In Vitro Fertilization and Micromanipulation

The History That Changed the Treatment of Male Factor Infertility

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Male Factor Infertility: Historical Aspects Before 1980

Not everything in medical science has a clear beginning. The first realization of infertility and putative remedies remain shrouded in contextual history, but likely goes back to the dawn of our species, well before there was a written record. Childlessness was, and is still, considered a burden in some communities. Early feminism and medical enlightenment in the nineteenth century, such as the discovery of the ovum in 1827 by Von Baer [1] and the observation of the mammalian fertilization process in sea urchins by Oskar Hertwig in 1876 and rabbit by Leopold Schenk in 1878 [2], laid the foundations for our understanding of the role of spermatozoa and oocytes in reproduction, although the concept of the infertile man was not deeply explored or reported until the late 1920s [3,4]. Sperm function studies commenced only after the Second World War, with semen analysis becoming a systematic and published diagnostic method using mathematical endpoints in a similar approach to that of blood chemistry laboratories [5–7]; mammalian spermatozoa being successfully cryopreserved by Polge and co-workers in 1949 [8] and later in the human by Bunge and Sherman in 1953 [9]; and the maturation processes which spermatozoa had to undergo after meiosis being described in some detail [10,11]. Since the early 1970s there has been an increase in the development of new assays studying seminal chemistry, male pronuclear formation, membrane fusion, genetics and complex morphology, but despite much progress, many aspects of spermatozoa and the fertilization process remain unexplored [12,13]. Microscopic semen analysis provided medical fertility specialists interested in andrology with tools for diagnosis and possible treatment, and even in the relative absence of a scientific basis and medical literature in the 1930s, some open-minded practitioners taught Obstetrics and Gynecology residents about the reality of male infertility. When Patrick Steptoe was a pre-war registrar at St. George's Hospital in London, his clinical supervisor in the National Health Service, consultant Mr. Gwillim, explained that as many as one-third of clinical infertility cases could be traced back to male factor [14], but demographic publications were hardly available. It would be another 40 years before a suitable and universal treatment was found in in vitro fertilization (IVF) and particularly once the egg was microsurgically prepared to promote fertilization using fledgling new micromanipulation procedures [15–20]. This culminated in the development of a definitive solution (although not a remedy) for male factor infertility, first successfully applied at the Free University of Brussels in Belgium: the direct injection of a single immobilized spermatozoon into the ooplasm, a procedure the authors described as intra-cytoplasmic sperm injection (ICSI) [21]. This chapter reviews the brief but intense period of the exploration of male factor treatment using IVF and derived micromanipulation technologies before the Brussels team's publication of ICSI in 1992.

Natural procreation is a dance that involves two partners, although historically, women were blamed for unwanted childlessness unless the male partner was impotent. The latter was often confused with infertility [22]. The existence of male infertility was abjured by public individuals such as George Washington, who decried his spouse's alleged infertility even though she had children from a prior marriage. Male factor infertility in contemporary reproductive medicine is considered to be either the trigger or a secondary factor of involuntary childlessness. Contemporary studies over the past 20 years show that infertility is directly or indirectly affected by male factor in as many as 30–50% of cases [23].

Before the advent of in vitro fertilization (IVF) in the late 1970s, male factor infertility was rarely taught in medical class. The field of andrology appeared to be

