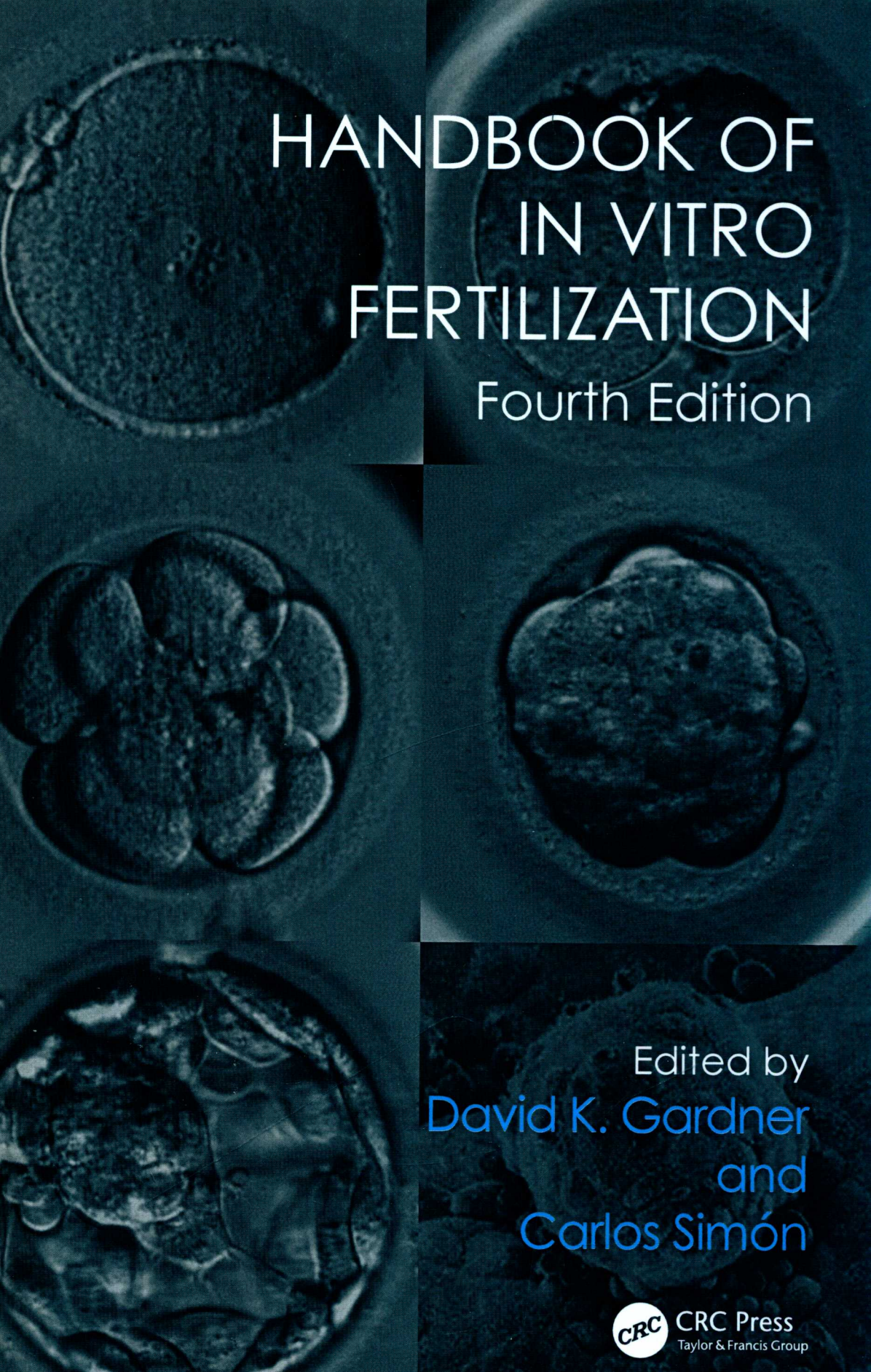

HANDBOOK OF IN VITRO FERTILIZATION

Fourth Edition

Edited by

David K. Gardner

and

Carlos Simón

CRC Press
Taylor & Francis Group

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Edited by

David K. Gardner, DPhil
School of BioSciences
University of Melbourne
Melbourne, Australia

Carlos Simón, MD, PhD
Igenomix
Valencia University
Instituto Universitario IVI/INCLIVA
Valencia, Spain

Department of Obstetrics and Gynecology
Stanford University
Stanford, California, U.S.A.

