Milestones in assisted reproduction and related technologies

Part 1: Developmental biology, preimplantation development, spermatozoa preparation, embryo culture and transfer

Jaffar Ali[1], Nafisa Ali[1,2]

[1]JRBF Online Publications, Shah Alam, Selangor, Malaysia
[2](Previously with: School of Archaeology and Anthropology, College of Arts and Social Sciences, The Australian National University, Canberra, ACT, Australia)

Abstract

The present effort is intended to document the milestones and events that contributed to the development of human assisted reproduction technology (ART). The authors endeavored to record every single milestone, whether grandeur or miniscule, and also ameliorate subtle imprecisions in historical narrations if detected. The search for these historical events was performed by extensive online search. One of the earliest milestones was the invention of the microscope in 1590 by Zaccharias Janssen, followed by the visualization of the human sperm in the 1670's by Nicolaas Hartsoeker and Antonie van Leeuwenhoek, independently. The next milestone was the identification of the mammalian oocyte in 1826 by Karl Von Baer, followed by the observation of fertilization of the mammalian oocyte in 1878 by Leopold Schenck. The first successful embryo transfer in the mammal (rabbit) was reported by Walter Heape in 1891. A major landmark development was the invention of the embryo culture medium in 1956 by WK Whitten. The ability to culture embryos outside the body was the single most significant development in ART which provided the impetus for the subsequent rapid development of not only human ART but also animal ART, gamete and embryo cryopreservation, in vitro maturation (IVM), preimplantation genetics, embryonic stem cell technology and cloning. Another landmark development was the in vitro fertilization, and successful embryo transfer in the mammal (rabbit) resulting in live young performed by MC Chang in 1959. The first human oocyte fertilization was reported by Robert Edwards in 1965 followed by the successful embryo transfer of a human embryo with the birth of the first ART baby reported by Patrick Steptoe and Robert Edwards in 1978. The first successful human livebirth from cryopreserved human embryo was reported by Mukherjee and co-workers (Mukherjee et al., 1978). Subsequent to this human ART developed at an exponential rate.

Disclaimer: The authors have no conflicts of interest.
J Reprod Biotechnol Biomed Sci 15 (Suppl 1): 3-39
Correspondence: Jaffar Ali; email: jrbf.online@gmail.com cc: jaffarali.abdullah@gmail.com
Compliance acknowledgment: This article was edited by the Australian Editorial Services (www.nativeenglisheditor.com)
Keywords: ART, development, Milestones, historical, inventions, innovations, landmark

Introduction

The technology of assisted reproduction and embryo transfer used in the alleviation of infertility in the human as we know it in present times was only developed in the last few decades but the science leading to its development commenced about five hundred years ago.

The authors have attempted to document the main milestones and events that contributed to the development of human ART as we know it at the present times including milestones that are likely to further augment its future development. The authors have noted that it has become increasing difficult for workers to keep track of the historical development of the ART due to the enormous amount of work that is being published over the last three decades such that some members of the profession are not aware of the contributions of individual workers or groups. The difficulty in accessing publications by workers of less endowed nations has also contributed to this

problem in large parts of the world. Confusion with regard to contributions of some workers also exists due to imprecisions in published works because of which credit is sometimes given to others such that the genuine efforts and hard work of the real contributor(s) is masked by inaccuracies. Furthermore, there appears to be progressively less time to impart historical accounts of ART evolution and development to students.

The present effort is intended to overcome this deficiency by documenting as comprehensively as possible but in a concise manner the significant contributions to the development of the ART. This endeavor attempts to record every single milestone or event, whether grandeur or miniscule, as accurately as possible, irrespective of its geographical origins. It also endeavors to ameliorate subtle imprecisions which has created confusion on the origination of research findings such that the genuine contributions of some are erroneously attributed to others.

We realize this task of chronicling the acquisition of knowledge that led to the development of the ART and related technologies will inadvertently and inevitably, in some instances, deliberately, leave out some contributions, in particular those not published in English mainstream scientific literature. In some instances, it is also due to a lack of resources at the time the present work was being collated and for a number of other reasons beyond the control of the authors. In reality, the enormity of this task dictates that a complete narration is unattainable by a small group of workers given the limited resources available to them within the span of a year of work nevertheless it is anticipated this work will become a useful reference material to the profession and is anticipated to be inspirational for the younger generation.