of no interest among most male specialists dominating gynecological research, and so it is perhaps not surprising that the causes and treatment for male infertility were not well studied until the routine application of IVF. In IVF and other derivatives since, the female partner is often the focus of the procedures, not because of the probable underlying etiology, but because it is much more complicated to obtain female gametes – at least in the vast majority of couples. Following the birth of Louise Brown, when embryologists were able to show that fertilization could be established in couples with male factor infertility, there was a surge of interest in sperm function and the physiological role of men in reproduction [24–26]. Though some male factor cases were successful, the method of IVF as an alternative treatment was not clearly shown to be more successful than artificial insemination or even natural procreation [27]. No randomized clinical trials were conducted comparing the various routes of alleged treatment, and prospective trials of male factor infertility treatment by IVF or modifications using micromanipulation were not performed. This was typical for the entire period of investigation of male factor treatment using assisted reproduction methods between 1980 and 1995. It is remarkable that the standard of the randomized controlled trial in the IVF laboratory was only explored from 1990 onward [28,29], and the first systematic reviews in infertility research also date from the early 1990s. A well-documented systematic review of infertility treatment and research, conducted by the University of Leeds in 1993, showed that randomized methodology was introduced relatively late in infertility practice compared to other branches of medicine [30]. An exponential rise in identified controlled trials with pregnancy as an outcome occurred only after 1990, alongside a simultaneous increase in the routine practice of assisted reproductive technology in multiple countries. Treatment of idiopathic oligo-astheno-teratozoospermia was investigated broadly without assisted reproduction, as was artificial insemination by partner or donor spermatozoa for male factor infertility. Only 60 trials were identified for male factor treatment without assisted reproduction till 1993, with fewer than 3000 patients tested [30]. Oocyte and embryo preparation or culture were tested in another 17 trials, mostly involving comparisons of embryo culture media. Sperm preparation trials were only reported in four investigations and none involved a comparison of IVF with either artificial insemination or micromanipulation of the oocyte. In the period between 1980 and 1992, preceding ICSI, there was a series of studies of IVF insemination in cases of male factor infertility and the first attempts at microsurgical fertilization [17,18], but without systematic comparison involving control groups and randomization. These studies included patients who either had failed fertilization during previous attempts of IVF or where the male partner had a diagnosis of abnormal semen analysis and so were considered inappropriate for further clinical treatment without some form of assisted reproduction. Results of the experimental procedures were compared with prior unsuccessful attempts, or the procedure was simply performed based on poor semen analysis, a very low yield of spermatozoa after semen preparation and male factor history. This approach may be considered archaic from an evidence-based point of view, but the use of a control group would have meant that participants had either no treatment or a treatment that was known not to work well in those years, such as artificial insemination in cases of extreme male factor infertility. As a consequence, the use of prospective series investigations of new experimental approaches seemed to be the only ethical alternative at the time. The relatively low outcomes following conventional IVF in most patient groups undoubtedly played a role as well.

In Vitro Fertilization: A Treatment for Male Factor Infertility?

When the genius population geneticist Haldane gave a private lecture for a group of Cambridge (UK) academics on the future of society and technology in 1923, he contemplated oddly on the history of the future of technology by projecting it retrospectively from hundreds of years into the future, and on the possibility of the processes of IVF, embryo culture and artificial gestation [31]. He branded the process as an apparent obvious solution for population planning and called it "ectogenesis," a name that surprisingly was not popularized. Not too many details were given, but his friend, the journalist and author Aldous Huxley filled in a few gaps a decade later with the publication of the novel *Brave New World* in 1932. Neither predicted that ectogenesis would have its origin by treating less fertile individuals, nor did the authors distinguish between preimplantation and postimplantation development.

The possibility of IVF as a treatment for infertility was first suggested in 1937, in a short editorial in *The New England Journal of Medicine* by Dr. John Rock, a highly regarded ObGyn at Harvard University who would later become a key scientist in the development of the anti-conception pill. He and his laboratory partner Miriam Menkin were presumably the first to isolate and attempt to fertilize human oocytes in vitro [32]. At the time of the 1937 editorial, however, the idea of IVF was perceived to be so outrageous that even the author avoided claiming it, and the editorial was published anonymously. The concept of assisted procreation had matured from being proposed as a futuristic method of general reproduction for anyone (Haldane and Huxley), into a clinical treatment for "barren" women with tubal disease. There was no mention of treating male factor infertility by in vitro fertilization in Rock's letter. The possibility of treating infertile men this way may have been contemplated by IVF pioneers in the UK, Australia and the USA in the 1970s, but there is no clear mention of it in Steptoe's and Edwards' book *A Matter of Life*, which tells the story of their 11-year collaboration prior to establishing the world's first purpose-built IVF clinic at Bourn Hall near Cambridge in the latter half of 1980 [33]. Nor was it suggested in Edwards' famous view of the future of assisted reproduction in 1965 [34] or in the short letter to the *Lancet* announcing the birth of Louise Brown [14], which described the case of Leslie Brown and her husband and characteristic tubal infertility. The entire series of cases performed before the birth of baby Louise was only evaluated in depth in a group of articles published in 2013 in *Reproductive Biomedicine Online* (RBMO) by Elder and Johnson, after the death of the pioneers, Purdy, Edwards and Steptoe. However, four of the pregnancies established in Oldham, of which only two led to births in 1978 and 1979, were described in a full-length paper by Edwards, Steptoe and Purdy in 1980 [35]. In characteristically understated prose they postulated that "*There seems no reason why similar methods of [IVF and embryo culture] treatment should not be applied in cases of infertility arising in some men with oligospermia, because so few spermatozoa are needed for fertilization in vitro.*" Some experts at the time reacted disapprovingly to this, although it does not seem that they confronted the authors publicly in writing. There was a fear among experts that morphologically and functionally abnormal spermatozoa could lead to congenital malformations, but this was

