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The periconceptional period and assisted reproduction technologies: a review of embryonic sex-specific adaptability and vulnerability

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Summary. In just over 40 years, more than 10 million people worldwide have been born with assisted reproductive technologies (ART); today in Europe they represent 3% of births, while reaching around 7% in some Countries. Despite the rapidly increasing of its use in infertility – and even beyond – and the progressive introduction of new technologies and procedures, the effectiveness/safety ratio remains far from that of natural procreation as observed in communities with a good health protection.

In addition to a low number of births compared to that of the procedures initiated, there are many concerns about the increased risk of pregnancy and neonatal diseases and above all about the risk of non-transmissible diseases – such as cardiovascular, metabolic, neurologic, immunologic, etc. – that can occur during lifetime and can also be inherited. Moreover, a different sex ratio at birth in relation to the different procedures and technologies has been observed, as well as the fact that the risk of diseases upon birth and during life is often different depending on sex.

To overcome the gap between the outcomes of the natural and the assisted reproduction, more solid scientific data is needed on gamete maturation and early embryo sex-specific development, adaptability and vulnerability, both in the natural tubal environment – able to respond continuously to the changes in the embryonic needs led by the mother-embryo cross-talk – and in the static, *in vitro* environment, to which the effects of the various procedures and manipulations should be added.

Many studies have been recently proposed; given the high sex dimorphism of the initial embryo, sex difference should be considered in the study planning as a systematic objective of research.

Keywords. Assisted reproductive technologies, embryo adaptability, embryo vulnerability, effectiveness/safety ratio, sex dimorphism.

Introduction

Since 1978, when the first baby was born after *in vitro* fertilization, we assisted to a worldwide rapid and progressive increase of babies born with assisted reproductive technologies (ART), with a progressive tendency to go beyond infertility therapy, towards societal demands for reproduction.[1] In the past few years, ART contributed in the various Countries to 1-3% of all births; in Europe the percentage in 2015 was 3%, with 6.6% in Denmark, 7.1% in Spain and 2.3% in Italy.[2] ART people today could be more than 10 million at global level.

Despite the rapidly increasing development and use of ART and the addition of new technologies and procedures, the successful outcomes of pregnancy – or better yet of live births ('baby in arms' or 'take-home-baby') have not increased, thus remaining low (around 30% of total procedures) and variable. Moreover, comparing the pregnancy outcomes after ART and those after spontaneous conception, we can observe a very high incidence of twins and monozygotic twins and an increased risk of adverse mother and fetal outcomes; there is also a growing concern about a higher risk of lifelong physical or psychic diseases in the offspring. In addition, when these events were evaluated, it was seen that they often occur in a sex-dimorphic way, and the more evident and challenging factor appears to be a different sex ratio at birth in relation to the different ART technologies.

The effectiveness/safety ratio and the balance between natural selection and plasticity[3] seem therefore distant from the natural ones we see in Countries characterized by good healthcare and, although many studies have been conducted, much more scientific evidence is needed to try real ART improvements, not least to provide couples with more complete and accurate information than the current one, so that they can choose how to achieve their parenthood in a truly free way.

The objective of this study is to review the current research data about the stress effects of the ART environment and procedures in the periconceptional period and the modes and limits of early embryo adaptability and vulnerability in the different situations and conditions when (and not least!) it is a female or a male.

ART pregnancy outcomes

After 40 years of ART, periodical national surveys and scientific reports show that the number of live births vs the number of cycle treatments appears very variable from one Country to another – and within the same Country from a fertility clinic to another – but nevertheless quite low. In a systematic review of all national and regional ART registries published worldwide from 2004 to 2013,

live births were 20.5% (range from 12.55% to 29.46% in the different world regions) of all embryo transfer cycles.[4] In the United States ART surveillance 2016, out of 197,706 ART procedure performed, 78.1% progressed to embryo transfer, and of these ones 52.4% resulted in pregnancy, with only 42% in live birth deliveries; live birth deliveries were therefore 33.4% of all procedures, and pregnancy loss was 18.5%,[5] higher than in the general population (overall 13.5%), but significantly related with an increase in maternal age in both cases.[6]

There is a very high rate of twins: 30.4%, versus 3.3% of all infants, 1.1% triplets and higher order multiples versus 0.1% of all infants;[4] moreover, among ART twins there is a two-fold risk of monozygotic and monochorionic twins also after only one embryo transfer,[7] mostly in blastocyst transfer.[8] All twin pregnancies are associated with higher risk to both the mother and the babies, but ART twin have even higher rates of preterm birth (PB), low birth weight (LBW), neonatal resuscitation,[6,9] even higher if they are monozygotic.[10]

A higher risk of birth defects has been also reported. In many systematic reviews and metanalysis, compared with spontaneous conceptions ART pregnancies were associated with a significantly higher risk of congenital malformations[11-13] and multiple blastogenetic defects[14], although with significant differences across Countries and across the types of assisted conception.[15]