Material and methods

The authors have chronicled the milestones that led to the development of the ART as comprehensively as possible using resources available to them. Online resources utilised were mainly the Google Scholar and the U.S. National Library of Medicine (Pubmed).

Results

The Charts 1 to 3 below chronicles the milestones pertaining to developmental biology, preimplantation development, sperm preparation for insemination, fertilization, embryo culture and embryo transfer. The milestones pertaining to cryopreservation, preimplantation genetics, application of artificial intelligence in ART, in vitro maturation, micromanipulation and embryonic stem cell technology are given in Charts 4 onwards which are to be published separately..

Discussion

The present documentation has provided an almost complete chronological narration of the events and milestones that led to the development of the science and practice of laboratory aspects of ART beginning from cellular and developmental aspects of the gametes, fertilization, embryo culture and embryo transfer. In subsequent publications the authors hope to address other areas of ART such as cryopreservation, preimplantation genetics, the application of artificial intelligence in ART, in vitro maturation, micromanipulation and embryonic stem cell technology.

We believe this endeavour has fulfilled in part the objective of overcoming the paucity of detailed narration of all the events and milestones that led to the development of ART. The authors stand in awe of the accomplishments of those that made the contributions to the advancement of knowledge given in Charts 1 to 3. More than anything else this work reminds us that we stand on the shoulders of the giants that came before us. It is imperative that their memories and contributions are not forgotten with the passage of time. It is hoped this work will become a useful reference material and help keep the contributions of these men and women that made what ART is today

Chart 1. Milestones in Developmental Biology and Preimplantation Development

Year	Milestone/Event	Citation/Reference
200's ACE	Galen postulated that women produced 'semen' from their female testes (ovaries), which travelled down the Fallopian tubes to the uterus, where it would encounter the male sperm to produce an embryo.	Galen (129-210 ACE). Cited in: Lopata A. History of the egg in embryology. J Mamm Ova Res. 2009; 26:2-9
1322	First successful artificial insemination in mammals (horse)	Performed by an unknown Arab chieftain or his spy. Cited in: Guttmacher AF. The Role of Artificial Insemination in the Treatment of Human Sterility. Bull N Y Acad Med.1943; 19(8) :573-591.
1460	Artificial insemination in the human – not documented but rumoured	King Henry IV of Castile was considered impotent, hence nicknamed the Impotent. He supposedly resorted to artificial insemination to impregnate his Queen Juana.
1590	The invention of the microscope was central to ART. The inventor of the microscope is thought to be Zaccharias Jansen in 1590. He was the first to use the word "cell" to describe the tiny structures he saw with the microscope he had invented. However, the usefulness of glass lenses has been known since the Roman age.	Zaccharias Janssen, 1590. Bellis M, 2018 History of the microscope. Cited In: https://www.thoughtco.com/history-of-the-microscope-1992146. Accessed 06 Sept2021
1608	Another contender as the inventor of the microscope is Hans Lippershey in 1608.	Hans Lippershey, 1608. Cited In: She EGA. From Stargazing to Space Travel. Our brief history into space. PowerPoint Presentation (semanticscholar.org). Accessed 03 Sep 2021
1665	Robert Hooke - description of the 'cell'.	Hooke R, 1665 Micrographia. Cited In: Mazzarello P. A unifying concept: the history of cell theory. Nat Cell Biol. 1999; 1: E13–E15.
1666	Jan van Horne and his student Jan Swammerdam stated that the human female testis is similar to ovaria of birds.	Thiery, M. Reinier De Graaf (1641–1673) and the Graafian follicle. Gynecol Surg. 2009; 6:189–191
1667	Niels Stensen/ Steno concluded that the female 'testes' are equivalent to the ovary (Stensen, 1668)	Niels Stensen N. 1667. Cited In: Medvei V.C. (1982) The 17th Century and the Microscopists. In: A History of Endocrinology. Springer, Dordrecht. The 17th Century and the Microscopists | SpringerLink . Accessed 5 Sept 2021
1672	Reinier de Graaf alluded to 'little balls' or the ova.	Thiery M. Reinier De Graaf (1641–1673) and the Graafian follicle. *Gynecol Surg* 2009; **6:** 189–191 (2009).

