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The pioneering of intracytoplasmic sperm injection: historical perspectives

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Abstract

Intracytoplasmic sperm injection (ICSI) has often been heralded as a ground-breaking technique that has transformed the treatment of couples with infertility. By injecting a single spermatozoon into the cytoplasm of the oocyte, ICSI bypasses the zona pellucida and increases the chances of fertilization and subsequent embryo development, independent of semen parameters. Ever since the first live births using ICSI were reported in 1992, ICSI has become the mainstay of treating male factor infertility as well as overcoming fertilization failure associated with conventional *in vitro* insemination. Today, ICSI is utilized in nearly 66% of all assisted reproductive treatments worldwide and has resulted in the birth of millions of babies. The primary goal of this review is to provide historical perspectives about the pioneering of ICSI. We begin by highlighting the scientific work of early investigators who elucidated the mechanisms central to mammalian fertilization. Furthermore, we briefly discuss how these findings contributed to the development of IVF for the treatment of infertility. We then emphasize the shortcomings of IVF in treating severe forms of male factor infertility and enumerate the micromanipulation techniques that were developed to circumvent these shortcomings. Finally, we indicate how the inadequacies of these micromanipulation techniques lead to the inception, application and popularity of ICSI.
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Introduction

The subject of infertility has stirred the interest of medical, social, religious and philosophical scholars from the beginning of human existence. Even though infertility was likened to a bad omen and was historically described using pseudoscientific and pagan terms, many civilizations documented its evidence at considerable length. For example, the ancient Egyptian *Kahoun* papyrus text, a medical textbook dating back to 1900 B.C., recognized several gynecologic conditions, including infertility (Morice *et al*. 1995). In fact, infertile women had their own goddess – Nephtys (Morice *et al*. 1995). Interestingly, not all cases of infertility were attributed to women, as evident from the story of Seth, lord of the desert, who was not able to conceive a child, even though his wife had a child with Osiris (Morice *et al*. 1995). The ancient Egyptians also proposed some simple methods of evaluating fertility in women, though much of it was permeated by magic and a limited knowledge of anatomy.

The Greeks, particularly Hippocrates and his pupils, wrote several treatises regarding infertility. Soranos of Ephesus, one of the greatest physicians of the Roman era, hypothesized that conception could occur only after the cessation of menstruation (Morice *et al*. 1995). While his reasoning was imperfect i.e. the uterus was overloaded at the time of menstruation, he was perhaps one of the first to suggest that conception could only occur during certain phases of the menstrual cycle. The basis of infertility was explored even more during the Middle Ages, the Renaissance and the early 20th century and was propelled by advances in anatomy and microscopy. However, most treatment methods were aimed at the surgical correction of reproductive tract anomalies.

In the 1890s, pivotal experiments were conducted by Walter Heape that would pave the way for human infertility treatment several decades later. Heape's embryo transfer experiments in rabbits were conducted between 1890 and 1897 and were subsequently published in the *Proceedings of the Royal Society of London* (Biggers 1991). In these experiments, Heape obtained two ova from the Angora doe rabbit, which had been fertilized approximately 32 h prior by an Angora buck. The ova were undergoing division into 4 segments. These ova were transferred into the fallopian tube of a Belgian hare doe rabbit, which had been fertilized by a buck of the same breed 3 h before. The Belgian hare doe rabbit then gave

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birth to 6 young ones – 4 were similar to her and the buck's breed, while the remaining two were of the Angora breed. Heape's work, therefore, marked the first occurrence of deliberate embryo transfer and reimplantation in animals.

It is interesting to note that the implications of Heape's work for treating infertility were perhaps not recognized in the 1890s. However, two literarians furthered Heape's ideas in their books, and even alluded to the technique of modern-day *in vitro* fertilization (IVF). John Burdon Sanderson Haldane first introduced the concept of 'ectogenesis' in his 1925 book *Daedalus, or, Science and the Future* (Haldane 1925). In this book, Haldane envisioned a future where humans could create other human individuals outside their body. Soon after, in 1932, Aldous Huxley published *Brave New World* in which he proposed that 'bottled babies' could be created in the near future, though the development would be 'technically and ideologically still a long way' (Huxley 1932). An editorial appeared in the *New England Journal of Medicine* on October 21st 1937 apprising the medical community about the scientific and ethical implications of Huxley's ideas (Editorial 1937). However, before any of these ideas could be realized or dismissed, it became imperative to address whether oocytes could undergo fertilization and normal development in *in vitro* conditions.