Department of Obstetrics and Gynecology
Baylor College of Medicine
Houston, Texas, U.S.A.

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Handbook of In Vitro Fertilization

To Our Families

Preface

The past four decades have witnessed many remarkable advances in the field of assisted human conception. Following in the footsteps of Robert Edwards, Patrick Steptoe, and Jean Purdy, numerous scientists and physicians around the world have worked tirelessly to develop more effective and safer procedures to treat infertile couples. Along with improvements in the areas of ovarian stimulation, embryo culture and cryobiology, we have seen the introduction of assisted fertilization through intracytoplasmic sperm injection, and the development of techniques to remove and perform genetic analysis on polar bodies, blastomeres, and the trophectoderm. More recently, with the advent of time-lapse microscopy, we now have the capacity to analyze embryo morphology and the kinetics of development like never before.

While no single volume can adequately cover the enormity of the diverse field of reproductive medicine, it is the aim of this book to review the achievements of the biomedical community involved in assisted human conception, and to highlight ongoing and potential future treatments and procedures. There can be no doubt that both basic and clinical research has improved clinical outcomes worldwide, not only increasing pregnancy rates, but also reducing the time to pregnancy.

This book provides considerable background to many areas of human-assisted conception, and much practical information, which can be readily translated into clinical practice. To facilitate this, the chapters found within have been written by acknowledged pioneers and experts in each area. To all of them we are indebted for sharing their expertise. This book will be of enormous value to clinicians, embryologists, scientists, and all students of biomedical sciences.

David K. Gardner
Melbourne, Australia

Carlos Simón
Valencia, Spain

Contributors

Michael M. Alper
Boston IVF
Waltham, Massachusetts

Basak Balaban
Assisted Reproduction Unit
VKF American Hospital
Istanbul, Turkey

José Bellver
Instituto Valenciano de Infertilidad
and
Department of Pediatrics, Obstetrics and Gynecology
Faculty of Medicine
University of Valencia
and
IVI Foundation
Valencia, Spain

Ernesto Bosch
Department of Reproductive Medicine
Instituto Valenciano de Infertilidad
Valencia, Spain

Frank J.M. Broekmans
Department of Reproductive Medicine and Gynaecology
University Medical Center Utrecht
Utrecht, The Netherlands

Antonio Capalbo
G.EN.E.R.A. Centers for Reproductive Medicine
Rome, Italy

and

GENETYX Molecular Genetics Laboratory
Marostica, Italy

Danilo Cimadomo
G.EN.E.R.A. Centers for Reproductive Medicine
and
Dipartimento di Scienze Anatomiche
University of Rome "La Sapienza," Istologiche
Medico Legali e dell'Apparato Locomotore
Sezione Istologia ed Embriologia Medica
Rome, Italy

Ana Cobo
Cryobiology Unit
Instituto Valenciano de Infertilidad
Valencia, Spain

Michael J. Davies
Robinson Research Institute
The University of Adelaide
Adelaide, Australia

Christopher J. De Jonge
Department of Urology
University of Minnesota Medical Center
Minneapolis, Minnesota

Alan DeCherney
Program in Reproductive and Adult Endocrinology
National Institute of Child Health and Human Development
National Institutes of Health
Bethesda, Maryland

Bart C.J.M. Fauser
Department of Reproductive Medicine and Gynaecology
University Medical Center Utrecht
Utrecht, The Netherlands

Daniela Galliano
Instituto Valenciano de Infertilidad
Rome, Italy

and

Instituto Valenciano de Infertilidad
Barcelona, Spain

David K. Gardner
School of BioSciences
University of Melbourne
Melbourne, Australia

Eva Gomez
Igenomix
Parc Cientific Valencia University
Valencia, Spain

Stephanie Hallam
Good Start Genetics, Inc.
Cambridge, Massachusetts

Roger Hart
Fertility Specialists of Western Australia
and
School of Women's and Infant's Health
University of Western Australia
Perth, Australia

Mae Wu Healy
Program in Reproductive and Adult Endocrinology
National Institute of Child Health and Human Development
National Institutes of Health
Bethesda, Maryland

Micah J. Hill
Program in Reproductive and Adult Endocrinology
National Institute of Child Health and Human Development
National Institutes of Health
Bethesda, Maryland

