In vitro fertilization – From Louise to 21st century luminosity

Kathryn J. Go, Ph.D., HCLD

This past summer marked the 40th birthday for Louise Brown, the world's first baby born via in vitro fertilization (IVF). Her parents' courage and her momentous, safe delivery and birth in 1978 heralded the arrival of a revolutionary therapy, born of the careful union of modern medicine and basic science.[1] At that time, IVF injected new and real hope into infertility treatment. It allowed for the development of embryos *in vitro* for intrauterine transfer, implantation and sought-after pregnancy and livebirth. Continued efforts to enhance fertility patient outcomes, improve efficacy of treatments and expand application have inspired scientific investigation and innovation that has led to present-day assisted reproductive technology (ART).

Since its introduction, IVF has benefited from ongoing efforts to improve its every facet and phase—maximizing the number of high quality eggs, achieving the highest fertilization rate with the most challenging sperm factors, producing the highest number of best-quality embryos, achieving freezing and thawing rates of embryos that compare to or surpass those of fresh embryos and attaining the highest implantation and livebirth rates.

Early days of IVF

Eggs were initially collected from mature ovarian follicles by laparoscopic aspiration. However, the physical space required for early IVF made the process more challenging. Hospitals had to allocate precious space for operating rooms and general anesthesia used in laparoscopic egg retrieval. Early IVF laboratories were relatively simple, but had specific instrumentation and engineering requirements for water, air, climate and gas systems. Despite their central role, many early IVF laboratories were housed in converted storage rooms or closets.

The limited space worked well enough for the basic laboratory equipment required for early IVF. Typical equipment included a deionizing system to generate water, a pH meter, osmometer, microbalance and laboratory glassware for making culture medium from scratch; a laminar flow hood to provide an aseptic workspace; one or more incubators with appropriate medical-grade gas tanks; a phase contrast microscope, a dissecting microscope and inverted microscope with high contrast optics; and a centrifuge.

The original IVF process was fairly straightforward:

- Collect the eggs (cumulus masses) from follicular fluid.
- Process the corresponding husband's/partner's/donor's semen to collect concentrated motile sperm.
- Inseminate the eggs with sperm and allow for overnight culture.
- Assess the eggs for fertilization.
- Evaluate the developmental stage and morphology of resultant embryos.
- Transfer multiple embryos (as many as four) on Day 2 or Day 3.

In the early to mid-1980s, clinics aimed for 15 to 20 percent clinical pregnancy rates. Bourn Hall in the United Kingdom, Monash University in Australia and Eastern Virginia Medical School in the United States were the standard-setting clinics at the time. Many of the earliest embryologist recruits were medical technologists who understood the confluence of medicine and laboratory science. Others were researchers from basic reproductive biology and physiology research laboratories. These individuals came from laboratories where IVF was routinely used to investigate the cellular and molecular mechanisms underlying fertilization and early embryo development in mammals such as mice, rabbits or guinea pigs. Many of these pioneers have seen the incredible trajectory of IVF from its earliest days through its evolution to becoming present-day ART.

ART is a pleasantly ironic acronym given the technical expertise and *craft* that are central to its effectiveness. Requiring both artistry and technical mastery, ART relies on a host of techniques, including egg retrieval, egg freezing through vitrification, fertilization by conventional insemination or intracytoplasmic sperm

injection (ICSI), assisted hatching, embryo/blastocyst biopsy for genetic testing, cryopreservation of embryos/blastocysts by slow-cooling or vitrification, and embryo transfer.

Advances in culture media design and conditions

There is perhaps nothing more important than the nutrient solution for egg, sperm and embryo culture that provides the environment for fertilization and development. These media must meet the different nutrient requirements of sperm, eggs and the developing embryos while avoiding the formation of toxins.

In the earliest days of IVF, laboratories performed the time- and effort-intensive task of making their own culture media. This required using purified water along with an array of individual chemicals that were carefully weighed or dispensed with subsequent adjustments to attain specific ranges of osmolality and pH. Quality control was achieved using a system such as the mouse embryo assay to identify acceptable batches of media for patient use.[2]

Some laboratories used commercially provided media such as Ham's F-10, a general tissue culture medium, or more IVF-specific media such as Menezo's B2 medium. A culture medium modeled after human tubal fluid became popular in the 1980s, a time when industry-sourced and quality controlled culture media, protein supplements and an array of other adjunct products were common.[3-4] These cultures afforded embryologists convenience and time, eliminating hours spent on making and quality control-testing in-house culture mediums.