In addition, many studies reported an association between ART and an increased incidence of normally rare imprinting disorders, such as Angelman syndrome, Beckwith-Wiedemann syndrome, Prader-Villi syndrome, Silver-Russel syndrome[16,17] and other pregnancy adverse outcomes, as those related to methylation disorders in the placental imprinted loci.[18,19]

Regarding the association between fertility treatments and cancer in ART children, many recent reviews and meta-analyses noted no – or a slight, but not significant – risk of increase for all cancers;[20-22] the risk was further increased, but was not significant, from age 18 onwards,[23] but not enough time has elapsed yet to have data on older ages; a systematic review and a meta-analysis have instead noted – although in different ways by the authors – an increased risk for specific cancers, many of which could originate in-utero as neuroblastoma, retinoblastoma, leukemia, hepatoblastoma, bone tumors, sarcomas;[22,24] among children born in Denmark, frozen embryo transfer compared to children born to fertile woman was associated with a small but significantly increased risk of childhood cancer.[21]

A higher risk of pregnancy-related complications has also been observed. In a meta-analysis of 161,370 ART and 2,280,241 spontaneously conceived singleton pregnancies, ARTs are associated with pregnancy-induced hypertension (RR 1.30), gestational diabetes mellitus (RR 1.31), placenta previa (RR 3.71), placental abruption (RR 1.83), antepartum (RR 2.11) and postpartum (RR 1.29) hemorrhage, polyhydramnios (RR 1.74) and oligohydramnios (RR 2.14).[25] The International Federation of Gynecology and Obstetrics places ART among the risk factors for pre-eclampsia.[26]

At the same time, the number of adverse perinatal outcomes is higher than in naturally conceived pregnancies. Infant born after ART in 2016 in USA were 1.8%, but contributed to 5% of all LBW infants and to 5.3% of all infants born preterm,[5] and the pooled estimated risk in singleton births was 10.9% for PB, 2.4% for very PB, 8.7% for LBW, 2.0% for very LBW, 7.1% for small for gestational age (SGA), 1.1% for perinatal mortality.[27] In addition, a four-fold increase has been estimated in the risk of stillbirth in women who conceived with *in vitro* fertilization (IVF) versus fertile women,[28] an increased risk in the offspring both for respiratory distress and infections requiring hospitalization in the first week and for delayed achievement of developmental milestones at nine months.[29] On the other hand, the transfer in utero of frozen embryos at the blastocyst stage is related to large for gestational age (LGA) and high birth weight (HBW) offspring.[30]

About the later life outcomes, considering the increased risk of not transmissible – sometimes inheritable – diseases suggested in the Developmental Origin Health and Diseases hypothesis (DOHaD),[31] ART children appear generally healthy, and ART people are still too young to manifest many non-transmissible diseases, and even more so to show their possible inheritability. In the DOHaD hypothesis, PB and LBW – more frequent in ART infants than in naturally conceived ones – are associated with the risk of cardiovascular, metabolic, immunologic, neurological morbidities in adulthood in a sex-specific way.[32,33] In ART children, moreover, subclinical changes have been observed. In a systematic review and meta-analysis, the blood pressure levels of IVF and intra cytoplasmic sperm injection (ICSI) offspring from childhood to young adulthood were significantly higher than those of the naturally conceived; cardiac diastolic function was suboptimal, vessel thickness higher, as higher were the fasting insulin levels, indicating a risk of cardiovascular diseases.[34] In another comprehensive review, ART offspring were associated with higher systemic blood pressure, diastolic dysfunction and an increase in systolic pulmonary artery pressure, as well as alterations associated with vascular ageing, such as a reduction in the arterial flow-mediated dilation, increased arterial stiffness and carotid intima-media thickness, also observed in ART singleton born at term with no signs of perinatal complication; moreover, LBW ART children and adolescents exhibit sex-specific increased peripheral adipose tissue mass, subclinical hypothyroidism, higher fasting glucose and higher triglyceride levels, which are highest in children from frozen embryos.[35] ART-induced prema-

ture vascular aging persists in apparently healthy adolescents and young adults, without any other detectable cardiovascular risk factor, and could progress to arterial hypertension.[36] Fetal echocardiographic studies show that ARTs are associated with in-utero cardiovascular remodeling and dysfunction (more globular heart, thicker myocardial walls, decreased longitudinal function, dilated atria, etc.) that persist after birth at 6 months[37] and 3 years,[38] also in tweens.[39]