Sujata Lalitkumar
Karolinska Institutet
Stockholm, Sweden

and

Igenomix
Parc Cientific Valencia University
Valencia, Spain

Michelle Lane
Robinson Research Institute
School of Paediatrics and Reproductive Health
University of Adelaide
Adelaide, Australia

Giulia Mariani
Department of Biomedical, Clinical and Experimental Sciences
Division of Obstetrics and Gynecology
University of Florence
Florence, Italy

David R. Meldrum
Reproductive Partners La Jolla
San Diego, California

Markus Montag
ilabcomm GmbH
Sankt Augustin, Germany

Vivienne M. Moore
School of Public Health
The University of Adelaide
Adelaide, Australia

David Mortimer
Oozoa Biomedical Inc.
Vancouver, Canada

Sharon T. Mortimer
Oozoa Biomedical Inc.
Vancouver, Canada

Zsolt Peter Nagy
Scientific and Laboratory Director
Reproductive Biology Associates
Atlanta, Georgia

Robert Najdecki
Scientific Director at Assisting Nature IVF Unit
Thessaloniki, Greece

Tassia K. Oswald
School of Psychology
The University of Adelaide
Adelaide, Australia

Evangelos Papanikolaou
Clinical and Scientific Director of Assisting NATURE Centre of Reproduction
Thessaloniki, Greece

Richard J. Paulson
Department of Obstetrics and Gynecology
Division of Reproductive Endocrinology and Infertility
University of Southern California
Keck School of Medicine
Los Angeles, California

Antonio Pellicer
Instituto Valenciano de Infertilidad
Rome, Italy

and

IVI Foundation
and
Faculty of Medicine, University of Valencia
Director of Instituto Valenciano de Infertilidad
Valencia, Spain

Nuria Pellicer
IVI Foundation
Valencia, Spain

Roger A. Pierson
Obstetrics, Gynaecology and Reproductive Sciences
College of Medicine
University of Saskatchewan
Saskatoon, Canada

Gregory Porreca
Good Start Genetics, Inc.
Cambridge, Massachusetts

Nina Resetkova
Boston IVF
Waltham, Massachusetts

and

Beth Israel Deaconess Medical Center
Department of Obstetrics and Gynecology
Harvard Medical School
Boston, Massachusetts

Laura Rienzi
G.EN.E.R.A. Centers for Reproductive Medicine
Rome, Italy

and

GENETYX Molecular Genetics Laboratory
Marostica, Italy

Carmen Rubio
Molecular Cytogenetics
Igenomix
Valencia, Spain

Maria Ruiz-Alonso
Igenomix
and
Parc Cientific Valencia University
Valencia, Spain

Alice R. Rumbold
Robinson Research Institute
The University of Adelaide
Adelaide, Australia

Carlos Simón
Instituto Universitario IVI/INCLIVA
Valencia University
Valencia, Spain

and

Igenomix S.L.

and

Department of Obstetrics and Gynecology
Stanford University
Stanford, California

and

Department of Obstetrics and Gynecology
Baylor College of Medicine
Houston, Texas

Sandra J. Tanahatoe
Department of Reproductive Medicine and Gynaecology
University Medical Center Utrecht
Utrecht, The Netherlands

Nathan R. Treff
Reproductive Medicine Associates of New Jersey
Basking Ridge, New Jersey

Filippo Maria Ubaldi
G.EN.E.R.A. Centers for Reproductive Medicine
Rome, Italy

and

GENETYX Molecular Genetics Laboratory
Marostica, Italy

Diana Valbuena
Igenomix
Parc Cientific Valencia University
Valencia, Spain

Felipe Vilella
Igenomix
Parc Cientific Valencia University
Valencia, Spain

Melanie L. Walls
Fertility Specialists of Western Australia
and
School of Women's and Infant's Health
University of Western Australia
Perth, Australia

Irene Woo
Department of Obstetrics and Gynecology
Division of Reproductive Endocrinology and Infertility
University of Southern California
Keck School of Medicine
Los Angeles, California

Contents

1

IVF: The First Four Decades

Mae Wu Healy, Micah J. Hill, and Alan DeCherney

Origins of Fertility and Reproductive Physiology

The concept of fertility has been linked throughout history to prosperity and womanhood. Dating back almost 40,000 years, cave paintings and sculptures depict females as well rounded figures showing importance on the ability to carry a child (1). Thus, the inability to procreate has caused much distress among many societies. Hippocrates, born in 460 BC, sought to logically explain different causes of infertility. He hypothesized infertility could be due to malposition of the cervix, softening of the endometrial cavity due to congenital origins, acquired from scarring after ulcers, obstruction of the opening of the uterus, excessive menstrual flow thus inability to fix the seed, or uterine prolapse (2).

