding to environmental signals, the embryo demonstrates a high degree of developmental plasticity by modulating its metabolism, gene expression and rate of cell division. The human embryo is exquisitely sensitive to cues from the environment, and its developmental plasticity has great implications for later health and wellbeing (Bateson *et al.*, 2004).

The importance of developmental plasticity for human health was highlighted by the work of David Barker who suggested that fetal adaptations to environmental cues might be the root cause of many chronic degenerative diseases (Barker, 1998). Environmental exposures during early embryo development may lead to adaptations in the anatomy, physiology and metabolism of various organ systems and thereby affect disease susceptibility. Depending on their timing, type and degree of environmental exposure, the developing embryo can be differentially affected and adapted, leading to metabolic and physiological alterations in the cells that subsequently form tissues and organs systems, and thereby influencing the developmental trajectory and the individual's susceptibility to disease. These adaptations are thought to increase short-term survival, as they reshape developmental trajectories, but may have adverse consequences for health later in life. Studies across the globe showing consistent associations of low birth weight with increased risks of cardiovascular disease have led to the hypothesis that adaptations to early environmental cues permanently affect disease susceptibility (Barker, 1998). The hypothesis is known as developmental origins of health and disease (DOHaD). Obviously, low birth weight is not the causal factor leading to diseases in later life, it is merely a crude indicator of fetal growth and gestational age. After the original discoveries, many studies have shown that programming can occur without affecting growth, and there are striking examples showing that environmental exposures during early development can have an impact on physiology and disease susceptibility without affecting size at birth. For instance, people exposed to the Dutch famine in early gestation had similar birth weights as those who had not been exposed to famine prenatally, yet their cardiovascular disease risk was doubled (Roseboom *et al.*, 2006). Similarly, studies of hundreds of babies from the earliest stages of gestation have shown that crown rump length in the first trimester is strongly correlated to later cardiometabolic health completely independently of size at birth (Jaddoe *et al.*, 2014).

Environmental stressors around conception, such as maternal infection, undernutrition or extreme stress have consistently been shown to be associated with increased risks of chronic degenerative diseases in the offspring. Studies of men and women whose mothers were exposed to the 1918 flu pandemic, for instance, showed increased cardiovascular mortality compared to offspring of mothers who had not been exposed (Mazumder *et al.*, 2010). Similarly, men and women whose mothers had been exposed to undernutrition during early gestation, in the Dutch famine or the Chinese Great Leap Forward famine, have consistently been shown to have increased rates of cardiovascular disease and type 2 diabetes as well as increased rates of schizophrenia and other mental health disorders (Susser and Lin, 1992; Roseboom *et al.*, 2006; Franzek *et al.*, 2008; Li *et al.*, 2010; van Abeelen *et al.*, 2012; Painter *et al.*, 2005). The effects of famine exposure are most severe when they occur in early gestation, suggesting that early gestation is an especially sensitive period for developmental programming. This makes biological sense since all organs and tissues are laid down in the first trimester and hence environmental exposures during this critical time window are likely to have the biggest impact on development and health. In fact, men who were exposed to undernutrition during the first trimester of pregnancy had smaller brains, reduced cognitive function and reduced participation on the labor market (de Rooij *et al.*, 2010, 2016; Scholte *et al.*, 2015). This shows that environmental exposures can leave lasting marks on the structure and function of organs, which can be detected decades after the initial exposure, and that these functional effects translate into societally relevant consequences. All of these findings from observational studies in humans point towards developmental plasticity and the lasting effects of early environmental cues on later development and health, however, it has been the animal experiments that have established causality.

Experimental studies in rodents and sheep have shown that environmental cues around the time of conception have permanent effects on the offspring's phenotype (Fleming *et al.*, 2011). Reduced protein intake during the first 3 days after fertilization (and thus even before implantation) retards cell proliferation and skews the balance of cell lineage differentiation in the blastocyst (Sun *et al.*, 2014). The effect of nutritional disturbance at conception persists through the implantation stage, and is able to influence placental development and nutrient transfer capacity (Fowden *et al.*, 2008), induce hypertension (Kwong *et al.*, 2000), alter the stress response (Gardner *et al.*, 2004; Gopalakrishnan *et al.*, 2004), increase anxiety levels (Sun *et al.*, 2014) and impair glucose tolerance (Oliver *et al.*, 2005).