1677	Dutch scientists Hartsoeker (1674) and Leeuwenhoek (1677) observed human spermatozoa independently under the microscope for the first time. Leeuwenhoek was convinced that sperm impregnated the ovum/ egg in procreation through his study of animal semen.	Nicolas Hartsoeker, 1674; https://embryo.asu.edu/pages/nicolaas-hartsoeker-1656-1725. Accessed 24June2021 and Antoni van Leeuwenhoek. 1677. Cited in: Lopata A. History of the egg in embryology. J Mamm Ova Res. 2009; 26:2-9 Also cited in: Howards SS. Antoine van Leeuwenhoek and the discovery of sperm. Fertil Steril. 1997; 67(1): 16-17. Antoni van Leeuwenhoek. Letter of June 12, 1685. In: Gilbert SF, Sunderland MA. https://www.ncbi.nlm.nih.gov/books/NBK10005/
1688	Francesco Redi (1626–97) stated that insects were born from eggs.	Redi F(1688) Cited in: Cobb M. An amazing 10 years: the discovery of egg and sperm in the 17th century. Reprod Domest Anim. 2012;47 Suppl 4:2-6
1780	It is believed that Lazzaro Spallanzani performed the first successful **artificial insemination** in 1780. Spallanzani inseminated a confined bitch in heat by depositing semen in the uterus with a syringe. This resulted in the birth of three pups 62 days later that resembled the dam as well as the semen donor (Herman, 1981).	Herman HA. Improving Cattle by the Millions: NAAB and the Development and Worldwide Application of Artificial Insemination. University, 1981. Cited in: Foote RH. The history of artificial insemination: Selected notes and notables J Anim Sci 2002. 80:1-10.
1784	A century after Leeuwenhoek's discovery, Italian scientist Lazzaro Spallanzani, performed the first successful artificial insemination in a dog in 1784.	Lazzaro Spallanzani, 1784. Cited in: Ombelet W, Van Robays J. Artificial insemination history: hurdles and milestones. Facts Views Vis Obgyn. 2015;7(2):137-43.
1789	First successful assisted fertilization in the frog with the generation of tadploes.	L'Abbé Lazzaro Spallanzani. L'Abbé Spallanzani 1789 Dissertations Relative to the Natural History of Animals and Vegetables. Volume II, Murray, London. https://catalog.hathitrust.org/Record/000912530 Accessed 17Aug2021 Also cited in: RML Winston. The 40th anniversary of human IVF: time to celebrate and time to reflect. Reproduction.2018; 156: E1–E3 (Special issue)
1790-99	First vaginal insemination in the human. Other investigators repeated the work of Spallanzani, on artificial insemination including John Hunter in 1799, which resulted in a human pregnancy .	Herman HA. Improving Cattle by the Millions: NAAB and the Development and Worldwide Application of Artificial Insemi nation. University, 1981.