Achieving fertilization in *in vitro* conditions

Early studies of fertilization were conducted in invertebrate animals such as the sea urchin and starfish (Yanagimachi 2012). These animals generally produced millions of mature oocytes at one time, with fertilization and subsequent embryo development occurring outside the body (Yanagimachi 2012). Furthermore, the early stages of development could be observed in a simple dish of sea water under laboratory conditions (Yanagimachi 2012). Although 40–50 mammalian oocytes could be obtained from mice or rabbits using superovulation, the process of fertilization occurred inside the body of a female (Yanagimachi 2012). This limited the number of *in vitro* observations and experiments that could be conducted in laboratory conditions using mammalian oocytes. However, the work of Pincus and Enzmann served as a major break-through (Pincus & Enzmann 1934). In their 1934 paper, the authors demonstrated normal development of mammalian oocytes that were subjected to *in vitro* experimental manipulation (Pincus & Enzmann 1934). In one of their experiments, an albino doe was made pseudopregnant via copulation with a vasectomized male. Ova were then obtained at the 1-cell stage from the fallopian tubes of an English doe that was mated with a fertile agouti male. These ova were cultured in Carrel flasks for 20 h, after which 5 ova were transplanted into the left fallopian tube of the pseudopregnant albino doe. Thirty days later, 2 gray English-spotted young were obtained.

Fertilizing oocytes with male gametes in *in vitro* conditions was the next logical step to follow Pincus' and Enzmann's work. Two successive papers published by Rock and Menkin in 1944 and 1948 described the early stages of oocyte fertilization in humans (Rock & Menkin 1944, 1948). In one study, 800 oocytes were retrieved from women undergoing various gynecologic surgeries, of which, 138 were exposed to spermatozoa (Rock & Menkin 1948). Two oocytes were found to be in the 2-cell stage, while two others were in the 3-cell stage after culturing with human blood serum. Although poor fertilization of oocytes was an acknowledged limitation of the aforementioned studies, the reasons for poor fertilization remained unknown. However, in a series of papers published in *Nature*, M C Chang described the vital process of sperm capacitation, which was imperative for successful *in vivo* fertilization (Chang 1951, 1953, 1955). A few years later, in 1959, Chang also provided the irrefutable evidence forIVF of gametes (Chang 1959). In his study, Chang collected mature unfertilized oocytes from albino female rabbits injected with sheep pituitary extract. Spermatozoa were collected from the uteri of albino females that were mated with albino males 12 h prior. The oocytes were inseminated with spermatozoa in a Carrel flask on a rocker. Approximately 4 h later, the oocytes were transferred to another flask containing 50% heated serum in saline and cultured until the 4-cell stage. Thirty-six cleaving oocytes were transferred into 6 black female rabbits; four rabbits delivered 15 young albinos. Chang's IVF experiments, in conjunction with improved oocyte collection techniques, microscopes, culture dishes and media, served as the harbinger of IVF attempts in humans (Edwards *et al*. 1966, De Kretzer *et al*. 1973, Steptoe & Edwards 1976), culminating in the birth of Louise Brown, the first IVF baby in 1978 (Steptoe & Edwards 1978).