This suggests that the early embryo is sensitive to its environment and raise the question of how the early environmental exposures in ART affect growth and development of the growing population of individuals conceived through ART.

Is DOHaD relevant to ART?

A few years before David Barker's initial discoveries in the UK, Steptoe and Edwards had realized their break-through in human reproduction, with the birth of the first baby conceived through IVF. With the reproductive revolution, the use of reproductive technologies increased, and new procedures were developed, involving increased embryo manipulation such as mechanical insertion of the sperm into the oocyte in ICSI procedures, removal of a blastomere in preimplantation genetic testing, and freezing and thawing of embryos, to name a few. Assisted reproductive technologies were initially developed to help women with tubal disease conceive, but have since been applied to an expanding range of other indications, resulting in an exponential growth in the numbers of ART children (Kamphuis *et al.*, 2014). Currently, an estimated 1 million ART children per year are expected to be conceived and born. From the perspective of developmental plasticity, it seems plausible that assisted reproduction techniques could influence the early embryo and thereby permanently influence the development and health of individuals conceived through these techniques. With the increasing number of ART children, the potential consequences of developmental plasticity in relation to ART also become relevant to public health.

Initially, studies in ART logically focused on pregnancy rates and live-birth rates. Subsequent studies have focused on congenital anomalies with much larger numbers of observations needed for sufficient power. Hence these studies were performed when many ART individuals had been born and the techniques were already well established in clinical practice. In the last decade, studies of ART have included other measures of growth, development and health. These studies indeed provide evidence that ART may have lasting effects. Children conceived through assisted reproduction techniques are more often born prematurely and small for gestational age (Cavoretto *et al.*, 2017), both of which are risk factors for poorer health outcomes in later life (Barker, 1998). These risks were initially thought to be due to

multiple pregnancies, but in fact also singletons conceived through ART are at increased risk of being born prematurely and small for gestational age. Apart from this increased perinatal risk, ART children also have an increased risk of congenital abnormalities (Davies *et al.*, 2012). Also, there is also evidence that ART may have long-term consequences for later health (Hart and Norman, 2013a, 2013b). The health risks associated with ART are relatively small, and the majority by far of IVF children are born healthy. But with the increasing numbers of children conceived through ART, also more subtle effects of ART on health are becoming detectable, and these may provide important clues to further improve clinical care and counseling.

ART is not associated with increased rates of childhood diseases, hospitalization, use of medical care or quality of life (Hart and Norman, 2013a). Nor is ART associated with problems associated with behavior, cognition, hearing or vision (Hart and Norman, 2013b). The available data to date suggest that despite initial reports of a potential increased risk of cancer among ART children (Moll *et al.*, 2003), there seems to be no increased risk of cancer among ART children (Hart and Norman, 2013a). However, adequately powered studies are still needed to provide more definitive evidence. The evidence of a potential link between ART and asthma and allergic diseases is inconsistent, with some studies reporting higher rates of asthma among ART children (Ericson *et al.*, 2002; Kuiper *et al.*, 2015), while other studies report lower rates (Ludwig *et al.* 2009b) and some report no association (Cetinkaya *et al.*, 2009). There is also limited evidence of increased neurodevelopmental delay, cerebral palsy or prevalence of depression in ART children (Hart and Norman, 2013b). Furthermore, there is an increasing body of evidence to suggest that ART may have consequences for cardiovascular and metabolic risk factors.

A recent systematic review of the literature analyzed data from 19 cohort studies, including over 2000 ART children and 4000 naturally conceived controls (Guo *et al.*, 2017). The analyses consistently showed small yet statistically significant differences in blood pressure, cardiovascular function, adiposity and metabolism between ART and naturally conceived children, with ART children having poorer outcomes than naturally conceived ones. ART children were more centrally obese, and had reduced glucose tolerance and higher blood pressures (Guo *et al.*, 2017). Although the absolute differences in blood pressure were relatively small, these differences are clinically important, since childhood blood pressure tracks into later life (Chen and Wang, 2008). Using non-invasive vascular imaging techniques and functional tests, recent studies have shown that ART children have endothelial dysfunction and stiffer blood vessels (Scherrer *et al.*, 2012). Carotid-femoral pulse wave velocity, a proxy of arterial stiffness, and carotid intima-media thickness were higher in ART children. These subclinical vascular alterations may predispose the individual to developing premature atherosclerosis and could in the future lead to cardiovascular morbidity and mortality (Urbina *et al.*, 2009). Furthermore, the higher prevalence of subclinical hypothyroidism among ART children (Sakka *et al.*, 2009) may further contribute to their predisposition for cardiometabolic disease as subclinical hypothyroidism is associated with metabolic syndrome (Uzunlulu *et al.*, 2007).