Early understanding of reproductive physiology was limited. For instance, spermatozoa were thought to come initially from the bones (3). Another example is Aristotle's proposal that humans came from the egg, but sperm was what gave the egg its shape (4). In 1672, De Graaf described the physiology of the ovary and follicular function (5). Mistakenly, he described the follicle to be the egg (5). Further, Von Horne claimed infertility was due to a vicious humour inside the uterine cavity and recommended uterine irrigation to treat infertility (1).

In 1677, Antonii von Leeuwenhoek invented the microscope to study human semen, by which he discovered the spermatozoa a year later (1). He described them as "living animalcules in human semen..." believing the tails were operated by muscles, tendons, or joints (6,7). He also thought a spermatozoa contained an already formed embryo (1). The contributions of the egg and the sperm remained poorly understood for almost another 200 years until the work of Matthias Schleiden and Theodor Schwann. The pair hypothesized the egg and sperm were both individual cells with hereditary factors to contribute to each other (8). In 1875, Oskar Hertwig showed sea urchin fertilization could be achieved when one sperm cell penetrated the egg, thus illustrating the basics of genetic inheritance (9).

The Evolution of Artificial Insemination

Introduction

Almost a hundred years later, in 1786, John Hunter from London described the first artificial insemination in humans. Treating a male patient with severe hypospadias, Hunter advised him to collect his semen in a warmed syringe and inject it into his wife's vagina (10). Another pioneer, American J. Marion Sims, attempted 55 inseminations in the mid-1800s, reporting one pregnancy. At that time, he believed ovulation occurred during menstruation and focused much of his work on post-coital testing (10). In 1863, he published his work in the *Clinical Notes on Uterine Surgery*, which was regarded as both controversial and ahead of its time with its emphasis on treatment of infertility, including artificial insemination (10).

It was not until the 1890s that the idea of embryo transfer was described. Walter Heape, a professor and physician at the University of Cambridge in England, performed embryo transfer experiments between 1890 and 1897. The results were published in the *Proceedings of the Royal Society of London*,

illustrating the first case of embryo transplantation in rabbits. Heape described transferring two ova from an Angora doe rabbit that had been fertilized by a male Angora rabbit 32 hours prior. He placed the embryo into the fallopian tube of a Belgian Hare doe rabbit that had been mated with a Belgian Hale male rabbit three hours prior. The Belgian Hare doe delivered six babies, two with angora phenotypes and four with Belgian phenotypes (11,12).

Ahead of his time in 1932, Aldous Huxley wrote his science fiction novel *Brave New World* that conceptualized the technique of in vitro fertilization (IVF). Two years later, in an attempt to bridge fiction in vitro fertilization with reality, Pincus and Enzmann from the Laboratory of General Physiology at Harvard University, removed mammalian oocytes from the ovary and watched them undergo normal development in vitro (13). In 1948, Miriam Menken and John Rock retrieved eggs from over 800 women who were undergoing gynecology surgeries (14). They further exposed 138 of these oocytes to spermatozoa, reporting cleavage of the human embryos (14).

Donor Sperm, Rabbit Surrogacy, and Mice Embryo Transfers

In 1953, the first successful pregnancy from artificial insemination with frozen and thawed sperm was reported (10). With growing concern of transmitting HIV/AIDs with use of fresh sperm, the U.S. government began to require infectious disease screening, with a time period of quarantine prior to use (10). Thus, donor fresh sperm samples became exceedingly rare. Also, in an attempt to regulate the chances of unknowing marriage of biological siblings, the government restricted the number of times a single donor's sample could be used (10).

That same year, surrogacy became a reality. Min Chueh Chang, a scientist born in Tai Yuan, China, who studied in Cambridge, demonstrated this in rabbit experiments (15). First, Chang described growing rabbit embryos derived from oocytes fertilized by capacitated spermatozoa in a small Carrel flask (16). Six years later, he showed an egg from a female black rabbit could be fertilized in vitro by sperm from a black male rabbit. The embryo was then transferred to a white female rabbit, resulting in the birth of a black offspring (17). Chang also performed numerous studies on IVF in the hamster, mouse, and rat ova, all of which would serve as a foundation for Patrick Steptoe and Robert Edwards almost 20 years later (15).