To address the differing biochemical requirements of gametes (eggs and sperm) versus early embryos and blastocysts, culture models using sequential media were developed. First, one medium was provided for egg and sperm culture for the insemination interval. Then, a second medium was specifically prepared for embryo development for fertilization through the eight-cell stage (Days 1 to 3). Finally, a third medium was used for the blastocyst development stage (Day 3 to Day 6 or 7). This design follows the changing requirements of the embryo and emulates the differing environments of the fallopian tube (early embryo development) and the uterus (morula to blastocyst).[5]

Complementing more favorable formulations for human culture media has been the adoption of low-oxygen atmospheres in incubators. Reducing the oxygen tension to physiologic levels in embryo culture alleviates physiologic stress and contributes to more favorable conditions for optimal development.[6] Advances in both culture medium design and culture conditions can contribute to a higher blastocyst formation rate in a patient's fertilized egg cohort and higher quality (i.e., implantation potential of the blastocysts).

Egg vitrification

Egg freezing by vitrification is the most recent addition to the ART portfolio. Successful pregnancies and livebirths using frozen eggs and slow-cooling methods had been reported from as early as 1986, though certain challenges prevented consistent outcomes.[7-8] The use of vitrification on eggs, as it had been applied to blastocysts, provided reliability that allowed for wider adaptation.[9] After multiple reports that reproducible methods had been described with favorable and acceptable results, the American Society of Reproductive Medicine (ASRM) advised its membership in 2013 that egg freezing was no longer considered "experimental."[10]

Egg freezing has two benefits to patients. The first is fertility preservation. In particular, this benefits women of reproductive age who receive cancer diagnoses requiring gonadotoxic treatments. With minimal or conventional ovarian stimulation, a patient's eggs can be collected prior to cancer therapy and frozen for use after treatment and recovery. Other candidates for fertility preservation might include women in the military and others purposefully delaying childbearing. This includes women who are mindful of advancing age and are, perhaps, waiting for the best time and/or partner for building a family. The second dividend of egg freezing is the opportunity to create donor egg banks, analogous to sperm banks, with the same breadth in variety of phenotypes.

Egg vitrification also allows expanded accessibility to donor eggs. It eliminates the potential inconveniences of waiting for a donor to undergo stimulation and retrieval and the necessary synchronization of the donor and recipient cycles to allow embryo transfer. A donor egg recipient can now select eggs from a bank's catalogue, have them shipped to a designated IVF laboratory for warming and ICSI, then await embryo development and transfer —all on the recipient's timeline.

Micromanipulation

The advent of micromanipulators—a system of microtools and controls installed on an inverted microscope—allowed embryologists to manipulate and perform operations on single cells or embryos. This catalyzed the development and refinement of procedures to enhance IVF outcomes. It is difficult now to think of IVF without the array of techniques made possible by micromanipulation. Ultimately, micromanipulation was key to improving embryo implantation, revolutionizing the treatment of male factor infertility and introducing preimplantation genetic testing.

Intracytoplasmic sperm injection

Partial zona dissection (PZD) and subzonal insemination (SUZI) were the hope for maximizing and achieving fertilization despite the most severe forms of male infertility, including limiting factors of sperm count, motility and morphology.[11-12] In PZD, an opening was created in the zona pellucida to allow sperm to bypass binding and penetration of the zona. In SUZI, the zona was also opened, but one or more sperm were deposited between the zona and the egg membrane to eliminate sperm transit through the zona pellucida. This optimized the opportunity for sperm and egg membrane fusion leading to fertilization.

Even with these important innovations, circumstances of severe oligo-, astheno- and teratospermia still precluded fertilization, signaling that proximity alone was inadequate to reliably overcome poor sperm quality. ICSI offered a decisive solution.[13] Injection of a sperm cell directly into an egg's cytoplasm could overcome the barriers of the mature egg (i.e., the cumulus oophorus, corona radiata, zona pellucida and egg membrane).