soon put to rest when the first studies of treating infertile men by IVF were published. This fear was founded in terms of anomalies of somatic cell morphology, as abnormally shaped somatic cells and cells with differing physiology have classically been associated with disease. Poikilocytosis and anisocytosis are examples of abnormally shaped blood cells associated with forms of vitamin deficiency, iron-deficient anemia and other malfunctions.

The first references to, and description of, treating male factor infertility by in vitro fertilization are included in the proceedings of the first human conception in vitro meeting (this was before the general terminology of IVF was accepted internationally). This meeting was held at Bourn Hall Clinic in 1981 and the proceedings, edited by Bob Edwards and Jean Purdy, were published in the spring of 1982 [24]. In this, the emerging possibility of using IVF to treat oligospermia and immunological infertility was described by Fishel and Edwards. The first two babies from oligospermic men were reported in a series of 182 infertile couples, and the likely limitations of treating severe oligospermia with IVF were also discussed by Mettler's team from Germany. In a series of eight oligospermic cases reported by Fishel and Edwards, five had one oocyte fertilized, but details of semen analysis were not presented, although sperm quality in the insemination droplets was provided, and one case was presented as having severe oligospermia, without further details. It is presumed that this patient had less than 5 million spermatozoa per mL in his semen. The general fertilization rate with "acceptable" spermatozoa was 85%. In 31 patients, observations of the spermatozoa in the insemination droplets identified features that were considered to reduce fertilization. It is unknown how many of those cases were classified as male factor infertility: formal diagnosis was not always evident in those days, routine semen analyses not universal, and most early IVF clinics did not have male reproductive specialists on staff. All of these patients had natural cycles or some clomiphene was given during the early follicular phase. Of the 33 mature oocytes, 14 (42%) were fertilized, and sperm agglutination was reported to be compatible with fertilization. During the discussion between the participants, Simon Fishel commented that fertilization was still possible at concentrations of 1 million spermatozoa, albeit at a much lower rate (it is possible that he referred to the prepared specimen and not the initial semen

sample). Fertilization was apparently normal in cases of unexplained infertility. In the contemporaneous series of 25 couples with abnormal semen analysis reported in the same book by Mettler, no embryo cleavage was observed after IVF, perhaps indicating that there were differences between laboratories and their ability to obtain motile spermatozoa for in vitro insemination from men with adverse semen analyses. The two teams apparently did not delve into the differences of their approaches. Regardless of these modest results during the early days of IVF, the establishment of pregnancy and live birth reported by Fishel and Edwards suggested that male factor could be treated successfully with IVF, and the ability to fertilize enhanced by optimizing sperm preparation technologies. Another small series of cases was reported by the Monash group from patients seen between 1980 and 1983, indicating that the Australian team also attempted to fertilize oocytes and transfer embryos from male factor cases early on [25,26]. Their work will be described in some detail below.

Major questions that remained unanswered, at least for a while, were how to optimize the process of removing seminal plasma, micro-organisms and somatic cells, and how to determine the lowest threshold values for reduced sperm count, reduced motility and elevated frequency of abnormally shaped spermatozoa, a class of anomalies collectively referred to as oligo-astheno-teratozoospermia. It was clearly on the minds of reproductive specialists to follow up the pregnancies and children from IVF-related procedures and, most importantly, to collect evidence by performing prospective trials or indirectly through retrospective analysis and comparisons with untreated control groups. However, studies performed over the following 10–15 years showed that there was little impetus to follow and consult the couples with male factor infertility; many of the patients in those early years traveled over long distances to attend the first few clinics, and hence after discharge, many patients did not stay in contact with their clinics. Also, there may have been a lack of funding for follow-up studies. Short- and long-term safety of offspring, details about miscarriage and rare birth defects were only first published in the 1990s and 2000s [36], well after the new experimental procedures involving microsurgical fertilization had been introduced to enhance the chances of fertilization during IVF beyond just insemination and fertilization