While Chang continued his work on rabbits, in London developmental biologists Anne McLaren and Donald Michie were similarly working on this idea with mice. The pair developed and described mouse embryo transfer techniques of blastocysts to uterine-foster mothers (18). These findings were published in *Nature* in 1956 and released to the public under the headline "Brave New Mice" by the London *Daily Telegraph* (19).

Steptoe and Edwards

Many say the true start of IVF began with the work of Patrick Christopher Steptoe, a gynecologist, and Robert Geoffrey Edwards, a developmental geneticist. Together, their work pushed the limits of their time.

Patrick Steptoe was a member of the Royal College of Surgeons, licensed in 1939. During World War II, he volunteered for the Royal Navy Volunteer Reserves, serving as a Naval Surgeon until his ship was hit off Crete in 1941. He was captured and for 2 years remained a prisoner of war in Italy. Reports state he helped prisoners escape and thus landed himself in solitary confinement until he was released in 1943. After the war, Steptoe focused his work on sterilizations and gynecologic surgeries. Like many surgeons in the 1950s and 1960s, he sought alternatives to laparotomies. Learning of laparoscopy from Raoul Palmer in France and Hans Fragenheim in Germany, Steptoe began performing laparoscopic sterilization in England in the mid-1960s (20).

Concurrently, Robert Edwards, an expert in genetics, immunology, and embryology, worked with colleagues in Glasgow, Scotland, to produce the world's first embryonic stem cells from rabbit embryos. Intrigued by the idea of stem cells, Edwards turned to investigating human oocytes in vitro as a source of stem cells. Over the next several years, Edwards tried unsuccessfully to collaborate with clinicians in England to help him retrieve human eggs. Frustrated with these barriers, Edwards came to the United

States in 1965 and joined Georgeanna and Howard Jones at Johns Hopkins Hospital where they were performing ovarian wedge biopsies (21). One of his major accomplishments during his time in Baltimore, Maryland, was discovering that complete oocyte maturation in vitro took 37 hours (22). Thus, he concluded insemination should be carried out 35–40 hours after ovulation (22). By 1969, collaborating with his PhD student Barry Bavister, Edwards was able to fertilize human oocytes with spermatozoa (21).

In 1968, Edwards came across Steptoe's article in the *Lancet* on "Laparoscopy and Ovulation" (23). A chance meeting at a London conference initiated the famous partnership of Steptoe and Edwards (21). In 1970, Steptoe and Edwards performed their first laparoscopic oocyte retrieval (24), with the start of human embryo transfers a year later (21).

The First IVF Pregnancies

In 1973, across the world in Melbourne, Australia, Professors Carl Wood and John Leeton formed the Monash IVF research team. The group reported the first human IVF pregnancy (25). They described treating the patient with clomiphene citrate for 5 days with an ovulation trigger of human chorionic gonadotropin (HCG) 5000 International units (IU) on day 9 of her menstrual cycle. This was followed by laparotomy to remove the oocytes. About 74 hours after fertilization with her husband's sperm, an 8 cell zygote was transferred back to the patient's uterus (25). The pregnancy, unfortunately, resulted in an early miscarriage (25).

In 1976, Steptoe and Edwards reported the second IVF pregnancy, resulting in an ectopic pregnancy (26). The patient was stimulated with human menopausal gonadotropin (HMG) with an ovulation trigger of 5000 IU of HCG. The embryo transfer was performed between the morula and blastocyst stage, with a positive pregnancy test several weeks later. The patient then presented with pelvic and abdominal pain with a laparoscopy at 13 weeks gestation revealing a right tubal ectopic pregnancy. Pathology confirmed degenerate chorionic tissue (26).

During this time, U.K. colleagues, the media, and the Archbishop of Liverpool met the pair with criticism and accusations of malpractice. Despite this, Edwards and Steptoe persisted and finally achieved their first live birth with Louise Brown (27). The announcement of the successful birth of Louise in Oldham, England, on July 25, 1978, was met with some acceptance, but more skepticism and doubts (21). After 1978, the progress of Edwards and Steptoe stalled for 2 years due to the retirement of Steptoe from Britain's National Health Service (21). By 1980, their private laboratory in Bourn Hall near Cambridge was up and running and served as the center to one of the most well-known laboratories in the world today (21).

