

commentaries

Safeguarding ART

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Assisted reproductive technologies (ART) are exceptional among clinical therapies, as unlike most medical procedures, ART have generational consequences. Further, human embryo research in the US has been sponsored solely by the private sector and, until recent biotechnology forays into human embryonic stem cell (hESC) and cloning research, exclusively by infertility clinics. Additionally, the relatively brief clinical history of ART has made it difficult for practitioners and researchers to agree on criteria for its safety and success. Against this backdrop, market pressure on biotechnology companies to create hESC lines and on clinical practices to occupy the innovative forefront has resulted in arguably risky experiments with human embryo cloning, as well as in unintentional germ-line genetic modifications during ART and perhaps during gene therapy. Reproduction, once governed largely by passions and instinct, now seems to need further governance. Some argue that it could now be time for the biomedical community, especially in the US, to take further steps to safeguard ART.

From its roots in *in vitro* fertilization (IVF), ART (Fig. 1) has matured over the past twenty-five years to include other sophisticated protocols, such as ICSI (intracytoplasmic sperm injection) and PGD (preimplantation genetic diagnosis). The high fertilization rate of ART now far exceeds the ~25% rate during natural human reproduction¹, but many challenges remain. Oocyte numbers sufficient for ART are usually obtained after ovarian stimulation, although egg quality, maternal age, environmental exposures and even the stimulating hormones themselves may compromise success.

Fertilization by ICSI differs from IVF in several fundamental ways (Fig. 1a). Men with abnormal sperm or a low sperm count, and even those with no sperm in their ejaculates, can now father children through ICSI. Unlike natural fertilization or IVF, in which the sperm is chosen by competition (for example, swimming, acrosome reaction, zona pellucida passage or fusion with the plasma membrane of the egg), the sperm for ICSI is selected by the subjective criteria of the embryologist. The ICSI needle is carefully inserted to avoid damaging the meiotic spindle of the egg, and membrane healing, egg activation and the trigger and propagation of the cortical reaction can be all expected to differ from IVF. Decondensation of sperm DNA during ICSI is non-uniform, the first DNA

replication cycle is retarded² and the orientation of the male and female pronuclei (decondensed sperm and egg nuclei, respectively) is perpendicular to that after IVF (C. Simerly and D. Takahashi, personal communication). Compelling discoveries on mammalian body axis specification have demonstrated that unfertilized murine and human oocytes have an intrinsic polarity^{2–4} and that the sperm entry site establishes the second axis in mice⁵. Extrapolation to humans suggests that the region of the egg chosen for sperm deposition determines the future left–right body axis.

A second partially understood area of human reproductive biology with important implications for ART is extranuclear genetic transmission. Mitochondria, inherited by exclusive maternal origin⁶, deviate from the norm during cytoplasmic transfer (CT)^{7,8}. Foreign DNA that adheres to the injected sperm is transmitted to transgenic mice⁹ and is introduced during ICSI (though perhaps not during IVF) in non-human primates¹⁰. The mitotic spindle of the zygote poles typically require the sperm centrosome, and centrosome dysfunction is one form of male infertility^{11–15}. Ooplasmic endomembranes, sperm RNA¹⁶ and epigenetic alterations of the nuclear genome, including genomic imprinting^{17,18}, are other unipaternal contributions worthy of further investigation.

PGD has expanded ART beyond infertility therapies: not only can potential parents bear offspring, but they can also ensure that the offspring do not carry genetic disease. PGD involves the removal of one or two cells from the early embryo (Fig. 1c) that then undergo chromosome analysis, usually by fluorescence *in situ* hybridization (FISH) or DNA analysis by PCR for specific genetic diseases (for example, cystic fibrosis, Huntington's disease, sickle-cell disease, X-linked disorders and Duchenne's muscular dystrophy). Recent improvements in embryo culture now permit routine *in vitro* blastocyst development. This provides an extra day or two for PGD, so that embryos with a normal chromosome complement are ultimately selected for transfer.