in vitro. It was not until the publication of the work by the Brussels group that serious attempts at understanding the potential consequences of artificially forcing the process of fertilization were evaluated [37]. The debate regarding possible consequences in offspring from infertile men is still ongoing nearly 40 years after the first few pregnancies were established in the UK and Australia.

Mahadevan and co-workers from Alan Trounson's and Carl Wood's laboratory at Monash University described the use of IVF in five different groups of infertility patients [25]. Several teams had discovered earlier that IVF was not only a treatment for tubal infertility but also for other types of patients, such as those with unexplained and male factor infertility [24,35,38–40]. A separate paper by the Monash team described the successful application of IVF in men who had persistently low-quality semen, with 58% of the 45 patients studied having at least one successful fertilization [26]. Almost all of those embryos were transferred, with seven patients becoming pregnant in 63 cycles, and four babies born from three pregnancies (one was a twin birth). Fertilization was highly dependent on sperm motility and morphology, with no fertilization observed when motility was below 30%. It was stated that once embryos were obtained pregnancy did not seem to be affected, so the success of the method would be highly dependent on fertilization rate. During the 1981 Bourn Hall meeting, Edwards had assured his colleagues that motility was less a concern and that an effect was only seen when motility was less than 10%, but he did not provide details of the sperm preparation. At least a quarter of the male factor patients treated at Bourn Hall before 1982 had total fertilization failure, but success rates very much varied according to etiology.

The first broad prospective case series of treating infertile men using IVF were published in relatively quick succession ([26,38,41]. The confidence of the Bourn Hall team was reflected in the title of their publication: "In vitro fertilization, a treatment for male factor infertility" [41], which suggested that the usefulness of IVF for cases of male factor was no longer in question. This was a limited series, reporting on 122 couples over a period of 20 months, rather than the entire experience between 1980 and 1985. Four groups were included: patients with clear fertility in the female partners and infertility in the men; couples in which both partners were considered

infertile; a small group of 13 couples with normal semen analyses before IVF but a clearly abnormal semen analysis during their IVF cycles; and seven couples with very high concentrations of spermatozoa (polyzoospermia), a now outdated and unlikely clinical condition.

New sperm preparation techniques were aimed at both removing seminal plasma, as it was considered toxic to gametes as well as the fertilization process [42], and also obtaining a high proportion of motile spermatozoa. Several sperm preparation methods were developed at Bourn Hall, partly based on prior work. The first of these was a fairly labor-intensive method of sample aggregation used in cases of extreme oligoasthenozoospermia, which relied on the collection of multiple samples over a one or two-week period [40]. For each sample, spermatozoa were prepared in a droplet of seminal plasma-free medium and stored at room temperature, as the loss of motility was lower than for storage at 37°C. Aggregated samples were then used for small volume insemination. The system for semen collection was considerably diverse during those days with the use of split ejaculates and collection directly into culture medium [41]. Collection in a single dry container in cases of male factor infertility was rare. Another preparation technique was the so-called sedimentation method, which involved the standard method of minimally two-step mild centrifugation and resuspension in 1–2 mL of fresh medium. The sample was then placed in a flat Petri dish covered with paraffin oil and left to sediment for 1–24 hours at room temperature under a 5% O_2, 5% CO_2 and 90% N_2 gas atmosphere in a glass desiccator. Cells and debris would fall to the bottom of the large droplet and spermatozoa would be removed from the top layer. These first two techniques were not reported in the early Monash work, but it is unknown if that would have made a difference. Another method involved swim-up and migration of motile spermatozoa into culture medium developed in the 1950s, according to Mortimer [43]. This technique was also described by the Monash team. It must be kept in mind that until the second half of the 1980s, sperm density gradients such as Percoll had not been tested clinically for sperm preparation [44]. Within a few years after the introduction of IVF for male factor infertility, density gradients would become a common type of sperm preparation and by 1990 many samples were treated that way. This may explain some of the rapid

improvement seen with widescale micromanipulation in the early 1990s. Again, we do not know the extent of improvement, because comparative studies were not performed or published until very recently [45]. This study showed that swim-up techniques are either equivalent or better for embryo quality than density gradients, but that retrospective trial though multicenter was not randomized and seemed underpowered, increasing the chance of bias. Even after nearly 30 years of using ICSI, the efficacy and optimization of adjunct technology remains unclear.