Sperm injection is preceded by egg preparation. This involves briefly exposing the egg-in-cumulus complexes to an enzyme solution (hyaluronidase), which disperses the cumulus and isolates the egg. The egg is microscopically examined to establish the presence of the first polar body that reflects the maturity of the egg at metaphase II and its appropriateness for ICSI. Immature eggs (germinal vesicle-stage or metaphase I) can be cultured to develop additional eggs for injection. This phase also allows characterization of an egg with respect to its zona pellucida and ooplasm. Eggs with morphologic features such as vacuolation or granularity, might be unfavorable for fertilization or embryo development.[14]

Once deposited in the prepared egg, the sperm can undergo all the postmembrane fusion events that comprise fertilization: sperm head decondensation, pronuclear formation and syngamy with the egg's pronucleus. Embryo development ensues, allowing completion of the treatment cycle with embryo transfer—an opportunity for pregnancy that could not previously be realized with conventional insemination, PZD or SUZI.[15]

Using surgical intervention to obtain sperm, ICSI expanded the possibility of biological fatherhood for patients with the most challenging forms of male factor infertility, including azoospermia. Sperm can be harvested for ICSI through collaboration with urologists performing testicular sperm extraction or epididymal sperm aspiration.[16-17] In the selection process for ICSI, nonmotile sperm from semen or surgically retrieved sperm can be tested for viability or function with measures such as the hypo-osmotic swelling test or hyaluronan-binding assay.

Assisted hatching

Before settling into the uterine lining, an embryo must hatch from the zona pellucida, its surrounding protein shell. There was an effort to address the hypothesis that in some patients, this step was impeded, leading to implantation failure. It was postulated that "zonal hardening" was a result of in vitro culturing, the patient's age or a characteristic of the embryo. A hardened zona might not experience naturally occurring zonal thinning, which allows an embryo to expand, and eventually, escape through an opening to implant itself into the endometrium.[18]

Assisted hatching (AH) was designed to aid the embryo in this process. An opening of optimal size in the zona pellucida was created either mechanically with a glass needle or chemically with an acidified culture medium. The expanding embryo was then assisted in exiting the zona pellucida through this opening, increasing the opportunity for implantation.[19] Lasers, customized for ART, have now largely supplanted other means of AH, offering more efficiency, greater consistency and reduced consumption of ART resources such as microtools and hatching solution.[20]

The technique bore out the expectation of higher implantation rates in some patients whose embryos received AH.[21] However, the practice committees of the ASRM and the Society for Assisted Reproductive Technology have reflected the procedure's limitations in various published reports that preclude drawing

conclusions about the efficacy of AH in increasing clinical outcomes.[22] Although there has been some evidence that AH slightly improves clinical pregnancy rates, particularly in poor prognosis patients or patients with prior failed IVF cycles, the limited findings provide insufficient evidence to conclude that AH improves livebirth rates.[22]

Preimplantation genetic testing of embryos and blastocysts

The union of IVF and genetics gave way to embryo biopsy and genetic testing of individual embryo cells.[23] In this application, IVF extended its reach beyond infertile patients to assist individuals at risk for transmitting genetic disease. The process generally begins via embryo biopsy at Day 3, when the blastomere number can be expected to be at least six, and ideally, eight.

The embryo is oriented such that the nucleated blastomere is readily accessible to a biopsy pipette. The zona is opened at a size that will allow removal of a blastomere without causing the entire embryo to exit the zona. To facilitate blastomere removal, the embryo is typically incubated in a calcium- and magnesium-free medium to minimize cell-to-cell adhesion. With gentle aspiration via the biopsy pipette, it is possible to extract a blastomere from each embryo for transfer to individual reaction tubes and shipment to a genetic testing laboratory.

There, the cells can undergo the requested testing (e.g., aneuploidy, translocation, chromosome structural anomalies or single gene mutation). The biopsied embryo is returned to culture for further development to Day 5, by which time genetic testing is complete. Once the testing report is received, appropriate embryos can either be transferred or cryopreserved.