Which factors could play a role?

Assisted reproduction techniques can involve manipulation of several steps in the process of reproduction, including down regulation of pituitary function, hormone stimulation to induce maturation of several follicles and superovulation, *in-vitro* culture of oocyte and embryos, washing of sperm, mechanical manipulation of gametes and embryos and cryopreservation of the embryo before its transfer into the uterus. All of these procedures have the potential to affect embryo development and hence future health.

There is limited understanding of the causes of adverse ART outcomes. It is currently unclear whether the identified risks are attributable to assisted reproduction techniques or to intrinsic parental characteristics affecting gamete quality and offspring health. Parental characteristics related to infertility are indeed likely to contribute to the risk of adverse outcomes, including preterm birth and birth defects (Raatikainen *et al.*, 2012). However, the treatment-related factors, particularly ovarian stimulation and the mode of fertilization, are also likely to play a role.

Patient characteristics

The different characteristics of couples undergoing ART and couples conceiving spontaneously could account for the poorer perinatal outcomes and health of their children. The older age of couples undergoing ART is likely to contribute to the higher rates of prematurity and low birth weight among ART offspring. Also, the metabolic features associated with polycystic ovary syndrome could contribute to poorer cardiometabolic health among ART children. Indeed, several studies have shown that the poorer outcomes of ART children are at least partly due to parental characteristics associated with infertility (Schendelaar *et al.*, 2014; Seggers *et al.*, 2014; Pontesilli *et al.*, 2015). For instance, time to conception, as a measure of the degree of subfertility, has been shown to be linked with the offspring's blood pressure (Seggers *et al.*, 2014) and poorer neurological outcomes at the age of four (Schendelaar *et al.*, 2014). But when comparing ART children with spontaneously conceived children from subfertile couples, there are still differences in outcomes between ART children and spontaneously conceived children, suggesting that the ART procedure itself also plays a role (Ceelen *et al.*, 2008; Seggers *et al.*, 2014).

Hormone stimulation

The hormone stimulation used in ART to induce maturation of multiple follicles has been shown to induce epigenetic changes in the oocyte (Fortier *et al.*, 2014), which might subsequently affect offspring health. Interestingly, IVF children from a manipulated natural cycle had higher blood pressures than children from IVF cycles with ovarian stimulation (Seggers *et al.*, 2014). Similarly, children whose mothers developed ovarian hyperstimulation syndrome (OHSS) had reduced systolic and diastolic function in their common carotid arteries compared to children whose mothers underwent IVF without developing OHSS (Xu *et al.*, 2014).

Laboratory procedures

The manipulation of gametes and embryos in the IVF laboratory could influence their physiology and thereby affect later health in the offspring. Various factors, ranging from the retrieval of the gametes, to the culture conditions, and to cryopreservation could all impact the developmental phenotype and hence the susceptibility to later disease.

Culture media

Culture media have changed considerably since the introduction of IVF. These changes have been associated with improvements in fertilization rates and clinical pregnancy rates. The early years of human IVF were characterized by the presence of serum in culture media, with patient's serum being the most commonly used. In natural conceptions, however, the embryo is never exposed to serum but to the fluids of the female reproductive tract. When research in animal models showed that serum induced changes in morphology and metabolism, the use of serum in human IVF was abandoned. Most studies of culture media have failed to report outcomes beyond pregnancy rates. There are, however, indications that the compositions of culture media affect embryo growth and fetal growth and result in differences in birth weight (Dumoulin *et al.*, 2010; Kleijkers *et al.*, 2014). The differences in growth can even be detected postnatally, at ages 2 and 4 (Kleijkers *et al.*, 2014). Not only is there evidence that the composition of the culture medium influences growth, it also affects development. A study in France showed that in a randomized design, the composition of the culture medium used in ART influenced development of children, leading to more or fewer developmental problems at the age of 5 years (Bouillon *et al.*, 2016). Together with evidence from animal experiments, these data suggest that the composition of the culture medium may have a lasting effect on growth and development (Grace and Sinclair, 2009). It is currently unclear what the exact composition of most commercially available culture media is, and which media can provide the best environment for the developing embryo.