Why was IVF not powerful enough for treating all cases of male factor? Which group benefitted and which did not? Without micromanipulation, fertilization rates were considerably lower than the results from ICSI as practiced nowadays, probably by a factor 2 or 3. In addition, with ICSI, samples from men with extremely low counts can still be used and azoospermic men can usually be treated using testicular or epidydimal biopsy. Samples with abnormal sperm morphology probably had the highest level of success, but reduced motility seemed particularly limiting as described by some groups [26]. This effect of motility was not seen by other teams [41], indicating that there may have been qualitative differences in sperm isolation and removal of seminal plasma. Rates of complete fertilization failure in obviously male factor couples were high, at 39% per cycle as reported by the Bourn Hall group [41], yet pregnancy rates after embryo transfer were comparable or better to those patients with other etiologies [46]. The worst results were seen when there was a combination of asthenozoospermia and oligozoospermia with a total fertilization failure rate of 60%, although deliveries of ongoing pregnancies were described for seven couples with extreme asthenozoospermia (< 3% motility) and sperm concentrations less than 1 million/mL, for whom the sample aggregation method of sperm preparation had been used. The major drawbacks of the IVF method were clearly the high incidence of complete fertilization failure and the labor-intensity of the sperm preparation procedures. The results quickly led to a realization that the methodology of IVF was not optimal for male factor infertility treatment, even though implantation rates were allegedly higher than in other groups of patients as many of the female partners were young and fertile. Micromanipulation of the fertilization process by direct injection of a spermatozoon into the ooplasm in the mouse was performed before publications of the first male factor

IVF series (Cohen and Zeilmaker, unpublished experiments; Trounson, personal communication), but progress in this area and live birth after assisted fertilization in the mouse was only first published by Gordon and Talansky in 1986 [15].

How Micromanipulation Became a Revolution for Male Factor Infertility Treatment

The micromanipulator, a device which aids in the microdissection and surgery of the living cell, was developed by Robert Chambers while at NYU (New York, USA) in 1912. His invention allowed dissection of the cell and separation of the chromosomes for the first time. Micromanipulation has been used in experimental cell biology for more than 100 years (https://utsic.org/2013/01/10/165/), and in experimental embryology and veterinary medicine for at least 50 years. The device and sub-components hook onto a microscope stage giving the observer a chance to precisely control small glass needles in three-dimensional space while visualizing the process through an inverted microscope. Combining the apparatus with an inverted microscope allowed free access to the slide, dish or setup containing the specimen above an array of changeable lenses. During the 1970s, this setup was a familiar tool in many experimental embryology laboratories and could also be equipped with real-time video and a monitor. These interphases gave biologists a system to fine-tune movements, removing or reducing the effects of normal hand tremor and permitting simple as well as complicated cell surgical interventions.

During the first years after the birth of Louise Brown, several embryologists suggested that the egg could be micromanipulated to come into very close contact with spermatozoa to promote fertilization, even when few spermatozoa were retrieved from seminal plasma. The initial experiments were rarely shared, but several investigators attempted to fertilize mouse oocytes microsurgically, using micromanipulation to directly inject spermatozoa into the ooplasm [47,48], although others did not publish their findings (Trounson, personal communication; Zeilmaker and Cohen, unpublished observations). There was very limited success in the mouse because of invasive membrane breakage and high rates of oocyte degeneration caused by piercing the egg with the relatively blunt glass needles used at the time. The reasons for