Reproductive ageing is a growing challenge for ART as more couples delay procreation. The ability of women in their sixties to deliver healthy children using oocytes obtained from younger donors suggests that reproductive ageing occurs within the cytoplasm of the egg *in vivo*. The meiotic spindles of oocytes from women in their twenties are better organized, with more tightly aligned chromosomes than those from the oocytes of women in their forties^{19,20}. Aneuploidy screening (AS; or preimplantation genetic screening (PGS); Fig. 1c, inset)²¹ shows promise for couples with a maternal age in excess of 38 years. In

these instances, embryos display higher rates of aneuploidy and can be screened for correct karyotypes before embryo transfer (ET). Pregnancy rates of 28.5% have been reported, results that are well in excess of controls^{22,23}. AS is applicable only in cycles with several viable embryos from which to select. As blastocysts must be cultured to allow the required time for data collection and interpretation, it is imperative to resolve any concerns regarding adverse outcomes from extended embryo culture. Possibly significant in this context are recent findings in mice of altered genomic imprinting after extended periods of culture¹⁷. The higher rates of identical twins born after extended culture also warrant further study.

Cryopreservation procedures for sperm, zygotes and embryos are now routine, but need to be optimized for unfertilized oocytes, and ovarian and testicular tissues (Fig. 1f). Approximately half of frozen sperm and embryos remain viable, but unfertilized oocytes are less tolerant. Improvements in ovarian or oocyte cryopreservation may permit females with cancers who are undergoing chemotherapy or radiation treatment to preserve their later reproductive options²⁴, as well as aid women suffering from premature ovarian failure. Despite the acceptance of cryopreservation in the clinic, the potential consequences of such storage merit comprehensive investigation.

The reliable transfer of a single human embryo remains a top priority. In cattle, implantation rates per embryo approach 80%. However, clinical rates are much lower and dependent on maternal age and embryo quality. This explains the practice of transferring multiple embryos, where the goal is to achieve a single pregnancy. However, ART pregnancies result in higher rates of triplet and higher-order multiples. One strategy to improve implantation rates is to culture oocytes and embryos with FF-MAS (follicular fluid meiotic activating sterol²⁵), a cholesterol biosynthesis intermediate. In mouse studies, 'protein paints', isolated from the extracellular surfaces of embryos with superior implantation rates, improved the implantation success of inferior embryos²⁶.