Oxygen tension

Levels of oxygen tension vary dramatically between IVF laboratories around the world. Atmospheric oxygen of around 21% is considerably higher than the level of oxygen to which the human embryo is exposed to *in vivo*. Animal studies have consistently shown that embryo development is significantly decreased when embryos are cultured under atmospheric oxygen tension. Human embryos develop more slowly under atmospheric oxygen tension compared to 5% oxygen, resulting in a lower percentage of embryos developing into blastocysts (Catt and Henman, 2000). Both implantation and pregnancy rates are reduced after embryo culture in atmospheric oxygen, resulting in lower live-birth rates. A systematic review of the literature has demonstrated that low oxygen concentrations during human embryo culture are associated with increased healthy live-birth rates (Bontekoe *et al.*, 2012). Whether oxygen tension during embryo culture has lasting effects on development and health after birth is currently unknown.

Cryopreservation

Children born following the transfer of vitrified embryos are heavier at birth than children born after fresh embryo transfer or slow frozen embryos (Wennerholm *et al.*, 2013). This might be due to the freezing itself or to the absence of a direct effect of hormone stimulation on the endometrium which is 'washed away' in frozen–thawed embryo transfer. The effect of cryopreservation on other perinatal outcomes and later health has not been evaluated in detail yet, with the most recent meta-analysis reporting that only one study had included congenital abnormalities as an end-point. No outcomes beyond live-birth rates have been reported.

Other laboratory factors that could influence development and health

Various other factors in the IVF laboratory could affect the development of the embryo. Initially, human embryos were cultured in groups, while currently, many IVF clinics culture embryos individually using microwell dishes. Whether or not this influences embryo development is currently unclear. Since the introduction of time-lapse incubators, the number of times that doors of incubators are opened has been reduced, leading to less variations in temperature and less light exposure. There are some indications that this could lead to better quality embryos and higher rates of implantation and lower rates of early pregnancy loss (Gardner and Kelley, 2017). Again, whether this affects development and health is currently unknown.

Is there a causal association?

Since most follow up studies of ART children are observational in nature, causality cannot be assessed. Apart from the confounding factors of age, subfertility, medical history and socio-economic status, bias may also be introduced because ART couples might be more likely to seek medical help for their children. Attrition is also associated with selection bias, and long-term follow-up studies are especially hampered by attrition bias. A further complicating factor is that variations in lifestyle contribute to differences in cardiovascular and metabolic health of children. This adds to the noise in follow-up studies and could introduce bias if the lifestyles of ART parents and children differ from that of those who conceive spontaneously. Another interesting confounder could be that even blinded researchers are able to successfully identify the mode of conception in the majority of participants without having access to their medical data, suggesting that unintentional bias cannot be completely prevented (Ludwig *et al.*, 2009).

Experiments in animals consistently show that effects are causal and that manipulating the environment of the embryo periconceptionally has significant long-term consequences for growth and metabolism and physiology of the offspring. Animal data demonstrate that even modest variations in individual component of embryo manipulation can have profound effects on gene methylation and expression, pre- and postnatal growth, physiology and metabolism. In mice, IVF alters later development and growth, which leads to metabolic and behavioral and physiological changes. Mice conceived through IVF have increased fasting glucose levels, impaired glucose tolerance and altered insulin signaling, compared to naturally conceived controls (Chen *et al.*, 2014) as well as vascular stiffness, higher blood pressure and endothelial dysfunction (Rexhaj *et al.*, 2013). Moreover, ART in mice has consequences on the life span; when challenged with a Western style high-fat diet, the life span of ART mice was shortened by roughly 25% compared with control mice (Rexhaj *et al.*, 2013).

Although the techniques used in ART in animal husbandry differ from those used in human assisted reproduction, there is clear evidence that ART in sheep, cows and horses affects development and health, and manipulations of the early embryo environment have successfully been used to optimize offspring health in animal husbandry (van Wagtendonk-de Leeuw *et al.*, 2008).

Animal models are advantageous not only for developing and improving ART techniques, but also for investigating the long-term consequences of these techniques. Importantly, animal studies have

several advantages, including the ability to remove subfertility as a potential confounder, assess causality and investigating underlying mechanisms, while they significantly shortening the time needed to assess lifelong health consequences. Although one obviously needs to bear in mind that extrapolation from mice to men cannot be made without any restrictions, closer links between animal and human researchers and harmonization of follow-up protocols in studies of animal and human ART would be helpful to accelerate scientific progress and reduce research waste.