		Cited in: Ombelet W, Van Robays J. Artificial insemination history: hurdles and milestones. Facts Views Vis Obgyn. 2015;7(2):137-43.
~1824	J. L. Prevost and J. B. Dumas reported seeing a sperm in the mucinous coat of the egg during their study of amphibian fertilization, which led to their conclusion that a single sperm fertilized an egg. They also observed the development of follicles in amphibians and concluded that follicles are not eggs, as they noted that the ovum and embryo in the uterus were much smaller than the follicle. They postulated that the follicle may have released the egg but could not describe the mechanism or describe the released body, thus losing the opportunity to discover the egg. J. L. Prevost and J. B. Dumas claimed that sperm were implements of fertilization rather than 'parasites' as they are not found in the semen of immature boys and elderly men.	Prevost JL, Dumas JB.Deuxieme mémoire sur la génération. Ann Sci Nat 1824; 2, 129–149 Cited In: Conner SJ, Lefièvre L, Hughes DC, Barratt CLR, Cracking the egg: increased complexity in the zona pellucida, Hum Reprod 2005;20(5):1148–1152 Lopata A. History of the egg in embryology. J Mamm Ova Res. 2009; 26:2-9 Also in: Eggs and Sperm as Germ Cells. Reproduction: Antiquity to the Present Day. Chapter 29 Cambridge UP Pblrs. Hopwood N, Flemming R, Kassel L.,Eds
1826/7	The mammalian (canine) oocyte and blastocyte were observed for the first time by Karl von Baer, 200 years after the observation of sperm.	Karl Ernst von Baer, 1826/7 . Cited in: Altmäe S, Acharya G, Salumets A. Celebrating Baer - a Nordic scientist who discovered the mammalian oocyte. Acta Obstet Gynecol Scand. 2017;96(11):1281-1282
1833	The nucleus of the orchid cell was identified and described by Brown.	Brown R. (1833). On the organs and mode of fecundation in Orchideae and Asclepiadeae. Trans. Linn. Soc. London. 1833; 16, 685–745. Cited in: Osorio DS, Gomes ER. The contemporary nucleus: A trip down memory lane. Biology of the Cell, 2013;105(9): 430–441.
1838/9	Schwann and Schleiden proposed the foundational "Cell Theory" which stated that all living things were formed of cells, which are the basis of structure of life. Schwann also suggested that the ovum is a cell.	Jones SL, Harris K, Geyer CB. A new translation and reader's guide to Victor von Ebner's classical description of spermatogenesis. Mol Reprod Dev. 2019; 86(11): 1462–1484. Thomas TA. Theodor Schwann: A founding father of biology and medicine. Curr Med Issues 2017;15:299-301
1841	Albert von Koelliker asserted that spermatozoa were formed in the testis, as well as that sperm and eggs are cells.	Koelliker RA (1841). Contributions to the knowledge of the sexual relations and the seminal fluidity of invertebrates. In. Berlin. In: Jones SL, Harris K, Geyer CB. A new translation and reader's guide to Victor von Ebner's classical description of

		spermatogenesis. Mol Reprod Dev. 2019; 86(11): 1462–1484.
1843	Martin Barry observed the presence of sperm that had penetrated the zona pellucida of rabbit ova removed from the Fallopian tube.	Lopata A. History of the egg in embryology. J Mamm Ova Res. 2009; 26:2-9
1848	Discovery of the chromosome by Wilhelm Hofmeister	Hoffmeister, W. 1848; Cited in: Kaplan DR, Cooke TJ. The Genius of Wilhelm Hofmeister: The Origin of Causal-Analytical Research in Plant Development Am J Botany. 1996;83(12):1647-1660
1850	The role of Leydig cells	Leydig DF. Zur Anatomie der Mannlichen Geschlechtsorgane und Analdrusen der Saugethiere. Z Wiss Zool. 1850; 2: 1-57 In: Puerta Suárez J, du Plessis SS, Cardona Maya WD. Spermatozoa: A Historical Perspective. Int J Fertil Steril. 2018;12(3):182-190.
1850	First report of the post-coital test; . the and First pregnancy (1 of 55) by artificial insemination using the husband's semen. AIH was usually performed at an inopportune phase of the menstrual cycle which led to failure.	Simms JM, 1850. Cited in: Ombelet W, Van Robays J. Artificial insemination history: hurdles and milestones. Facts Views Vis Obgyn. 2015;7(2):137-43. Also In: Guttmacher AF. The Role of Artificial Insemination in the Treatment of Human Sterility. Bull N Y Acad Med.1943;19(8):573-591.
1853	Newport observed the penetration of frog spermatozoa into frog eggs.	Newport G. 1851. On the impregnation of the ovum in the amphibia (first series). Philosophical Transactions of the Royal Society of London. 1851; 141:169–242. Cited in: Birkhead, T. R., & Montgomerie, R. (2009). Three centuries of sperm research. Sperm Biology, 1–42.
1856	The penetration of an egg cell by a sperm cell by observed by N. Pringsheim.	Pringsheim, N. 1855. U¨ ber die Befruchtung der Algae. Monatsberichte der Akademie der Wissenschaften zu Berlin, 1855, 133–165 Cited in: Birkhead, T. R., & Montgomerie, R. (2009). Three centuries of sperm research. Sperm Biology, 1–42.
1857	Mitchondria was described by Kolliker in 1857.	Kölliker A. Einige Bemerkungen über die Endigungen der Hautnerven und deu Bau der Muskelzellen. Z Wiss Zool. 1857; 8:311–325 Cited in: Allen C.A., Van Der Giezen M., Allen J.F. (2007) Origin, Function, and Transmission of Mitochondria. In: Martin W.F., Müller M. (eds) Origin of Mitochondria and Hydrogenosomes. Springer, Berlin, Heidelberg.
1858	"omnis cellula e cellula"- The famous conclusion by Rudolf Virchow (physician, pathologist and anthropologist), that cells develop only from pre-existing cells	Cited In: Chamnanwong P, Yuenyoung C. The Possibility of Historical Approach in Cells Teaching for Explicit Nature of Science. Research Journal of Applied Sciences,