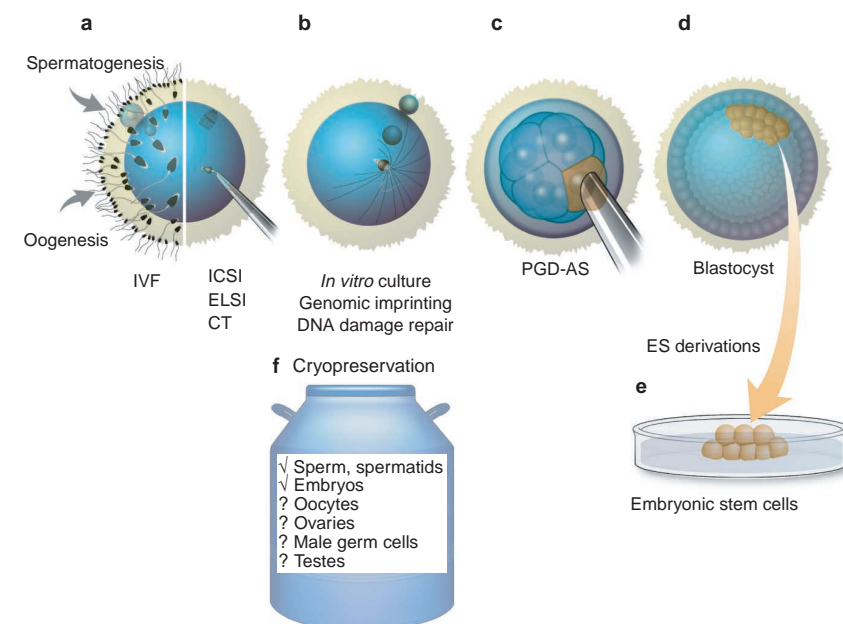


Figure 1 The State of ART. **a**, IVF mirrors natural reproduction, as sperm are selected competitively (left). ICSI (right) differs from IVF in that the chosen sperm are microinjected into the oocyte at a cytoplasmic region distal from the maternal chromosomes aligned on the metaphase-arrested second meiotic spindle. Ejaculated, epididymal and testicular sperm are used for either ICSI or elongated spermatid injection (ELSI). CT is a variation of ICSI, in which ooplasm from a donor oocyte is introduced with the sperm. **b**, Zygote development. ICSI (shown) differs from IVF through delayed DNA decondensation of the male pronuclear apex (conical nucleus with adjacent sperm tail), due to sperm structures discarded at the egg surface during IVF (for example, perinuclear theca and SNARE membrane proteins). DNA replication is also delayed. The sperm aster is the radially arrayed microtubule that translocates the male pronucleus (sperm nucleus) towards the centre of the egg while simultaneously pulling the female pronucleus (egg nucleus; upper sphere) to its centre. The function of the sperm aster seems to be similar in both ICSI and IVF. The aster is organized by the sperm centrosome (introduced at fertilization), which recruits and retains maternal microtubule binding proteins and motors. **c**, Preimplantation human development now permits genetic testing (PGD, AS), as well as biopsy of blastomeres for hESCs, without compromising the reproductive potential of the embryo before freezing. **d**, Blastocysts are comprised of two cell types: the interior inner cell mass cells (ICM; bathed with blastocoelic fluid) and the exterior trophoblast (TE) cells. Only some ICM cells contribute to the foetus and could be considered the true human embryo. The TE contributes to placental and other extra-embryonic tissues. **e**, hESCs are derived from the ICM after immunosurgical removal of the TE cells. **f**, Cryopreservation routinely succeeds with sperm, zygotes and embryos (checked). Active research is underway to optimize freeze–thaw technologies for unfertilized oocytes, ovarian and testicular tissues, and male germ cells.

An increase in implantation rates, such that only one or two eggs are needed per cycle, might reduce or even eliminate the need for hormonal stimulation. Many questions still remain about preimplantation development, implantation, pregnancy establishment and the post-implantational events that shape the nascent human²⁷.

Many adult and paediatric diseases may have foetal origins; thus, investigating the consequences of ART could directly test the 'Barker Hypothesis'²⁸, which proposes that embryonic and foetal environmental

exposures may have life-long consequences for health²⁹. Diseases already cited include diabetes, obesity, cardiovascular disease, stroke, depression and schizophrenia³⁰. A comprehensive series of investigations on ART outcomes and environmental exposures during ART could be crucial, not only for evaluating ART, but also to understand cross-talk between foetal/maternal genetic factors and environmental regulators^{31–33}. Some of the oldest IVF children have now conceived naturally, the oldest ICSI child has just turned ten and the oldest ART

primates range from ~18 years (IVF)³⁴ to 4 years (ICSI)². These human and non-human primates, in conjunction with mice³⁵ and domestic species^{36,37}, comprise an invaluable research resource that should be responsibly investigated to determine whether clinical ART have any health, behavioural or reproductive consequences.