these failures are easy to determine in hindsight: the needles were not sharp enough, the glass relatively thick, an aperture/glass thickness ratio that remained the same after pulling (miniaturization of the capillary dimension), and the mouse oocyte membrane was a poor model as its oolemma is relatively fragile. The mature mouse oolemma breaks easily upon piercing with any instrument larger than a simple fluid injection needle such as those used in early gene injection experiments. Those very thin injection needles were made by pulling thin capillaries and gently breaking the closed tip on a holding pipette [49]. However, a larger diameter needle was necessary to accommodate the fairly large mouse spermatozoon. The first successful penetration of the oolemma and formation of a male pronucleus was demonstrated by early procedures in Yanagimachi's pioneering laboratory in Honolulu, following the injection of human and hamster spermatozoa into hamster oocytes [47]. This work was performed at the same time as the zona-free hamster egg test for human sperm function was being developed in the same laboratory [50]. Hamster oocytes are still a model for testing and training in the ICSI procedure, but other than demonstrating male pronucleus formation, the zygotes do not develop in vitro.

A pre-clinical model for microsurgical fertilization was required and the mouse oocyte was considered to be the most obvious system, as mouse in vitro fertilization and development to full-term had been established years earlier [51]. However, mouse oocytes are not easily microinjected with spermatozoa and often degenerate upon injection. Experimental embryologist Clement Markert (1983) [48] commented ironically when showing that fertilization could be established after single sperm microinjection "*The principal problem encountered in injecting sperm directly into the egg is that most of the eggs die at once from the microsurgical injury.*" Nevertheless, Markert showed that even phenotypically challenged spermatozoa could fertilize a denuded mouse egg after injection. He also demonstrated, as did Yanagimachi before him, that all outer investments such as cumulus cells and zona pellucida were apparently unnecessary for fertilization once the sperm cell was directly exposed to the ooplasm. The fertilized eggs rarely developed to blastocysts and presumably because of this, embryos were not transferred to recipient foster females. Survival, fertilization and development rates were not provided in

Markert's 1983 paper, and it would take another decade before it became technically feasible for micro-injected mouse eggs to routinely survive. The problem was related to the structure and behavior of the mouse oolemma during mechanical injection, and Kimura and Yanagimachi (1995) showed that a high rate of success with ICSI was only possible using a piezo-mediated drive of the needle into the ooplasm [52]. This was the first time that offspring was obtained after ICSI-derived technology in the mouse. Microsurgical fertilization is a rare example of embryology research where the clinical experiment usually preceded animal work [15–18,21].

The concept of sub-zonal sperm insertion – deposition of one or more motile spermatozoa into the perivitelline space – was first introduced by Alan Trounson's team at Monash University [53,16], but it was Jon Gordon and Beth Talansky at Mt. Sinai University in New York City who popularized pre-clinical microsurgical fertilization in the mouse, with the concept of allowing spermatozoa to naturally traverse the zona pellucida through an artificial hole, a process they referred to as zona drilling [15]. The artificial opening was made using very small amounts of acidified Tyrode's solution (ATS) released immediately adjacent to the zona pellucida, and this approach was often used to open or remove the zona pellucida until the early 1990s. The first non-contact non-toxic laser for opening the zona pellucida in the mouse was developed in 1993 during a collaboration between the Beckham Laser Institute at UC-Irvine and the IVF team at Cornell University Medical Center [54]. The earlier ATS procedure used a holding pipette on one of the micromanipulators to firmly grip the oocyte, with a small-bore injection pipette containing ATS on the opposite micromanipulator. The fine tools were made out of glass capillaries and prepared in the laboratory using a forge to shape the glass, a glass-puller to break the capillary, and a small grinding instrument to create an angled tip, if needed. Microtools, including holding pipettes, were not commercially available until the 1990s and had to be produced by each clinical laboratory that set up a microsurgical fertilization program. This may have been one of the aspects causing technical differences between laboratories and limiting some clinical embryologists from participating in the early research.

The first paper on zona drilling was not just a presentation of pioneering technology or proof that oocytes could survive after micromanipulation, fertilize and develop in vitro, but also showed that the procedure was compatible with implantation and live birth after the embryos were transferred using a surgical intra-uterine transplantation procedure [15]. The authors also demonstrated that with zona drilling the concentration of spermatozoa could be diluted to 100 times below the threshold required for normal in vitro fertilization in the mouse, although fertilization diminished from 75% to 15%. Polyspermy in zona-drilled mouse oocytes was not greater than in zona-intact controls, demonstrating that the block to polyspermy was active on the oolemma. Earlier experiments showed that rat and mouse oocytes from which the zona pellucida were removed could be fertilized resulting in monospermic fertilization [50]. Mouse oocytes fertilized with a significantly higher rate (75%) after the zona drilling procedure compared to zona-intact controls (22%), and when embryos were transplanted into recipient-foster females, term development was at the same rate (36.7%) as mouse IVF zona-intact controls (44.3%) [15].