Aneuploidy analysis, a frequently requested test, was initially carried out by fluorescent in situ hybridization (FISH). This technique used chromosome-specific probes to enable visualization and enumeration of the differently colored signals for each chromosome to identify trisomies, for instance. But FISH was limited in that it could only detect a subset of chromosomes (e.g., 13, 15, 18, 21, 22, X and Y). Further, research found that biopsy at Day 3 had negative effects on embryo development.[24] The limitations of FISH analysis and blastomere biopsy indicated the need for a new method of preimplantation genetic testing (PGT).

The current and prevailing method for PGT encompasses the innovations of blastocyst culture and biopsy and applies them at a more resilient stage of multiple cells per embryo. Current PGT also harnesses state-of-the-art techniques from molecular biology (e.g., array comparative genomic hybridization, quantitative polyamerase chain reaction or next-generation sequencing) for more sensitive genetic testing and detection of all 24 chromosome types.

Current screening methods have revealed the incidence of mosaicism, or the presence in an individual blastocyst of more than one cell line and chromosomal constitution.[25] The understanding and management of mosaic blastocysts is currently evolving through ongoing investigations and analyses in genetics laboratories. Researchers note that identifying mosaicism might help facilitate more accurate categorization and ranking of embryos for transfer.[25] Pending test results, all biopsied blastocysts are vitrified with an optimized cryopreservation method to optimize frozen embryo transfer (FET).

Cryopreservation

Even with higher numbers of embryos transferred in early IVF cycles, many patients had supernumerary embryos available from their treatment cycles. How could these be preserved for future cycles? An immediate solution was to cryopreserve them for FET. This eliminated the need for multiple cycles of ovarian stimulation and egg retrieval. The first pregnancy achieved by transfer of a thawed eight-cell embryo was reported in 1983.[26]

For many years, embryos were frozen on Day 3, at the six- to eight-cell stage. The embryos were first equilibrated in specifically formulated cryoprotectants to achieve dehydration. The process replaced the embryo's water with antifreeze-like cryoprotectant prior to slow cooling in a programmable cell freezer. The process replaced the embryo's water with antifreeze-like cryoprotectant prior to slow cooling in a programmable cell freezer at a rate of minus 0.3 degrees C° per minute.[27]

The thawing process entailed rapid warming with a stepwise rehydration of the embryo. The cryoprotectant was replaced with water through equilibration in a series of warming solutions, culminating in the embryo's return to culture medium for transfer. It was not unusual for an embryo to endure some cell loss at thaw, and pregnancies were still achieved even when some blastomeres within an embryo did not survive warming.[28] To avoid cell attrition in thawing, some clinics preferred to freeze fertilized eggs at the one-cell or dipronucleated (2PN) stage.[29] These could be thawed and placed in culture to proceed through early embryo development and, ultimately, transfer at Day 2 or Day 3.

The efficacy of embryo cryopreservation was significantly improved by the introduction of vitrification, or the creation of a glassy or vitreous state within the embryo to avoid damage that can result from ice crystal formation incurred during slow-cooling methods.[30-31] Vitrification involves the rapid equilibration of blastocysts (or zygotes or cleavage-stage embryos) in high concentrations of cryoprotectant followed by immediate immersion into liquid nitrogen, providing an effective cooling rate of minus 20,000 degrees Celsius per minute. Unlike slow cooling, vitrification requires no programmable freezer, and the processing time—from first equilibration to placement in liquid nitrogen—is only minutes per blastocyst. Its application in mammalian embryos was first described in 1985.[32]

Vitrification relies on adherence to strict time limits for exposing the blastocyst to the cryoprotectants and loading it into the cryo carrier. Meticulous technique is rewarded by high rates of recovery, survival and implantation, and these attributes have been central to 21st century ART. By providing an ideal method for cryopreserving blastocysts—the increasingly preferred developmental stage for freezing embryos—vitrification has enabled more frequent cryo-all cycles and facilitated blastocyst biopsy for preimplantation genetic testing.