Regarding neurodevelopmental disorders, there are fairly homogeneous reports on the increase in cerebral palsy in ART children,[40] mostly mediated by preterm and multiple births.[41] Neurodevelopment and psycho-social health in ART children, compared with natural conceived ones, did not reveal any significant differences – or only small ones – in school performance score,[42] vision and hearing,[43] cognitive, motor, language development[44] and autism spectrum disorders.[45] In the UK Millennium Cohort Study, at age 3 ART offspring showed a higher incidence of psychosocial problems than natural conceived children, but the differences decreased with age, and were negligible at 14.[46] In a retrospective population-based cohort study, including live births, conducted in Western Australia from 1994 to 2002, with at least 8 years of follow-up, there was a small increase in the risk of intellectual disability in ART children, more than two-fold for those born very preterm and after ICSI: the highest risks were seen for ICSI conceived girls.[47] A low, but significantly increased risk of autism spectrum disorders, hyperkinetic disorders, emotional or social disorders and tic disorders was observed in children conceived after ovulation induction.[48] In a retrospective Finnish population-based register study on live births between 1990 and 2013, ART subjects, compared with non-ART, presented an increased likelihood of received – and receiving earlier – a psychiatric diagnosis until young adulthood; the outcomes were similar in boys and girls.[49]

Sex ratio in ART newborns

In studies about ART pregnancy outcomes, sex differences were evaluated only occasionally, and when they were considered, it was almost often among matching factors,[50] and not among the research goals; a number of studies evaluated instead the male-female ratio at birth, or secondary sex ratio (SSR). SSR is the result of a complex series of events that regulate on the one side the fertilization and on the other embryo and fetal loss during pregnancy.[51] In gender neutral Countries, the male-female ratio at birth, although variable as for place and time, is estimated to range between 105 and 107 male per 100 female births.[52] In spontaneous pregnancy, parental obesity or hypo-nutrition, aging, sub-fertility status[53,54] as periconceptional exposure to adverse natural events,[55,56] environmental or occupational hazards,[57] radiations[58] and severe life events[59] have been associated with a lower birth sex ratio, and the closer the exposure was to conception, the lower the sex ratio,[59] confirming the periconceptional period as a very sex-specific sensitive window.

In ART infants, SSR could differ in relation to the different fertilization as well as embryo transfer techniques and methods. Male predominance was seen in fresh IVF cycles, and female predominance in ICSI.[60-62] Blastocyst transfer (BT) was positively associated with male infants (aRR = 1.03) and ICSI was negatively associated with male infants (aRR = 0.94).[63] In BT, IVF and ICSI maintain their diversity, but both result in 6% more males than the after cleavage-stage embryo transfer (CT).[64] A recent, large Japanese nationwide longitudinal birth cohort study found 51.3% males in spontaneous conception, 50.7% in not-ART infertility treatments, 48.9% in CT and 53.4% in BT.[65] A review and meta-analysis of 26 studies conducted between 2001 and 2017 established a significantly higher M/F ratio at birth and a higher risk of monozygotic twins after BT than with CT.[66] A higher M/F ratio has been observed in frozen-thawed embryo transfer, but after controlling for related factors in a multicentric analysis, it was prevalently BT that could be responsible for the SSR.[67]

Moreover, 53.5% of the infants resulting from cycles with embryo biopsy for pre-implantation genetic diagnosis (PGD) or screening (PGS) were male, versus 50.6% in the non-PGD/PGS group.[63,68]

All this suggests a wide sex difference in the embryo's response to stress during the various developmental steps.[69]

Early embryo sex dimorphic development in the tubal cradle

Spontaneous conception occurs in the Fallopian tube at the infundibulum, and the embryo's initial development occurs during the 4/5-day journey to the uterus, where it will be implanted.

Crucial processes take place during this journey, as maternal zygotic transition (MZT) completed at 8-cell stage, zygotic genome activation (ZGA) completed at blastocyst stage and, in female, inactivation of an X chromosome (CXI) also completed at blastocyst stage, when the first cell differentiation emerges; their abnormal development could cause embryonic arrest. These crucial processes – regulated through a series of carefully orchestrated steps – are carried out concomitantly with a dramatic genomic re-organization and reprogramming of gene expression, and a series of cellular and molecular events, including degradation of maternal factors, methylation process, histone post-translational modification, chromatin remodeling, genome spatial reorganization.[70-72]

DNA methylation-demethylation waves preceding and accompanying the fertilization have been the most studied phenomena, which highlights their crucial role in reprogramming the two haploid parental genomes and in the major global regulatory mechanisms, like epigenesis and imprinting,[73] allowing the 1-2-cell zygote to gain the totipotent state required to regain in turn its own *de novo* DNA progressive re-methylation, establishing the first lineage decision and differentiation and implementing its own sex-specific developmental program.[74-76]