		Engineering and Technology . 2014;7(22): 4685-4689
1865	Sertoli described the three stages of male germ cell development (mitotic, meiotic and post meiotic; the mammalian spermatogonia, the blood-testis barrier and more. He also described the function of testicular cells which went on to bear his name, Sertoli cells.	Sertoli E. Dell'esistenza di particolari cellule ramificatenei canalicoli seminiferi del testicolo umano (About the existence of special branched cells in the seminiferous tubules of the human testis). Morgagni 1865; 7:31–33. Cited in: Geyer CB. A historical perspective on some "new" discoveries on spermatogenesis from the laboratory of Enrico Sertoli in 1878. Biol Reprod. 2018 Sep; 99(3): 479–481.
1868/9	Freidrich Meischer discovered nucleic acids and DNA in 1868. He called the material 'nuclein' as it was found in the nucleus.	Miescher F (1869a) Letter I; to Wilhelm His; Tübingen, February 26th, 1869. In Die Histochemischen und Physiologischen Arbeiten von Friedrich Miescher – Aus dem Wissenschaftlichen Briefwechsel von F. Miescher, His W et al (eds), Vol 1, pp 33–38. Leipzig, Germany: FCW Vogel Miescher F (1869b) Letter IV; to Miescher's parents; Tübingen, August 21st, 1869. In Die Histochemischen und Physiologischen Arbeiten von Friedrich Miescher – Aus dem Wissenschaftlichen Briefwechsel von F. Miescher, His W et al (eds), Vol 1, p 39. Leipzig, Germany: FCW Vogel Miescher F (1869c) Letter V; to Wilhelm His; Leipzig, December 20th, 1869. In Die Histochemischen und Physiologischen Arbeiten von Friedrich Miescher – Aus dem Wissenschaftlichen Briefwechsel von F. Miescher, His W et al (eds), Vol 1, pp 39–41. Leipzig, Germany: FCW Voge Cited in: Dahm R. (2010). From discovering to understanding. EMBO Reports. 2010; 11(3), 153–160.
1876	The fusion of sea urchin egg and sperm at fertilization was observed by German biologist, Oskar Hertig.	Hertwig O: Beiträge zur Kenntniss der Bildung, Befruchtung und Theilung des thierischen Eies (Contributions to the knowledge of the formation, fertilization and division of the animal egg). Morphol Jahrb.1876; 1: 347–434.Cited In: Jarvis GE. Early embryo mortality in natural human reproduction: What the data say [version 1; peer review: 1 approved, 2 approved with eservations]. *F1000Research* 2016, **5**:2765
1878	Schenck observed and described mammalian fertilization (rabbit).	Leopold Schenck, 1878. In: Rines, George Edwin, ed. (1920). "Schenk, Leopold" . Encyclopedia Americana.