Single embryo transfer

A sure indicator of how far IVF has advanced since Louise Brown's birth is the ascendance of the elective single embryo transfer (eSET). In the earliest IVF treatment cycles, and with only modest adjustments as recently as the early 2000s, the transfer of multiple embryos—as many as four —was not only recommended but actively sought as a way of optimizing a cycle. Several factors have contributed to the arrival and application of eSET.

Great strides in culture medium systems allowed for the maximal developmental potential of embryos. Advances in ovulation induction, operative methods of egg retrieval and embryo transfer also helped set the stage for eSET. Additionally, the sheer benefit of experience and practitioner networking have contributed to the procedure's advancement.

The transfer of one, high-quality embryo became sufficient to give a patient an optimal chance of becoming pregnant. This new option addressed the vital concern of obstetric and maternal-fetal medicine clinicians that IVF resulted in too many multiple gestations and births, which could bring clinical complications, medical risks and negative effects on a family's financial health and quality of life. The expanding application of eSET may continue to gain traction with the array of techniques used for selecting the best embryo.

Culture to blastocyst, preimplantation genetic screening for aneuploidy and noninvasive methods can provide information about the embryo beyond its morphology. One noninvasive method is time-lapse imaging. With this technique, the developmental course of each embryo can be examined through continuous image capture over multiple days of culture, allowing the development of algorithms to identify developmental trajectories associated with implantation. Another method employs metabolomics, or an analysis of metabolic products in the culture medium of each embryo that might yield information about embryonic vigor and developmental potential.

Mitochondrial therapy and gene editing

Finally, two other methodologies receiving attention for potential application and inclusion in ART are mitochondrial transfer (or mitochondrial replacement therapy) and gene editing.[33-34]

Mitochondrial transfer is the placement of mitochondria from a donor egg into the cytoplasm of a recipient's egg. This method might be applied if a recipient is a carrier of mitochondrial disease or otherwise has poor egg quality. In either case, the donor mitochondria are the therapy to overcome deficiencies and, as the sources of cellular energy, provide the metabolic resources to drive normal embryo development. However, the introduction of a donor's mitochondrial DNA into a recipient's egg might entail some controversy, as it results in three sources of DNA in the embryo: the maternal DNA, the paternal DNA and the donor mitochondrial DNA.[33]

Gene editing, mediated through an enzyme system called CRISPR-Cas 9, envisions the deletion of errant genes and their replacement with correct DNA sequences. Genetically transmitted disease from one or both parents could be addressed at the embryonic level through DNA correction. Like mitochondrial transfer, gene editing raises ethical concerns that clinicians and patients will need to consider.[34]

Summary

Clinicians, caregivers and other ART professionals have consistently shown resolve in acquiring and mastering the ever-changing knowledge and skill needed to make fertility treatments more comprehensible and

accessible to those who must use them to build their families. Their mission for patient care has been to continuously improve ART. Louise Brown's birth was the genesis of decades of insightful research and innovative techniques that have supplanted the original IVF method. As we thoughtfully survey the constellation of technology that informs modern life, such as molecular techniques and nanotools, information and telecommunication technology, robotics, personalized medicine or microfluidics, it is exhilarating to contemplate how ART will continue to evolve.

About the author

Kathryn J. Go, PhD, HCLD, is the director of the embryology laboratory of Boston IVF, the Maine Center and the clinical laboratory director at ReproSource Fertility Diagnostics. Go previously served as the scientific and laboratory director at IVF New England (formerly the Reproductive Science Center of New England) for 11 years and as the laboratory director at Pennsylvania Reproductive Associates for 21 years. She has been active in the Society for Assisted Reproductive Technology executive council and the American Society of Reproductive Medicine (ASRM). Go has also served as a U.S. Food and Drug Administration consultant, course chair and faculty for ASRM and New England Fertility Society (NEFS) postgraduate courses and chair of the ASRM reproductive biology professional group. She previously served as NEFS president and is currently the NEFS member-at-large for industry. Go was a lecturer at the Medical College of Pennsylvania, now Drexel University College of Medicine and has served on the faculties of the University of Pennsylvania, Thomas Jefferson University, the Experimental College of Tufts University and the University of Massachusetts. She holds a doctorate in molecular biology from the University of Pennsylvania.

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