By 1978, Howard and Georgeanna Jones had retired from Johns Hopkins to start their own IVF program at the Eastern Virginia Medical School in Norfolk. During that year, out of 41 laparoscopic oocyte retrievals, only 13 patients had embryos that underwent cleavage, and no pregnancies were achieved. By 1981, after many failed cycles, Georgeanna Jones altered their protocol from a natural cycle to one with ovarian stimulation with HMG in hope of obtaining more oocytes. Following this change in protocol, they achieved America's first IVF pregnancy, the world's fourth IVF baby. This was their 13th attempted stimulated cycle. Elizabeth Jordan Carr was born in December 1981 at Norfolk General Hospital in Norfolk, Virginia.

Advancing the Field

Bourn Hall Meeting

During this time of rapid advancements in IVF, Edwards called together a gathering at Bourn Hall with the top physicians and scientists in the field (21). During this meeting, held in September of 1981, a consensus was formed, with five important concepts that have guided IVF protocols even to today. Agreed upon were stimulated cycles were better than natural cycles, to increase number of oocytes retrieved and allow for better prediction of ovulation timing. Secondly, ultrasounds should be used to monitor growth of follicles. Thirdly, progesterone supplementation should be used for luteal support. Two additional areas of weaknesses were identified, one of which was quality control in culture media and laboratory

processes. The other concern was the effect of gas on oocyte quality with laparoscopy (21). Over the next several decades, research and changes to the field were focused on improving each of these five concepts.

Pharmaceutical Advancements

The rising use of ovarian stimulation led to increased pharmaceutical development of stimulation medications. Starting in the 1950s, Gemzell described using human pituitary follicle-stimulating hormone (FSH), obtained at time of human autopsies, to make partially purified FSH preparations (28). In the early 1960s, Lunenfeld introduced HMG extracted from post-menopausal urine (29).

From here, the idea of making a more consistent preparation pushed pharmaceutical companies to isolate just the FSH from urine by treatment with polyclonal antibodies to remove the luteinizing hormone (LH), leading to urinary human FSH. In the 1990s, monoclonal antibodies specific to FSH were used to further refine the product, leading to highly purified urinary FSH. Keene and his colleagues described the first recombinant FSH produced from genetically engineered Chinese hamster ovaries (30). These described preparations are still being used today.

Furthermore, in 1981, Trounson and his colleagues described the use of clomiphene citrate with HMG in IVF treatment protocols (31). In Vienna, Feichtinger and Kemeter reported the first live births of twins using clomiphene citrate in August 1982 (32). Within the next 10 years, the first papers describing the use of letrozole, an aromatase inhibitor, for ovulation induction was published (33,34). Today, clomiphene citrate and letrozole are the most common oral agents used for controlled ovarian hyperstimulation (COH).

Technological and Laboratory Advancements

Around the world, groups worked on advancements in the laboratory that continued to improve the IVF process. In 1976, Menezo developed the B2 culture medium, known as the "French medium," that mimicked the follicular, tubal, and uterine environments of the sheep, rabbits, and humans (35). Simultaneously, the Melbourne group worked on improvements to the culture medias (36,37) in addition to developing Teflon-lined catheters to help improve embryo transfers (38). There were also new developments in laboratory assays. Frydman and Testart from the University Hospital in Clamart, France, created an assay to test for the initial rise of LH in plasma (39). This more accurately predicted the LH surge, thus helping to predict the ideal time for retrieval of the oocytes (39).

Better fertilization techniques continued to be investigated throughout the late 1980s. In 1987, Laws-King and colleagues described a micromanipulation method that involved taking a single spermatozoa and placing it under the zona pellucida (40). This helped overcome severe male factor infertility. Authors reported a high rate of fertilization with minimal damage to the oocyte (40). Shortly after this technique was described, the first birth using the subzonal sperm insemination (SUZI) method was described from the National University of Singapore (41).

In 1992, Gianpiero Palermo reported a significant breakthrough for male factor infertility. While attempting to perform subzonal injection of sperm for fertilization, Palermo noted if the sperm was instead injected through the zona pellucida and into the ooplasm of the metaphase-II oocyte, fertilization would also occur. His group published this as intracytoplasmic sperm injection (ICSI) in *The Lancet*, describing the first four pregnancies with this technique (42). Studies since have demonstrated better fertilization and pregnancy rates with ICSI over SUZI (43,44). Thus, ICSI has remained the standard of care, successfully treating cases of severe male factor infertility.