At the same time, the female embryo is engaged in CXI, which happens gradually, through a progressive dampening mechanism, until, just before implantation, the roughly equal ratio of X-linked maternal/paternal genes has been reached;[77] however, in reality there is a more or less wide variability in the balance of this ratio, so much so that a skewed ratio with the same allele (paternal or maternal) can be present in 75-80% or more of the cells.[78] Moreover, the inactivated chromosomes X could be not completely silent: about 20% of the genes escape inactivation, and are expressed by the two X, even if they are expressed minus and in a variable way in the inactivated X across cells, tissues and individuals.[79-81] The definitive activity of the female X chromosome with paternal and maternal genes, – and with more than 20% of escape genes, – is very different from that of the male X, with only maternal genes, and is crucial in the sex difference and in introducing phenotypic diversity[82] and sex different susceptibility to diseases.[83]

During the CXI process, X gene expression in females is twofold, even if progressively decreasing until the CXI is completed; autosomal chromosomes are also involved in a sex-specific manner, through a continuous sex chromosome-autosome cross-talk,[84-87] leading to a genome-wide sex-specific transcriptional regulation in the same autosomal genes;[88,89] even imprinted genes are indeed expressed in a sex dimorphic way, regardless of the original parent;[90] they have several key functions, including growth, metabolism, behavior and stem cell regulation,[90] and are crucial for normal sex-specific development and trans-generational epigenetic inheritance.[74]

Already at the embryonic activation, and well before gonads have been formed and sex-specific hormones have been produced, sex differences are transcriptionally expressed and epigenetically regulated, and therefore are running at biochemical, metabolic, cellular and morphologic level.[88,91] Growth paths and trajectories are sex-different and in males they are faster:[92] while the female embryo is engaged in CXI, the male one is growing;[93] apoptosis rate appears sex regulated,[94] like also the protein and glucose metabolism pathways.[95,96]

Sex dimorphic epigenetic regulation has been observed in global methylation in different development stages[97,98] and in the DNA methylation of some sequences or of the differentially methylated region (DMR), like for instance the lower methylation and higher transcriptional levels of the insulin-like growth factor 2 (IGF2) gene in females.[99]

Sex dimorphic differences also occur in embryo-maternal signalling: recognition molecules, like interferon tau (IFNT) or a progesterone receptor (PGRMC1), are up-regulated in the female; there is a sex dimorphic developmental response to maternally derived cytokine CSF2 (colony-stimulating factor 2)[100,101] and, at the implantation in the embryo-maternal interface, sex different metabolites[102] and the sexual dimorphic role of miRNA have been observed.[103]

During these processes, the Fallopian tube and the uterus are not only a comfortable cradle, but they play an active, sex-different role in the embryonic nutrition, growth, transport and protection against the stress and the maternal immune system. The morphological and functional diversity of the epithelia along the tubal path and the modification of the maternal ovarian hormones allow to respond adequately to the gradually changing female or male embryo needs. The tubal fluid provides different substrates for the evolving energetic metabolism, which initially is oxidative, but turns into glycolytic as the embryo assumes its metabolic autonomy, as well as embryo-trophic and growth factors and non-enzymatic and enzymatic systems and protective factors against the very dangerous oxidative and heat stresses.[104] In addition, the Fallopian tube regulates the gas composition – in which the O_2 concentration is lower than the atmospheric (2-8% vs 20%) – to protect the embryo from oxidative stress; the CO_2/HCO_3 balance is fine-tuned for optimal pH, RNA synthesis, normal cleavage, while H_2S and NO are regulated and involved in tubal contraction and embryo transport.[104]

Early embryo sex dimorphic plasticity and vulnerability

During the tubal journey driven by the fine-tuned continuous cross-talk with the mother, the early embryo adapts – and learns to adapt – to normal environmental variations, but in case of excessive stress it can even arrest its development or undergo modifications, which can become harmful when the stimulus disappears. There is growing evidence that these early days could be the most vulnerable period of pregnancy, as observed in famine,[105] maternal undernutrition or overnutrition and obesity, parental dietary regimen and/or lifestyle[106-109] and environmental pollution, with sex-specific effects.[110]

Studies in animal models confirm this short developmental window as critical for the embryonic interaction with external and maternal factors, which can program the course of pregnancy and the risk of postnatal disease;[106] the evidence of these effects is now so convinc-

ing that it is recommended to begin to protect the health of the offspring even before the onset of pregnancy.[107,111]

Moreover, in the periconceptional period the combination of the highest developmental plasticity during the epigenetic reprogramming events and the greatest transcriptional sexual dimorphism could result in higher, stable, sex-specific epigenetic alterations, leading to sex-specific consequences for the offspring.[88] Sex dimorphic sensitivity to oxidative stress has been observed in the pre-implantation mouse embryo, whereas female were more resistant;[112] cardiovascular, metabolic and behavioral disease in the adult offspring – with females being more susceptible – were observed in the rodent model of pre-implantation maternal low-protein diet;[113] in the sheep, periconceptional restriction of folate, B12 vitamin and methionine had no effects on pregnancy or birth weight, but led to adult offspring obesity, insulin-resistance, elevated blood pressure, altered immune responses (more severe in males) and to an altered methylation status, where more than half of the affected loci were male-specific.[114]