The first detailed pre-clinical experiments with microsurgical fertilization by sub-zonal insertion of a single human spermatozoon were reported by Alan Trounson's laboratory from Monash University in Australia [53] after the first pilot experiment had been reported a few years earlier [55]. Jeff Mann, also from Monash University (Trounson Laboratory) subsequently reported on the first birth in the mouse after sub-zonal injection of a single spermatozoon [16]. It was not surprising that those who were interested in achieving live human offspring in cases of male factor infertility investigated less invasive technologies first, given the poor outcomes from animal models with ICSI-like procedures [48,56]. Even though the first offspring from mammalian ICSI in the rabbit were born in Japan in Dr. Iritani's laboratory in 1988, only 2/72 transfers leading to live births were reported in the first few papers [57]. This and early clinical work performed at the Jones Institute [58] resulting in poor fertilization outcomes, discouraged most fertilization specialists from performing ICSI clinically and transferring embryos for a few years. When considering the body of work from the pioneering microsurgical fertilization groups, it is not surprising that both zona drilling and sub-zonal insertion were the leading procedures pursued in clinical assisted fertilization during the first years, even though their

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application in the mouse led to unacceptable fertilization outcomes. It is perhaps a prime example of how the mouse model can elude clinical decisions.

The first babies born after microsurgical fertilization were conceived using an alternative method of zona drilling, as the acidified solution which was so successful in the zona drilling of mouse oocytes was found to be detrimental in the human [59,60]. Despite the relatively successful and routine use of acidified solution in human embryos for zona drilling, assisted hatching and biopsy procedures, the unfertilized egg clearly demonstrated a particular vulnerability [61,62]. Once there was a technical change in zona drilling from chemical to mechanical dissection, the partial zona dissection procedure became clinically successful and the first healthy babies were born [17]. Babies born from sub-zonal insertion were reported in quick succession [18,20], but both methods yielded modest monospermic fertilization rates. It was also determined that even at modest sperm concentrations outside the zona pellucida the use of partial zona dissection increased polyspermy. Unlike in the mouse, the block to polyspermy of human oocytes is regulated by the zona pellucida and not the oolemma [63]. In the human, polyspermy mechanisms resemble those seen in bovine and hamster eggs. This fundamental finding demonstrated the modest suitability of partial zona dissection and sub-zonal insertion for treating infertile men. What was needed was a method using a single spermatozoon directly inserted into the human ooplasm. Although the method of direct mouse egg injection existed, fertilization rates were modest and degeneration occurred frequently. The reasons for the disappointing observations made between 1988 and 1992 can be debated, although it seems likely that major technical differences existed in the microtool-making processes. The standard injection microtool developed for sub-zonal insertion (Monash University, Fishel and Antinori's team in Rome and Cornell University Medical Center) differed considerably from a much sharper, thinner enhanced model separately developed by Hubert Joris in Palermo, DeVroey and Van Steirteghem's laboratory (DeVroey, personal communication). The enhanced model tool could easily break the human oolemma with minimal indentation of the zona pellucida. The Belgian team also optimized suction control and visualization of membrane breakage as well as a meticulous setup process aligning the tools at high magnification and using some of the standards developed by Lanzendorf et al. (1989) such as reducing sperm motility and applying tail breakage to each spermatozoon [58]. The highly technical aspects are what separated the first attempts at ICSI from those developed later in Brussels. The fertilization rates reported by the Brussels team were two to three times higher than seen with the prior approaches. The comparisons were so obvious that no further evidence appeared to be needed and within a year, ICSI became the dominant methodology for treating male factor infertility, and this has remained so for almost 30 years. The prospect of sterility for most men diagnosed with male infertility turned for the better in a timespan of 12 years. It was a remarkable and swift medical revolution. A feat accomplished by several groups publishing their technology and findings in a few dozen papers.

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