Is it getting better?

The evolution of assisted reproduction technologies over the past decades has been very rapid. Often, new technologies have been introduced into the clinic without evidence on the long-term consequences of the technique. At the same time, the indications for ART have broadened. As a consequence, the long-term safety of new techniques can only be assessed when the 'new' technique is already outdated, and more modern and potentially less harmful technologies are available. Because of the a lack of reliable contemporary evidence on safety and effectiveness, it is virtually impossible to inform ART policy and practice internationally, based on long-term evidence.

There are, however, indications that ART has indeed become safer. Not only are there indications that the numbers of multiple pregnancies have gone down with the adoption of the single embryo transfer policy, but also, the rates of prematurity are declining. Furthermore, in the most recent systematic review of the long-term consequences of ART, the increased cardiometabolic risks were mostly found in the older studies, whereas the studies of ART children born after 2000 suggest no difference in blood pressure between ART and naturally conceived controls (Guo *et al.*, 2017). This might suggest that ART has indeed become safer. However, it might also be that the effects are only observed with increasing age. The widening of the indications for ART, and hence that change in case mix, might also contribute to the seemingly better outcomes of more recent ART treatments.

Discussion: how to proceed?

The biological phenomenon of developmental plasticity is relevant to reproductive medicine. The early embryo is sensitive to its environment and the many techniques applied in reproductive medicine affect the early embryo environment and thereby their development, growth and health potential. It seems clear that the techniques applied in ART do actually affect the growth, development and health of offprin, but it is unclear to what extent each of the techniques does so, what the effects are on short and long-term health and what the optimal treatment would be.

Since longitudinal studies are expensive, extend well beyond the usual funding period, and are complicated due to selectively follow up, the evidence we currently have on the health of the millions of ART children is very limited. We could make faster progress in several ways. First, registries of ART should include information on the details of the patient characteristics, the hormone stimulation and the laboratory procedures used, and they should be linked to already available data from perinatal registries for neonatal outcomes, child health clinics for growth and development, school administration for school performance, and medical data for medication use, illnesses and hospitalizations. Second, animal models should be expanded to not only develop new ART techniques, but should include standardized follow up of the animals to obtain information of the potential long-term consequences of the new technique for growth, development and health. A closer link between experimental studies in animals and human reproductive medicine would be very helpful as experiments in mice and rats would allow us to get a glimpse of potential effects of new ART techniques on long-term health without having to wait for decades. Performing the same standardized test battery for ART follow up in animal experiments and in follow up of ART children would enable translation of findings from animal experiments to the human situation. Also, animal experiments could provide important information on underlying mechanisms and provide clues as to potential prevention of adverse consequences of ART-induced pathologies. Finally, biomarkers should be identified which should be measured both in animal studies as well as in human studies of ART, so that the translation from experimental studies in animals to human studies is facilitated further.

With ART techniques firmly established in clinical care and the fast development and refinement of the techniques, it seems impossible to evaluate their long-term safety. One of the big challenges is that many new techniques are rapidly introduced in clinical care while the long-term consequences of these techniques cannot be observed until decades later. Concerns have been expressed about overusing ART (Kamphuis *et al.*, 2014), as the indications for ART continue to be broadened, without solid evidence of ART being effective and safe. Knowing the relevance of developmental plasticity for human health, however, we cannot continue providing fertility care without appropriate follow-up registries. Using the clinical information from the many ART treatments that are being performed every day around the globe, we could learn more about how to optimize the early embryo environment and maximize safety of future ART treatments. Medical interventions administered within a sensitive window of development simply warrant specific investigations of future development, growth and health. Caution in the use of ART is mandatory, as we need to make sure we do no harm. In order for those considering the use of ART to make well informed decisions, information about the potential consequences of ART for their own health and that of their potential child is needed.

Conclusion

Innovation is a fundamental component of improving health care, just as the early embryo environment is of fundamental importance for later development and health. Therefore, ART registries should not only register the number of ART cycles performed and their success rates, but they should also register the specific laboratory techniques used to achieve pregnancy and link this information to data on growth, development and health of ART children (Kissin *et al.*, 2014). This will allow the scientific field of reproductive medicine to move forward and obtain the knowledge needed to provide ART children of the future the best possible start in life.

Author's role

T.R. conceived and wrote the article.

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Conflict of interest

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