We are far from knowing sex dimorphism in the early embryo development and adaptability, but these findings increasingly confirm the presence of a strong sex difference before the specification of gonads. Furthermore, we know little regarding whether, how and which of these sex differences can persist, and for how long, but a recent research on mouse cardiac development showed the existence of sexually dimorphic gene expression profiles and regulatory networks at every stage of cardiac development, some of which were established before CXI and before the appearance of sex hormones and are epigenetically perpetuated.[115]

The ART periconceptional pathway outside a continuous mother-gamete/embryo cross-talk

ART is now unanimously considered very stressful in animals and humans; the crucial steps of fertilization and early sex dimorphic development must face 'extreme exposure' both to the static culture environment and to the procedures and the effects of both could be added.[35,107,116,117] In human, moreover, ART are often employed on infertile/sub-fertile and aged women and men where gamete quality and oviduct functions could not be optimal. As difficult and intriguing as it could be to assess the contribution of ART and of these internal and external conditions to the pregnancy and offspring outcomes, many studies, even if not all, highlight the contribution of each of these different situations and how, when they often coexist, effects can coexist and add up.[117,118] In the various steps of the ART path there are many challenging questions still open and far from resolving answers.

The preconception period

Gamete maturation, selection and acquisition of competence to the fertilization are the crucial processes of the pre-conception period.

The strict selection and activation of the highest quality spermatozoa from the heterogeneous pool of ejaculated ones takes place during the journey to the tubal fertilization site, when they have to overcome the acidic nature of the vagina, the cervical mucus (which removes non-motile sperm), the phagocytosis in the uterus (which continues to remove weaker sperm) and the counter current in the fallopian tube (where the tube itself guides the sperm swimming with rheotaxis, thermotaxis and chemotaxis mechanisms tuned by a continuous sperm-tubal cross talk).[104,119,120] ART technologies, as the swim-up or the passage through differential gradients, only attempt to mimic – while being very far from reproducing – the natural fine-tuned sperm selection and activation,[120] even more in the cases of male infertility where the only chance of having a genetic child is to collect male gametes from the epididymis or testis;[121] moreover, old – and also new 'omic' – techniques of sperm quality assessment have not yet provided a clear evidence of an improved success of ARTs.[120]

Unlike paternal ones, maternal gametes acquire their mature specification only after oocyte recruitment in the post-pubertal cycling driven by follicular hormonal regulation and engaging multiple physiological systems to ensure the release of a mature oocyte, with the highest developmental potential at the right time for fertilization and establishment of pregnancy.[122] Controlled ovarian hyperstimulation (COH) seems to be an almost obligatory step in ART, in order to have better success, but – leading to the development of multiple, often not fully mature oocytes and interfering with the natural follicular steroid[123] and other biochemical substances production[124] – it suppresses the meticulous tightly regulated natural maturation and selection of oocytes;[71,125,126] also, since it occurs during the methylation wave of the oocyte genome specification, it could increase the risk of epigenetic and imprinting defects,[35,127,128] especially in aged women.[129] Methylation alterations associated with superovulation were found at specific DMRS loci and genes involved in glucose metabolism, nervous system development, cell cycle, cell proliferation and embryo implantation.[127] It has been shown that the oocyte DNA methyltransferase deficiency observed in women over 35 exacerbates the genome-wide DNA methylation abnormalities induced by ART in a sex-specific manner (preponderance of hypomethylation in female), and plays a role in mediating a poor embryonic outcome.[130]

In addition, the hyper-estrogenic environment induced by COH can negatively impact endometrial re-

ceptivity and interfere with the success of the embryo implantation and a good quality placentation. In ART pregnancies, the risk of LBW, PB, SGA are higher in IVF with COH than in IVF with natural ovulation,[131,132] in particular in women who are stronger responders to hormonal stimulation (pick up >20 oocytes),[133] and the risk of SGA is associated with follicular E2 supraphysiological level on ovulation trigger day.[132]

Fertilization

In vivo fertilization is carried out through the gametes meeting, recognizing each other, fusing and contributing to their mutual activation; sperm provides DNA for the male pronucleus (essential for egg activation), the centrosome for the first mitotic spindle and a correct chromosomal segregation,[134] and small non-coding mRNAs, contributing to early embryonic development;[135] egg provides DNA for the female pronucleus and a suitable environment for sperm-egg recognition, prevention of polyspermy, paternal genomic remodeling and embryonic genome activation, ensuring a successful transition from maternal control to a shared responsibility.[136]