Germline gene transfer (GLGT) in humans now demands increased attention because gene therapy vectors have been found in the seminal plasma of patients³⁸ and because of accidental transmission of mitochondria during cytoplasmic transfer (CT; Fig. 1a). CT was developed to treat couples with repeated ART failures and designed to improve oocyte quality. It involves the aspiration of cytoplasm from a donor oocyte (typically from a younger woman) into the same micropipette in which the sperm for ICSI will be drawn, followed by a single injection of sperm and donated ooplasm. CT has inadvertently resulted in children with maternal mitochondrial DNA (mtDNA) from both the nuclear and the ooplasmic source^{39,40}. An unusually high rate of Turner's syndrome has also been reported after CT. In cases of large deletions in the mtDNA genome⁴¹, mitochondrial heteroplasmy (that is, dissimilar mitochondria within the same cell) can result in disease. It is not known if the form of heteroplasmy caused by CT, in which both mtDNA genomes remain intact⁴², will have any clinical consequences.

The donation of clinically discarded human embryos from informed consenting parents has allowed the derivation of hESCs, which hold the promise of providing new therapies for devastating diseases and disorders^{43–45}. Obtaining novel or customized hESC cell lines by therapeutic cloning has provoked national and international discussions. Extravagant claims about reproduction by somatic cell nuclear transfer (SCNT; that is, reproductive cloning) have been announced, but not published by peer-reviewed journals. These widely publicized ventures are among the gravest threats to ART. The resulting controversies could lead to restrictions on ART programmes that might be devastating for infertile patients. Furthermore, biotechnology and

pharmaceutical enterprises may shift efforts overseas to avoid such restrictions. ART investigators may continue to leave the US or abandon essential studies, even those that are privately funded, for fear that their careers and livelihoods are threatened by ambiguous regulations. Bringing the promises of ART to fruition will require dedicated and well-coordinated research. However, this approach can potentially address fundamental aspects of human stem cell biology.

ART outcomes and future directions

In this supplement, Lord Robert Winston and Dr. Kate Hardy⁴⁶ have posed the question, "Are we ignoring potential dangers of *in vitro* fertilization and related treatments?" Their thoughtful commentary calls for more comprehensive research on all aspects of ART, including paediatric outcomes. Clinical reports indicating that ART may have unforeseen consequences cannot be ignored. Peer-reviewed publications attesting to either the safety or risks of ART are irreconcilable by professionals, let alone the public. The scientific progress of ART demands conclusive findings that are confirmed independently. A learned consensus on the evidential foundations of ART would allow research priorities to be clearly set.

In addition to knowledge, present-day ART also demands wisdom and balanced judgement. In common with other medical procedures, clinical ART has risks. These risks may no longer be to the patients, but to their prospective children. Infertile couples cannot postpone ART for years while complete data is garnered. Differences between ART practices and variations in data collection and reporting complicate interpretations. Anecdotal reports suggest that even variations in how frequently the incubator is opened may impact embryo development and correlate with adverse ART outcomes⁴⁷. Innovations in clinical ART are introduced swiftly, typically without animal studies, and the accuracy of extrapolations from research models remains unclear⁴⁸. Many ART procedures are technically demanding and expertise and instrumentation vary between programmes. Consequently, suc-

cess rates differ significantly, due in part to steep learning curves as embryologists and clinicians master the new protocols. This is further complicated by the speed with which these innovations are subsequently abandoned, to be replaced by more up to date approaches. All of these issues combine to complicate the predictive value of retrospective evidence-based outcomes.

Safeguarding ART in the US: a call for ART-silomar?

The primary objective of ART clinicians and their patients — namely, overcoming infertility — has been achieved, as evidenced by the millionth child born through assisted reproduction. Remarkably, at the same time, ART has made invaluable contributions to science by providing precious scientific resources, fundamental insights and clinical rationales for basic investigations that result in unanticipated discoveries. Breakthroughs in embryonic stem cells, cloning, and genetic modifications have highlighted ART beyond the realms of the infertility community. Consequently, the flexible freedoms previously enjoyed may now be in need of proactive efforts to continue to safeguard ART.