Success and limitations of IVF

Although Edwards and Steptoe reported the first IVF birth in a woman with bilateral tubal occlusions (Steptoe & Edwards 1978), many investigators envisaged the application of IVF for other infertility diagnoses as well. By inducing the growth of multiple follicles with clomiphene citrate (Trounson *et al*. 1981), IVF was no longer limited to a single oocyte retrieved during the natural menstrual cycle as described originally (Steptoe & Edwards 1978). Several good-quality embryos could be generated after fertilization of the retrieved oocytes, following which two or more embryos could be transferred (Edwards *et al*. 1984, Muasher *et al*. 1984). Such a strategy boosted the success and efficacy of IVF by several-fold. Studies from Monash University in Australia indicated that pregnancy rates had increased from 4–13% to 18–22% before and after using clomiphene citrate, respectively (Trounson & Wood 1981, 1984, Trounson 1982). Similar protocols were used at the Bourn Hall clinic in England, resulting in increased

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implantation rates i.e., 16.5–30% (Edwards *et al*. 1984). At Norfolk, USA, comparable protocols resulted in 105 pregnancies (319 patients) between 1981 and 1983, with pregnancy rates of 19% and 25% per cycle and per transfer, respectively (Jones *et al*. 1984).

Despite its early success, several clinics began to note significant limitations of IVF. In fact, about 40% of IVF cycles were complicated by poor fertilization or complete fertilization failure (CFF), even when an adequate number of oocytes were retrieved (Cohen *et al*. 1984, Mahadevan & Trounson 1984). The aforementioned fertilization issues were especially pronounced in couples where the male partner had moderate-to-severe abnormalities in semen parameters. Laboratories attempted to reduce the incidence of poor fertilization and CFF by increasing *in vitro* sperm concentrations (Palermo *et al*. 2014*a*,*b*). For example, when ejaculated specimens with sub-optimal semen parameters were obtained, the volume of the insemination medium was decreased, so as to increase the overall concentration of sperm. This facilitated the *in vitro* co-mingling of gametes (Palermo *et al*. 2014*a*,*b*). Multilayer density gradients or swim-up techniques were also utilized to increase the fraction of motile and morphologically normal sperm available for *in vitro* insemination (Alper *et al*. 1985). Sperm motility enhancers such as pentoxifylline were shown to increase fertilization rates in some couples undergoing IVF for severe male factor infertility (Yovich *et al*. 1990). Although the aforementioned methods increased the fertilization and pregnancy rates of IVF cycles for mild-to-moderate asthenozoospermia or oligozoospermia, their success remained limited in IVF cycles for severe oligo-asthenozoospermia or complete teratozoospermia.

Assisted fertilization

Given the incidence of poor fertilization and CFF in IVF cycles with sub-optimal sperm concentration and motility, investigators began to develop assays that could predict the fertilization potential of spermatozoa. The hemizona assay (HZA) was one such example where maximal binding of sperm samples to matching halves of a human zona pellucida (ZP) from a nonfertilizable and nonliving oocyte was evaluated after 4–5 h of coincubation (Burkman *et al*. 1988). Studies demonstrated that tight zona binding was significantly correlated with the percentage of motile sperm and normal morphology, as well as sperm concentration (Franken *et al*. 1989). Further studies also confirmed that patients with poor fertilization rates in IVF had significantly lower binding than patients with successful fertilization, with a sensitivity, specificity and positive predictive value of 83, 95 and 83%, respectively for fertilization (Oehninger *et al*. 1989). However, due to the limited availability of zonae, the HZA was limited to only a few specialized laboratories.

Although the HZA served as a valuable tool to evaluate sperm-ZP binding, it offered no therapeutic benefits i.e., it did not increase the fertilizing potential of poor-quality spermatozoa. Thus, the focus switched to assisted fertilization, which aimed at increasing the fertilizing potential of sperm by bypassing the cumulus cells and the thick glycoprotein coat (ZP) surrounding the oocyte. Early investigations aimed at complete removal of the cumulus cells to aid fertilization. In one such study (Lavy *et al*. 1988), 88 oocytes from 13 patients with male factor, immunological and idiopathic infertility were randomly assigned to cumulus removal with hyaluronidase or non-treatment. While cumulus removal did increase fertilization rates in some cases, only 1 clinical pregnancy was achieved.