In ART fertilization, intrauterine-insemination only – which is effective merely in a few types of infertility – preserves an important part of the natural sperm selection and spontaneous fertilization; among the most applied, totally *in vitro* techniques, IVF could still allow at least a reciprocal choice between the two gametes, and spontaneous fertilization; conversely, in ICSI the male gamete does not enter the egg through natural biological mechanisms, but is mechanically inoculated, and male and female gametes are chosen in the laboratory with criteria still being refined (indeed, we are very far from effectively evaluating their competence to a good fertilization).[121,137-141] ICSI has been applied initially in cases not solvable otherwise, like poor sperm quality, or according to precise clinical indications;[121] today, its use has been extended far beyond, to represent in some centers over 75% of total cases; in ICSI offspring, however, in addition to the female skewed sex ratio at birth,[60-63] the following has observed: a higher rate of *de novo* chromosomal anomalies,[142] congenital malformations[143] including blastogenesis defects,[144] an increased predisposition to sex dimorphic cardiometabolic disorders later in life,[145-147] a lower salivary cortisol concentrations in the pubertal female (not in the male),[148] and decreased semen quantity and quality in young adult men, probably partly related to father infertility.[149] Until research shows how to improve ICSI outcomes not only as live birth rate, but also as offspring safety, many authors state that there is no evidence that ICSI could be more effective than IVF in couples with non-male infertility.[150-152]

Development in the tubal cradle or in a Petri dish

The *in vitro* handling of gametes and early embryos in a culture environment, out of the tubal cradle and the gamete/embryo-maternal cross-talk, could produce direct cellular and extracellular damage, or it could affect the epigenetic regulation and its molecular events, especially in crucial developmental steps as MZT, ZGA, CXI and implantation. [104,153-155]

The main sources of stress in the *in vitro* culture closed system, also sequential, are on the one side the lack of feedback mechanisms to fulfil the sex-specific evolving requirements of energy substrates, gas, pH and temperature gradients, cytokine, growth and protective factors support, and on the other the accumulation of harmful factors, such as reactive oxygen species, metabolic derivatives and pollution substances, and also many physical stresses due to the handling, the Petri dish stiffness, and the exposure to visible light. All these factors could alter the developmental competence, interfering with cellular and extracellular structures and functions and compromising the embryonic quality, or even viability.[35,116] It has also been observed that the current culture media do not support the correct maintenance of the essential methylation processes; the majority of them lack both methyl donors and protection against the methylation anomalies induced by oxidative stress, resulting in epigenetic and imprinting disorders.[156,157]

The addition of one or the other factor to the culture medium has not produced significant improvements – sometimes even yielding contradictory results – and the first attempts of bioengineered dynamic culture platforms, currently being studied in order to try to continue *in vitro* development beyond the implantation time, appear for now very far from the complex dynamism of the *in vivo* tubal environment able to continuously meet the individual embryo's needs.[158,159] Given the sex different growth trajectories, metabolism and apoptosis rate, some authors also wonder if appropriate culture conditions for females or males are needed.[160]

Other 'elective' ART procedures

There are also other procedures, in themselves not indispensable, such as freezing or biopsies, that are totally new events, towards which gametes and embryos could have poor or no adaptability.

Frozen embryo transfers versus fresh transfers are associated with an increased risk of hypertensive disorders in pregnancy, LGA and HBW fetus[50,161] with no significant difference in gender distribution.[30]

In the last few years, gamete and embryo cryo-preservation is becoming a central pillar in ART, with an exponential increase in 'freeze all cycles', while at the same time vitrification is replacing the slow-freeze technique,

with a significant decrease in cryo-damage and a moderate increase in gametes and embryo survival.[162,163] The post-cryopreservation recovery of competent spermatozoa is still critical; loss of sperm motility and viability, acrosomal damage, mitochondrial membrane depolarization, nuclear and DNA damage have been described, and there is much concern not only about the increasing 'live birth' rate, but also about preserving fertility in a safe form.[164-166] In a rabbit model, the offspring from embryo vitrification – versus fresh embryo transfer and natural conceived embryo – seemed healthy at birth, albeit with a male skewed sex ratio and an increased birth weight, but showed lower growth rate and a reduced body weight in adulthood.[167] This data agrees with the observation, in other mammal models, that vitrification exposure could affect the epigenome and lead to the abnormal expression of imprinted genes, but it's difficult to draw definitive conclusions, due both to the low number of loci analyzed and the few studies conducted on the long-term impact on postnatal development.[168]

Introduced to select healthy embryos, pre-implantation embryo biopsy would improve the ART outcomes. Blastomere biopsy (BB) at day 3 (4-8 cells embryo) revealed an impaired developmental dynamic in human and mouse, with reduced implantation rates, increased risk of LBW or PB, and possible postnatal development impairment.[169] Trophectoderm biopsy (TEB) at day 5, removing cells destined to the placenta, shows better outcomes than BB, but not when compared with no-tested embryos, except in special cases, such as a very high risk of aneuploidy.[170] TEB could impair the blastocyst development, especially that of the lower morphologic grade ones, as suggested by a decrease in HCG in relation to the number of cells removed, and could increase the risk of preeclampsia and placenta previa;[171] it also seems to further increase the SSR observed in embryo transfer at blastocyst stage;[68] about the effects in adult life, today there is a lack of sufficient long-term follow-up studies, and at present there are no hypotheses on the effectiveness and safety of its use as a systematic screening test.[172]