The human fertilization and embryology authority (HFEA) in the UK, and its counterparts in other countries, meet their own sets of challenges within the context of their health systems and cultural sensitivities. By contrast, the private system in the US affords greater flexibility for individual couples and independent ART programmes, and the regulation of ART issues in the US is a unique challenge. First, ART in the US is independent of federal funding; second, the healthy competition between ART programmes would be undermined by premature disclosures of therapeutic innovations in a public forum; third, biotechnology and pharmaceutical companies recognize the lucrative potential of hESC and cloning research; finally, once enacted, laws are uncompromising.

The lessons from Asilomar on safeguarding recombinant DNA (rDNA) research are worthy to consider. Thirty years ago, just as IVF was perfected, the scientific community grappled with a different thorny issue:

rDNA. Coverage in the press fostered alarm in some communities at the possible threats to public safety posed by rDNA. In 1975, a now historic meeting at the Asilomar conference centre reached a consensus regarding appropriate safeguards for rDNA. These guidelines were approved by the National Academy of Sciences, published in respected journals⁴⁹ and formed a framework constructed by knowledgeable physicians and scientists, who established guidelines that alleviated safety concerns while allowing scientific progress.

One pertinent question concerns whether it is appropriate to consider an Asilomar-type approach for ART. Currently, ART has many more involved parties with differing and frequently opposing interests, as well as complicated funding and regulatory requirements. 'ART-silomar' might be a forum where clinicians and scientists from private and academic programmes, as well as from professional societies and biotechnology, meet with national authorities and international counterparts. Objectives could be limited to: first, ART glossary (for example, facts of ART, a nomenclature and identification of critical knowledge gaps); second, accepted ART procedures; third, ART education (for example, clear communication of ART facts and the separate fostering of on-going conversations regarding ethical, legal and social implications). Unambiguous consensus statements from renowned practitioners and scholars could be encouraged so that the ART protocols are identified as clearly appropriate, worthy of continued experimentation, or inappropriate without further knowledge. This might prove timely in the light of current hESC, cloning, transgenic and gene therapy initiatives. Needless to say, whether or not ART is appropriate cannot be judged on the basis of scientific merits alone. The ethical, legal and social implications that comprise the higher tier of consideration might well be informed by the ART scientific facts, so that important conversations are not side-tracked by science fiction (for example, human reproductive cloning).

Some question the need for ART-silomar now. Indeed, in the US to date, ART has been safeguarded through continuous, serious

and responsible conversations and management by federal, state and institutional authorities; professional societies and patient advocacy groups. Federally empanelled advisory boards (National Bioethics Advisory Commission (NBAC), President's Council on Bioethics, National Academy of Sciences (NAS)/Institute of Medicine (IOM)) have deliberated thoughtfully and prepared specific and detailed recommendations. These recommendations remain to be implemented, perhaps due to the thorniness of federal involvement in human reproductive research.

Critics might argue that scientific consensus cannot be achieved, let alone implemented, without legal enforcement. Laws and regulations to restrict ART are pending, although ironically, other laws proposed 25 years ago might have ended American ART. Congressional, presidential and FDA attention to ART might provide the momentum necessary to proceed with ART-silomar. Congress will probably ban human reproductive cloning on the basis of incontrovertible evidence that it is just not safe. Their outlawing of cloning research for stem cell applications remains in the balance, threatening scientific enquiries with profound therapeutic potentials. The responsibility of the FDA to ensure safety and effectiveness may include additional aspects of ART. Commerce in ART might benefit from guidelines that clarify human oocyte donations, especially for biotechnology. It might also benefit from clarifying the status of ~100,000 cryopreserved human embryos at ART clinics. The NIH must continue to sponsor frontier biomedical research while not allowing federal mandates to usurp its leadership and stewardship roles.

National and international attention is now focused heavily on the ART biomedical community and we must not miss this crucial opportunity to responsibly safeguard ART. Achieving consensus through better-informed conversations on ART facts, significant concerns and governance (if appropriate) is a first step. The second step — wide and accurate global communication of an ART-silomar consensus — will help promote responsible, effective stewardship for ART, both now and in the future. □

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