Different investigators then attempted to remove the ZP completely; however, such attempts invariably resulted in polyspermy and abnormal embryo development. Milder variants of zona removal were also used – for example, mild trypsinization of oocytes prior to *in vitro* insemination (Kiessling *et al*. 1988). Yet, polyspermy was still noted in 1 out of 3 treated patients. Localized chemical treatment of oocytes with acidified Tyrode's solution prior to sperm exposure, also known as zona drilling, was another method of introducing deliberate gaps in the ZP to facilitate sperm entry (Gordon & Talansky 1986). However, its application was limited by the 50% rate of polyspermy in drilled oocytes (Gordon *et al*. 1988). In addition, zona drilling involved exposure to a solution of pH 2.3, thereby increasing the risk of chemical damage to oocytes (Gordon *et al*. 1988). Zona drilling was refined further by creating a partial opening in the ZP with mechanical force. This technique was called partial zona dissection (PZD) and was considered less traumatic than zona drilling (Cohen *et al*. 1988, Malter & Cohen 1989). Also, to avoid ooplasmic damage, the oocytes were exposed to sucrose to shrink the ooplasm during micromanipulation (Cohen *et al*. 1988, Malter & Cohen 1989). In one study, 11 male factor infertility couples had some of their oocytes treated with PZD prior to *in vitro* insemination, while the remaining non-PZD-treated oocytes served as controls (Cohen *et al*. 1989). The investigators reported that the rates of monospermic fertilization and cleavage (23/34; 68%) with PZD were almost double when compared to the control oocytes (10/30; 33%) (Cohen *et al*. 1989). Furthermore, three PZD embryos also progressed to the blastocyst stage. Despite these encouraging results, investigators noted that polyspermy continued to occur with PZD (Cohen *et al*. 1988, Malter & Cohen 1989).

The limitations of PZD gave rise to another micromanipulation technique called subzonal insemination (SUZI) (Ng *et al*. 1988). In this technique (Fig. 1, Panel A), up to 3 spermatozoa were brought with an injection pipette through the ZP and deposited into the perivitelline space of a metaphase-II oocyte

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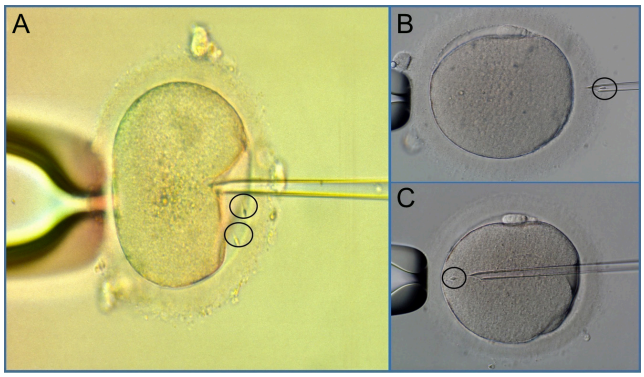

Figure 1 Panel A – subzonal insemination (SUZI) of oocytes in which 2–3 spermatozoa (black circles) are brought with an injection pipette through the zona pellucida and deposited into the perivitelline space; Panel B – a single spermatozoon (black circle) is seen in the injection pipette prior to performing sperm injection; Panel C – a single spermatozoon (black circle) is seen in cytoplasm of the oocyte immediately after sperm injection.

(Ng *et al*. 1988, Palermo & Van Steirteghem 1991). The initial results of SUZI were encouraging. For example, when SUZI was performed in 771 oocytes from 131 patients with very low sperm density and motility, the rates of monospermic fertilization and polyspermy were 16.6% and 2.3%, respectively (Ng *et al*. 1991). In another study of 43 couples with a history of CFF with IVF, subzonal insemination of 433 metaphase-II oocytes achieved a fertilization rate of 30.9% and subsequent cleavage rate of 80% (Palermo *et al*. 1992*a*). The overall pregnancy rate with SUZI, however, remained relatively low, ranging from 2.9% to 16.3% (Palermo *et al*. 1992*a*, Sakkas *et al*. 1992). Given the collective limitations of zona drilling, PZD and SUZI, systematic efforts were undertaken to develop a micromanipulation method that would optimize the fertilizing potential of a single spermatozoon and increase pregnancy rates, but also decrease the incidence of polyspermy and poor fertilization.