1879	Chromosomal behaviour during mitosis was described by Flemming in 1879.	Flemming, W. Ueber das Verhalten des Kerns bei der Zellteilung und über die Bedeutung mehrkerniger Zellen. Arch. Pathol. Anat. 77, 1–28 (1879). Cited in: Paweletz, N. Walther Flemming: pioneer of mitosis research . *Nat Rev Mol Cell Biol* **2,** 72–75 (2001).
1881	The naming of DNA and 'nuclein' as a nucleic acid is credited to Albrecht Kossell in 1881. He isolated the five nitrogen bases as the building blocks of DNA and RNA; adenine (A), cytosine (C), guanine (G), and thymine (T) (thymine is replaced by uracil (U) in RNA).	Kossel A, 1881 Cited In: Editorial. Timeline of Genomics (1865-1900). Geno. Prot. Bioinfo. 2004;2(1):64-68 And Dahm R. Friedrich Miescher and the discovery of DNA. Dev Biol. 2005;278(2):274–288.
1883 1892 1893	Development of germ cell theory	Weismann. A. (1893). The Germ Plasm. Cited in: Winther RG. August Weismann on Germ-Plasm Variation. *J Hist Biol. 2001;* **34,** 517–555 Stanford P. (2005). August Weismann's theory of the germ-plasm and the problem of unconceived alternatives. Hist Phil Life Sci. 2005; 27(2), 163-199.
1888	The centrosome and centriole were discovered, and prediction of its paternal origin from the sperm. Boveri observed meiosis and the reduction in the number of chromosomes to half during oocyte maturation. Through examination of the worm *Ascaris megalocephala*, Boveri was able to observe fertilization, and predicted the inactivation of the maternal centrosome during oogenesis.	Boveri T (1888). Cited in Sathananthan AH, Ratnasooriya WD, de Silva A, Randeniya P. Rediscovering Boveri's centrosome in Ascaris (1888): its impact on human fertility and development. Reprod Biomed Online. 2006 Feb;12(2):254-270.
1890	Discovery of Mitochondria, by Altman. He called it "bioblast". Its role in cellular oxidations was discovered by Lazarow and Cooperstein in 1953	Altmann, R. 1890 . Die Elementarorganismen und ihre Beziehungen zu den Zellen . Veit, Leipzig. Cited in: Lars Ernster L, Schatz G. Mitochondria: A Historical Review. J Cell Biol. 1981;91(3):2276-255s Lazarow A, Cooperstein SJ. Studies on the enzymatic basis for the Janus Green b* staining reaction. J. Histochem. Cylochem. 1953;1:234-241.
1898	Description of the Golgi Apparatus by Golgi in 1898.	Golgi C (1898a) Intorno alla struttura della cellula nervosa. Boll Soc Med Chir Pavia 13:1–14 (also: Arch Ital Biol 30: 60–70;Opera omnia, vol II. Hoepli, Milan pp:643–654) Golgi C (1898b) Sulla struttura della cellula nervosa dei ganglispinali. Boll Soc Med Chir Pavia 13:60–70 (also: ArchItal Biol 30:278–286; and 31:273–280; Opera omnia, vol II.Hoepli, Milan, pp 655–665)

		Cited In: roles: Dröscher A. Camillo Golgi and the discovery of the Golgi apparatus. Histochem Cell Biol. 1998;109:425–430 and Mazzarello, P., Bentivoglio, M. The centenarian Golgi apparatus. Nature 1998;392:543–544
1902	Boveri and Sutton (1902) proposed (independently) chromosomes as the carriers of genetic material- Chromosomal Theory of Inheritance.	Sutton WS. On the Morphology of the Chromosome Group in Brachystola Magna. Biol Bull. 1902; 4 (1): 24-39 Boveri, T. Das Problem der Befruchtung (Gustav Fischer, Jena, 1902). Cited In: Satzinger H. Theodor and Marcella Boveri: chromosomes and cytoplasm in heredity and development. Nature Rev Genet, Jg 9 2008; 9(3): 231-238.
1902	The chromosomal basis of sex determination was reported by CE McClung (1902)	McClung C . 1902 The accessory chromosome-sex determinant? Biol. Bull. (Woods Hole). 1902;3:43–84. Cited In: Portin P. The birth and development of the DNA theory of inheritance: sixty years since the discovery of the structure of DNA. J.Genet.2014; 93:293–302
1905	Wilson and Stevens independently discovered the x- and y- sex chromosomes.	Wilson EB, 1905; Stevens N, 1905 In: Moses MJ. Chromosomal structures in crayfish spermatocytes. *J Cell Biol* 1956; **2**(2):215–218.
1937 1949a 1949b	Polge and co-workers are credited with cryopreservation of sperm (1947). However others have reported the same independently, viz: Bernstein and Petropavlovski in 1937; Smirnov (1949) also cryopreserved sperm in the same year as Polge but he published his work in Russian. ***(Milestones in sperm cryopreservation are given elsewhere in this manuscript)***	Bernstein AD, Petropavlosky VV. The influence of nonelectrolytes on survival of spermatozoa (message III). Byull. Eksp. Biol. Med. 1937;3(1):41-43.Russian In: I. I. Katkov II, V. F. Bolyukh VF, Sukhikh GT. KrioBlast™ as a New Technology of Hyper-fast Cryopreservation of Cells and Tissues. Part I. Thermodynamic Aspects and Potential Applications in Reproductive and Regenerative Medicine. Cell Technol Biol Med. 2018;4:530-535 Polge C, Smith AU, Parkes AS (1949) Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 164 (4172):666 Smirnov IV. Preservation of domestic animal semen by deep cooling.