Implantation

The last step of ART is the embryo transfer from the culture medium to the uterus. The natural embryo implantation into the uterine endometrium is a finely regulated process, where a variety of factors (cytokines, growth factors, hormones, prostaglandins, adhesion molecules, enzymes, extracellular matrix, etc.) – produced by the receptive endometrium in response to the presence of the blastocyst and vice versa – are able to synchronize the development of the embryo to the competent blastocyst stage, as well as the differentiation of the uterus to the receptive state.[173,174] The efficiency of this process is essential in order to obtain a good placentation, which in turn is crucial for the health of the offspring and the mother,[175] but, also in natural conception, not all embryos are competent enough to overcome it; in ART, only about half of the transferred embryos results in a pregnancy, and about 20% of these hesitate in pregnancy loss;[5] moreover, as reported above, pregnancies that proceed until live birth have an increased risk of adverse maternal and neonatal outcomes, and also of offspring lifelong diseases, in a sex dimorphic way.[175,176]

In this highly selective stage, the cumulative effect emerges of many factors, some past – such as infertility, parent diseases or high age – others related to the extreme exposition to the ART itself and to low immunotolerance, especially in egg donor and in host surrogacy cases with totally allogeneic embryo.[177] In a retrospective cohort study, the risk of abnormal implantation – defined as biochemical pregnancy, ectopic/eterotopic pregnancy and first trimester pregnancy loss – resulted in a percentage of 31.8%, but different in relation to the embryo development stage at transfer and the use or not of cryopreservation: the lowest risk resulted in fresh blastocyst transfer (22%) and the higher in frozen non blastocyst transfer (57%), with cryopreservation a more significant factor than embryo stage.[178] Suboptimal embryonic or uterine conditions could allow the implantation, but could result in a suboptimal placentation with varying degrees of abnormality in a sex dimorphic way.[175,176,179] Suboptimal placenta derived from the cumulative effects of the ART pathway could develop adaptive compensative responses that, if unbalanced, could result in pathological features such as LBW, PB and preeclampsia.[180] In a murine model, different ART groups versus natural developed an increased placental weight and a reduced fetus-placental ratio; moreover, IVF placentas displayed global lower DNA methylation levels and hypomethylation of imprinting control regions of select imprinted genes.[118] A study of DNA methylome in human placenta showed imprinted gene hypomethylation significantly associated with ART, but present only in about 25% of ART pregnancies, the majority with female fetuses. Female conceptus could be more susceptible than the male to the induction of epigenetic abnormalities by ART, especially when the culture time is prolonged and the transfer in utero takes place at the blastocyst stage, when the in vitro culture extension coincides with the CXI.[181]

Discussion

Assisted reproduction technology allowed many infertile couples, persons with hereditary diseases, oncologic patients, but also single or aged women and same-sex couples, to conceive a child, and many million such people are already born worldwide, but the proportion of 'babies in arms' in relation to the number of proce-

dures or cycle treatments remains low, not only because of the high proportion of low-prognosis couples, but also because of the deep gap between the path of spontaneous and *in vitro* conception, with a high proportion of non-fertilization, embryonic development arrest, pregnancy loss, without forgetting the hardship of the procedures, with their physical, psychological and financial cost.[1] Also, there are concerns about the health of mothers and children alike, and even about the offspring's long-life non-communicable diseases, with their possible trans-generational inheritance, whose risk (challenging preclinical vascular ageing signs in children, adolescent, young adults) is continually emerging, but has not yet been well defined, both because the first subjects born are currently only 40 years of age and because there were no systematic longitudinal surveillance systems.

All this, and the morphologic, molecular, genetic researches, conducted mainly in animal models, show that conception and early embryo development outside the comfortable tuba environment and without the gamete/embryo-mother cross-talk happen within a hostile path, exposed to a wide range of risks, resulting in direct and indirect damage to intra- and extra-cellular systems, of which oxidative and thermal stress are important actors, and take place just when crucial steps take place, such as the first genetic and epigenetic lineage decisions and early development of a female or male embryo.

The scale of the consequences could range from developmental arrest to survival with morphological and/or functional damages, or to epigenetic only modifications in apparently healthy babies, who however, later in life, could manifest a disease that could be inherited by subsequent generations.