Additionally, other novel laboratory techniques continued to be investigated. Jacques Cohen, an embryologist who worked with Steptoe and Edwards in the 1980s at Bourn Hall, pioneered many of these new techniques. In 1990, Cohen, who was then at Emory University School of Medicine, introduced a new concept to assist in implantation. At that time, the rate of embryonic implantation, or babies born per embryo transferred, in the United States was <5% (45). Cohen's group hypothesized a substantial number of healthy IVF embryos failed implantation due to inability to hatch from the zona pellucida (46). Thus, they described piercing the zona pellucida with a microneedle until the needle tip was seen in the perivitelline space, otherwise known as assisted hatching (46). The result was a doubling in incidence of embryonic implantation from 11% to 23% (46). Since the technique was first described, there has been controversial debate of its contribution to increasing live birth rates. In 2012, the most recent Cochran

Review showed an increased chance of achieving a clinical pregnancy with assisted hatching; however, there is still insufficient evidence on live birth rate (47). Thus, since Cohen's group first introduced this technique, fertility clinics today still continue to use this for specific patient populations.

Ultrasound

In 1972, Kratochwil described ultrasonic tomography of the ovaries, overcoming poor visualization of the ovarian follicles during stimulation (48). This led to more accurate predictions of oocyte maturity and thus better timing of oocyte retrievals. Improvements in ultrasound technology also vastly improved both the number of oocytes retrieved during a cycle in addition to decreasing complications during the retrieval (49–52). Lenz and Lauritsen first described ultrasound guided trans-abdominal and trans-vesical aspirations under both local anesthesia or general anesthesia (49). In their group of 30 patients reported, oocytes were obtained in 57% of the cases. The only complication noted was transient hematuria (49). The technique was concluded to be atraumatic and inexpensive, thus was the recommended technique at that time over laparotomy and laparoscopic retrievals (49).

In 1983, Gleicher and his colleagues described the trans-abdominal ultrasound guided trans-vaginal needle oocyte aspiration. Their technique involved using a metal speculum with a tenaculum placed on the posterior lip of the cervix to manipulate the cervix upwards, exposing the posterior cul-de-sac. A 16 G spinal needle was passed through the cul-de-sac into the ovaries under ultrasound guidance. The benefits of this technique was a decrease in trauma to abdominal organs, notably the bladder, upon entry, in addition to not requiring general anesthesia (53). With this less invasive technique, their hope was to eventually make this an office procedure.

The introduction of vaginal ultrasound probes further changed many practices. In 1985, Wikland et al. reported the first trans-vaginal ultrasound-guided oocyte retrieval (TVOR) (54). Additionally, the vaginal probe needle introducer was introduced by Yale University to improve accuracy in follicular puncture and aspiration of oocytes. The benefits of TVOR were shortened operating time (55), no general anesthesia needed, less complications including pain, infection, and bleeding, in addition to being more cost effective (56). This described technique has thus remained the standard of care.

With improvements in oocyte retrievals under ultrasound guidance, investigators and physicians alike began looking into trans-abdominal ultrasound-guided embryo transfers. This was compared to transferring with the "clinical feel." This technique, described by Strickler et al. (57), allows for accurate positioning of the catheter tip in the uterine cavity, avoiding pressing the catheter tip in the fundus, allowing for better navigation through difficult utero-cervical junctions, and reassurance for both patient and physicians of proper placement of the embryo at the completion of an IVF cycle (58–60). This technique remains the recommended method to assist in embryo transfers today (61,62).

Cryopreservation

During the 1980s, there were increasing IVF cases of multiple gestations due to the high number of embryos being transferred back. The first triplets from IVF was reported in 1983 (63), followed by quadruplets in 1984 (64). The idea of cryopreserving embryos would allow patients to save additional embryos for the future, thus decreasing the number of embryos transferred back in the original fresh cycle. In 1983, the Monash group reported the first cryopreservation of a human embryo using the slow freezing method (65). More specifically, Trounson and Mohr described the techniques that would allow a four and eight cell embryo to survive the freeze, storage, and thaw process. Out of 15 patients described in their study, one became pregnant. The pregnancy resulted in a fetal loss at 24 weeks gestation after premature membrane rupture with chorioamnionitis (65). In 1985, Cohen's group reported the birth of the first term baby from a cryopreserved embryo (66).