		Sovetskaja Zootechnia 1949; 4:63–65 In: Isachenko V, Isachenko E, Katkov II, Montag M, Dessole S, Nawroth F, Van Der Ven H (2004) Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapor: effect on motility, DNA integrity, and fertilization ability. Biol Reprod 71(4):1167–1173
1931	The first (transmission) electron microscope, was invented in 1931 by Ernst Ruska and Max Knoll at the Berlin Technische Hochschule or Berlin Technical University.	Ruska E. The development of the electron and of electron microscopy. The Nobel Lecture. Biosci Reports. 1987;7(8):607-629 Also cited In: Robinson AL. Electron Microscope Inventors Share Nobel Physics Prize: Ernst Ruska built the first electron microscope in 1931; Gerd Binnig and Heinrich Rohrer developed the scanning tunneling microscope 50 years later. Science. 1986;14;234(4778):821-822.
1937	Manfred von Ardenne developed the scanning electron microscope.	Von Ardenne M, Beischer D. 1940 Untersuchung von Metalloxyd-Rauchen mit dem Universal-Elektronenmikroskop" [Investigation of metal oxide smoking with the universal electron microscope]. Zeitschrift für Elektrochemie und Angewandte Physikalische Chemie (in German).1940; 46 (4): 270–277. Cited In: Palucka T Overview of electron microscopy. History of electron microscopy, 1931-2000 (caltech.edu) Accessed 10Sept2021
>1938 -1948	Rock and Menkin in the USA attempted human IVF on experimental basis. Of the 800 oocytes they chanced to work with only 2 to 3 embryos cleaved (The eggs/embryos could be fragmented? This is because the eggs were left at room temperature after recovery for 3 hours and they performed insemination at room temperature and subsequently left the inseminated egg and sperm on the microscope for 1 to 2 hours also at room temperature. This methodology could have contributed to the failure experienced by these workers. Of course they had no way of knowing of the inherent errors of their technique at that time)	Rock J, Menkin MF. In vitro fertilization and cleavage of human ovarian eggs, Science 1944; 100(2588): 105-107 Menkin MF, Rock J. In vitro fertilization and cleavage of human ovarian eggs. Am J Obstet Gynecol. 1948;55(3):440-452 Also see: Yovich J. History of IVF from the Indian Perspective. 2020. (PDF) History of IVF from the Indian Perspective (researchgate.net) Accessed 14Sep2021
1942	Viability Stain for sperm	Lasley JF, Easley GT, McKenzie FF. A staining method for the differentiation of live and dead spermatozoa. I. Applicability to the staining of ram spermatozoa. The Anatomical Record. 1942; 82(2): 167-174.
1943	MacLeod suggested oxidative stress affects sperm function .	MacLeod J. The role of oxygen in the metabolism and motility of human