Inception of ICSI

Fertilization of an oocyte by injecting a single spermatozoon into the ooplasm was previously achieved in sea urchin (Hiramoto 1962*a*,*b*), mouse (Lin 1966) and hamster models (Uehara & Yanagimachi 1977*a*,*b*). However, replication of this technique in other mammalian models was often complicated by oocyte damage and lysis, with only 30% of oocytes surviving the injection procedure (Thadani 1980, Markert 1983). Much of the oocyte damage was attributed to the crudeness of the micromanipulation instruments (Pereira *et al*. 2016). Furthermore, because sperm injection bypassed ZP–sperm fusion, oocyte activation was required in most species, which was generally achieved by vigorous suction of ooplasm just before sperm injection (Perreault & Zirkin 1982) or with compounds such as A23187 (Palermo & Van Steirteghem 1991).

The first offspring using sperm injection into oocytes were achieved in rabbits (Iritani *et al*. 1988) followed by bovine species (Goto *et al*. 1990). Microinjection of a spermatozoon directly into the ooplasm of human oocytes was attempted in 11 patients as early as 1987. While this was the first description of successful fertilization of human oocytes with sperm microinjection, no zygotes were transferred in this study (Lanzendorf *et al*. 1988). Evidence mounted that injection of a single spermatozoon into a human oocyte would require intricate micromanipulation tools and holding dishes, as well as a standardized technique. Extensive work was carried out to improve the pre-existing micromanipulators i.e. refining of the injection needles, newer holding and injecting pipettes, delivery of a constant pressure through a 5 µ diameter micro-needle, as well as standardization of intracytoplasmic sperm injection (ICSI) dishes. It was while performing SUZI, with the aforementioned modifications, that the oolemma and ooplasm of an oocyte was inadvertently pierced with a single spermatozoon. The oocyte was re-examined the following day; two pronuclei were noted, which confirmed fertilization (Palermo *et al*. 1992*b*). This was perhaps the inception of ICSI (Fig. 1, Panels B and C). Figure 2 provides a simplified timeline of micromanipulation techniques leading up to the pioneering of ICSI.

Application and popularity of ICSI

The first four pregnancies using ICSI were reported in couples with severely impaired sperm characteristics and failed IVF and SUZI attempts (Palermo *et al*. 1992*b*). Initial comparisons of ICSI and SUZI demonstrated fertilization rates of 44% and 18%, respectively (Palermo *et al*. 1993). However, further research demonstrated that the fertilization rates with ICSI could be increased with aggressive sperm immobilization i.e., crimping the sperm flagellum between the midpiece and the rest of the tail prior to sperm injection (Palermo *et al*. 1996). For example, in a study of 837 cycles, ICSI after aggressive sperm immobilization was associated with fertilization and pregnancy rates of 82% and 82.4%, respectively compared to fertilization and pregnancy rates of 48.3% and 51.4%, respectively with standard sperm immobilization and ICSI (Palermo *et al*. 1996). Early adopters of ICSI were also able to improve fertilization and pregnancy rates by following specialized technical steps and adhering to stringent laboratory conditions while performing ICSI (Palermo *et al*. 2015). Similar to PZD and SUZI, ICSI allowed precise identification of fertilization by the appearance of the pronuclei and the first embryonic cleavage (Palermo *et al*. 2009, 2015). ICSI was also able to shed light on the genetics of abnormal human fertilization as well as the involvement of the

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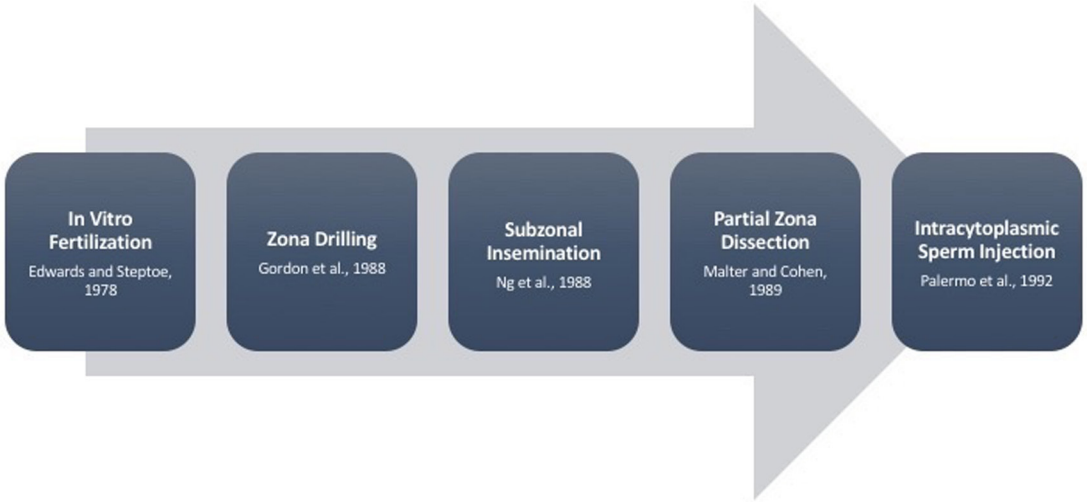