The original slow rate freezing method was associated with low survival of the embryos once thawed and low pregnancy rates. This, with the high cost of cryopreservation, pushed others to search for better techniques. In the late 1980s, the ultra-rapid freezing protocol was described (67). This technique, known as vitrification, is based on the idea of applying a higher concentration of cryo-protectants and a rapid cooling speed to prevent the formation of intracellular ice crystals (68). This technique is faster to perform, offers better control of the process, and more importantly offers a high post-thaw survival rate

with improved pregnancy rates and live birth rates (69). Thus, since vitrification was described, embryologists have continued to view this as the gold standard. In addition, since further improvements on oocyte vitrification technique have been made, American Society for Reproductive Medicine (ASRM) in 2013, has declared egg freezing no longer experimental (70).

Since 2000, there have been many changes in the field of onco-fertility, a big part due to the advancements in cryopreservation. One strategy for fertility preservation is to take the patient, prior to their gonadotoxic therapy, through an IVF cycle, oocyte retrieval, and vitrify their oocytes or embryos (71). Another strategy is to remove a portion of ovarian tissue prior to therapy with a re-transplantation later on. Oktay and Karlikaya reported the first ovarian transplant after cryopreservation of the tissue (72). Four years later, Donnez and colleagues achieved the first live birth after orthotopic transplantation of the cryopreserved ovarian tissue (73). In the last decade, groups have been investigating fertility preservation among cancer patients both with oocyte cryopreservation and the use of in vitro maturation (74,75). In 2008, Porcu reported the first birth of healthy twins after oocyte cryopreservation and bilateral ovariectomy in a cancer patient survivor (76). Per ASRM in 2013, oncology patients should have discussed with them options of fertility preservation and future reproduction after gonadotoxic treatment prior to treatment (77).

Donor Oocytes

In 1983, the Monash team reported the first successful pregnancy in a woman using a donor embryo, resulting in a spontaneous abortion (66). Across the world, at the UCLA Medical Center, John E. Buster's group described the first successful pregnancy after oocyte donation, opening up possibilities to infertile women with diminished ovarian reserve or primary ovarian insufficiency (78,79). Since then, egg donation has become a viable option, with either vitrified eggs through a cryobank or fresh donor eggs, with good pregnancy and live birth rates.

Protocol Improvements

Ovarian stimulation protocols have improved throughout the years. During the first decade of natural and stimulated cycles, physicians were noting a high cancellation rate due to premature ovulation prior to oocyte retrievals. Thus, in 1982, Fleming and his colleagues described using gonadotropin-releasing hormone (GnRH) agonist to help eliminate the premature luteinization and to help better control ovarian stimulation (80). In 1991, Frydman and his colleagues described the use of a gonadotropin releasing hormone antagonist to help prevent both LH and progesterone rises during the stimulation (81). Both these techniques are integrated into common standard protocols today.

The benefit of a GnRH agonist was found to be more than solely suppressing premature luteinization. In the early 1980s, most fertility centers were using clomiphene citrate and gonadotropin with only two to four oocytes retrieved per cycle. In 1984, focusing on increasing the number of oocyte recruited and thus retrieved and fertilized, Porter and his colleagues reported the idea of inducing multifollicular development by pituitary desensitization (82). They were the first group to propose that GnRH agonist also increased the number of oocytes recovered. Per their study, they reported a total of 87 oocytes over 11 cycles with GnRH agonist as compared to 21 oocytes retrieved in 20 cycles without GnRH agonist (82).

Other groups continued to brainstorm ideas for improved follicular recruitment prior to ovarian stimulation. Gonen and colleagues in 1990 described using gonadotropin suppression with oral contraceptives before IVF (83). The idea was studied among 181 stimulation cycles compared to 113 stimulation cycles without pituitary suppression. Their data demonstrated oral contraceptive pills (OCPs) significantly helped recruit more follicles with more oocytes retrieved than the nontreatment group. In addition, it helped prevent spontaneous LH surges (83). Both Porter and Gonen's groups laid the foundation for pituitary suppression prior to a stimulated cycle.

In 1990, Gonen's group introduced the use of a gonadotropin-releasing hormone agonist for triggering follicular maturation (84). They compared using 0.5 mg of leuprolide acetate to 5000 IU of HCG in 18 cycles of IVF among 14 patients. One significant finding was LH and FSH serum levels remained elevated for only 34 hours after the GnRH agonist. Comparably, mean HCG levels stayed elevated for 6 days after administration. The mean number of oocytes retrieved and the number and quality of the