The sex dimorphism in outcomes detected by significant clinical and experimental researches and the different SSR in relation to the different techniques strongly highlight the problem of whether there are differences between male and female early embryos as for stress response. At the beginning of ART, it was commonly believed that the manifestations of sex difference were related to the different gonadal hormones, and did not therefore concern the initial embryo; but in the last twenty years or so it has been increasingly more evident that, since embryonic activation, the differences expressed by sex chromosomes involves also autosomes, are epigenetically regulated and transcriptionally expressed, thus manifesting themselves in all aspects of the normal and abnormal embryonic development, and this difference, until the completion of the CXI, is the greatest of the entire life. The knowledge of the sex dimorphic responses to ART stressors – as well as the when, why and how they happen – should be one of the essential starting point for the improvement of the results of ART; however it is still lacking.

ART data come from large amount of national registers and prevalently observational clinical researches, but they are variable, and sometimes contradictory, probably due both to the great difference among the couples' characteristics, the procedures applied in the different clinics at different times and the choice of the data to be collected and how to collect it; by nature, register outcomes data fails to reflect the complexity of the early embryo development in relation with the multiple ART procedures, and the clinical research which accompanied the onset and evolution of ART can hardly focus on every step of a complex process, with many pre- and post- conceptional variables related to the embryo, its parents, the environment and at the same time to the multiple possible procedures, which are often chosen more on an empirical rather than scientific basis, so that even a recent comparison between systematic reviews revealed some discordant conclusions and methodological weakness.[182] Human embryonic randomized or invasive studies are not possible, for obvious ethical reasons, and at present recent data from the application of new non-invasive technologies does not appear to be able to produce a significant breakthrough.[154,183] Important contributions come from randomized, prospective animal studies, which make it possible to examine molecular, biochemical, morphological mechanisms in the various developmental phases, as well as their modifications in different situations, but animals are usually fertile, and there are some differences in terms of conception and early embryo development between animal species and humans. Ultimately, the result is that in ART the evidence is still shaky, and the choice of old and new procedures has been – and still is – based more on the "right to try" philosophy than on solid scientific data,[184] and could be or have been also affected not only by the pressure of those who suffer from being unable to have children, but also unfortunately by business and speculation.[1]

In recent years, however – in view of the several million people who were born, and the fact that even more could be born in the future, as well as the arrival of new emerging technologies and the drive to go beyond the confines of infertility[185] and of implantation,[158,159] but also in view of the strong demand for sustainability that ART cannot escape – the background of assisted reproduction is reflecting more on itself, while ethical questions are increasingly being posed.[186-191] The scientific concerns that emerge while looking forward are whether and how to overcome the deep gap between *in vivo* and *in vitro* conception and early embryo development, with the awareness by now that effectiveness and safety arise from the same factors, are closely related and can improve only together.[190,192] To approach in ART the effectiveness and safety of the natural conception that we have in communities characterized by good healthcare, well-designed studies on the effects of ART on the offspring are warranted,[193-195] with particular reference to life-long health consequences[196] and how stressors act

and can cumulate, and whether and how their effects can be avoided[190] and even whether appropriate culture conditions for females or males are needed;[160] the complex processes of embryo pre-implantation and their refined regulation require a more precise approach, based on solid scientific data, and the great sex dimorphism of the initial embryo should not be overlooked, but rather regarded – in the epidemiological, clinical and basic studies – as a systematic objective of research, and not just a matching factor to be considered occasionally.

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Abbreviations

ART	Assisted reproductive technology
BB	Blastomere biopsy
BT	Blastocyst transfer
CT	Cleavage-stage transfer
COH	Controlled ovarian hyperstimulation
CXI	Chromosome X inactivation
DOHaD	Developmental origin health and disease
DMR	Differentially methylated regions
HBW	High birth weight
HCG	Human chorionic gonadotropin
ICSI	Intra cytoplasmic sperm injection
IVF	*In vitro* fertilization
LBW	Low birth weight
LGA	Large gestational age
MZT	Maternal to zygotic transition
PB	Preterm birth
PGD	Prenatal genetic diagnosis
PGS	Prenatal genetic screening
SGA	Small gestational age
SSR	Secondary sex ratio (male/female ratio)
TEB	Trophectoderm embryo biopsy
ZGA	Zygotic genome activation

Key messages

- People born through ART are now well over 10 million worldwide, and ART is going beyond the confines of infertility, acquiring new emerging technologies and looking beyond the current confines of implantation time.
- The problem of ART effectiveness – but above all that of safety – remain largely unsolved, as demonstrated by birth and later-in-life outcomes, and a sex difference in outcomes is clearly emerging.
- In ART the highest periconceptional developmental plasticity during the epigenetic reprogramming events and the greatest transcriptional sexual dimorphism must face an 'extreme exposure', both to the static culture environment and to the procedures, and could result in sex dimorphic adaptability and vulnerability.
- Only solid scientific data could help understand whether and how it could be possible to overcome the deep gap between the natural and the *in vitro* paths, and to bring the effectiveness and safety of ART closer to those of the natural conception we observe in communities with good healthcare.
- The great sex dimorphism of the initial embryo requires sex difference as a systematic objective of research in all the ART epidemiological, clinical and basic studies.

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