Figure 2 A simplified timeline of micromanipulation techniques leading up to the pioneering of intracytoplasmic sperm injection (ICSI).

human sperm centrosome in controlling the first mitotic division after fertilization (Palermo *et al*. 1994, 1995*a*).

With the accumulation of more scientific evidence, it became apparent that ICSI was capable of fertilizing nearly every mature oocyte injected, irrespective of sperm source (Palermo *et al*. 1995*b*, 1998, Schlegel *et al*. 1995*a*,*b*, Su *et al*. 1999) and spermatozoa characteristics (Alikani *et al*. 1995, Chung *et al*. 1998, Palermo *et al*. 1999, Chan *et al*. 2001). In their recent publication, the International Committee for Monitoring Assisted Reproductive Technologies (ICMART) reported that ICSI was utilized in 66% of >4,461,309 cycles in 61 countries between 2008 and 2010 (Dyer *et al*. 2016). Interestingly, geographic variations in ICSI utilization were also reported by ICMART – 55% in Asia, 65% in Europe and 100% in the Middle East. As quoted in the ICMART report, 'the reasons behind the high use of ICSI in some regions are not fully understood and are outside of the scope of this report' (Dyer *et al*. 2016). In the USA, the use of ICSI has increased from 36.4% in 1996 to 76.2% in 2012 (Boulet *et al*. 2015). Of note, ICSI is being increasingly utilized even in the presence of normal semen parameters (Jain & Gupta *et al*. 2007). For example, the use of ICSI in the USA for non-male factor infertility increased from 15.4% in 1996 to 66.9% in 2012 (Boulet *et al*. 2015). The increased utilization of ICSI in the USA may correspond to the increase in preimplantation genetic testing, use of cryopreserved oocytes or *in vitro* maturation (Practice Committees of the American Society for Reproductive Medicine and Society for Assisted Reproductive Technology 2012, Boulet *et al*. 2015). However, it is still imperative to analyze the trend of increasing ICSI utilization in non-male factor settings, without any apparent benefits. In one recent study, the reproductive outcomes of 490 and 255 women >40 years of age undergoing ICSI and conventional IVF, respectively were compared (Tannus *et al*. 2017). Despite similar numbers of total oocytes retrieved, the conventional IVF group had more mature oocytes. The conventional IVF group also had a statistically higher number of zygotes. The fertilization rates, fertilization failure rates and live birth rates were similar between the ICSI and conventional IVF groups. In another study, the reproductive outcomes of couples with a history of tubal ligation and no male factor were compared by method of insemination i.e. ICSI vs conventional IVF (Grimstad *et al*. 2016). The authors compared 3189 ICSI cycles to 3956 IVF cycles and found no significant improvement in the fertilization rate. Furthermore, the odds of clinical pregnancy and live birth were lower in the ICSI group. While these and other previously published studies pertaining to the use of ICSI in non-male factor settings have led the American Society for Reproductive Medicine and the Society for Assisted Reproductive Technology to confirm that there is insufficient evidence to support the routine use of ICSI in patients without male factor infertility (Practice Committees of the American Society for Reproductive Medicine and Society for Assisted Reproductive Technology 2012), it is important to highlight that the use of ICSI should be individualized to the reproductive history of the couple (Palermo *et al*. 2015).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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