		spermatozoa. American Journal of Physiology-Legacy Content. 1943; 138(3): 512-518
1943	First human artificial insemination	Guttmacher AF. The Role of Artificial Insemination in the Treatment of Human Sterility. Bull N Y Acad Med. 1943;19(8):573-591.
1944	1944 — Oswald Avery and co-workers suggested that the DNA is the transforming agent responsible for heredity	Avery OT, Macleod CM, Mccarty .Studies on the chemical nature of the substance inducing transformation of pneumococcal types Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. J Exp Med. 1944;79(2):137-158.
1944-50	Erwin Chargaff discovered that DNA is the primary constituent of the gene, and that the amount of adenine in DNA was identical to thymine and likewise cytosine equalled guanine. This helped in the eventual discovery of the double helical structure of the DNA	Chargaff E. Heraclitean Fire. Sketches from a life before nature. The Rockefeller University Press. New York· 1978. Library of Congress Catalogue Card Number 77·95216.ISBN 87470·029·9
1950	Sperm acrosome reaction	Dan JC. Studies on the acrosome. I. Reaction to egg-water and other stimuli. Biol Bull. 1952; 103: 54-66.
1951	Roslind Franklin's 1951 work on X-ray crystallography showed the double helical structure of DNA. Her contribution was acknow-ledged posthumously, with the confirmation of her find-ings by Watson and Crick in 1953.	Franklin RE. (1951) Crystallite Growth in Graphitizing and Non-Graphitizing Carbons. Proceedings of the Royal Society A: Mathematical, Physical and Engineering Sciences. 1951;209(1097:196–218. Franklin RE. The structure of graphitic carbons. Acta Crystallographic.1951; 4(3):253–261. Maddox, B. The double helix and the 'wronged heroine'. Nature 421, 407–408 (2003). https://doi.org/10.1038/nature01399
1951, 1952	Capacitation, one of the final steps in the sperm maturation process, was independently discovered by Austin in Australia and Chang in the USA in 1951. Chang initially referred to this process as 'maturation', which Austin altered to 'capacitation' to prevent confusion with sperm maturation.	Austin CR. Observations on the penetration of sperm into the mammalian egg. Aust J Sci Res (Series B) 1951; 4:581-596. Austin CR. The 'Capacitation' of mammalian sperm. Nature 1952; 170:326. Chang MC. Fertilizing capacity of sperm deposited into the Fallopian tubes. Nature 1951; 168:697-698
1952	Gey and co-workers established a continuous human cell line in 1952. The cells were from the patient Henrietta Lacks. It was Gey's assistant Mary	Gey GO, Coffman WD, Kubicek MT. Tissue culture studies of the proliferative capac ity of cervical carcinoma and normal epithelium. Cancer Research. 1952; 12: 264–265.

	Kubicek who first noticed that HeLa cells remained viable in a nutrient solution of chicken plasma (see reports of Sharrer, 2006 and Lucey et al.,2009. Apparently the use of HeLa cells by the scientific community was not made known to Henrietta Lacks nor her family. Henrietta Lacks died in 1951 but her cells were immortalized. The cell line was derived from the adenocarcinoma of the cervix. Her cells were used initially in the development of the polio vaccine and subsequently for a vast array of medical and scientific research, which led to advancement in medicine and saved millions of lives. It was only decades later that the family of the late Ms Lacks came know of this matter.	Sharrer T. HeLa Herself. Celebrating the woman who gave the world its first immortal-ized cell line. The Scientists. 2006; 20: 22. Lucey BP, Nelson-Rees WA, Hutchins GM. Henrietta Lacks, HeLa cells, and cell culture contamination. Archives of Pathology & Laboratory Medicine. 2009; 133: 1463–1467. Cited in: Jedrzejczak-Silicka, M. History of Cell Culture. New Insights into Cell Culture Technology. 2017, p1-41. doi:10.5772/66905 Henrietta Lacks' Family To Sue Pharmaceutical Companies Continuing To Use Her 'HeLa' Cells Without Their Permission – CBS Baltimore (cbslocal.com). Accessed 29 July 2021
1953	1953 — Watson and Crick confirmed the DNA's double helix structure originally described by Roslind Franklin in 1951 using x-crystallograpy. DNA's double helix structure was confirmed by Watson and Crick in 1953, which was originally described by Roslind Frankin using X-Ray crystallography in 1951.	Watson, J., Crick, F. Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid. Nature 171, 737–738 (1953). https://doi.org/10.1038/171737a0
1953	First human birth from artificial insemination of stored frozen sperm.	Bunge RG, Sherman JK. Fertilizing capacity of frozen human spermatozoa. Nature. 1953; 172(4382): 767-768.
1953 1955	Ribosomes were discovered by Robinson and Brown discovered the ribosomes in (1953) in plant cells and and two years later Palade (1955) discovered them in animal cells by Palade (1955). It was Palade that called it "Ribosomes".	Palade GE. 1955. A small particulate component of the cytoplasm. J. Biophys. Biochem. Cytol. 1(1):59–68. Cited in: Zhen Shi and Maria Barna. Translating the Genome in Time and Space: Specialized Ribosomes, RNA Regulons, and RNA-Binding Proteins. Annu. Rev. Cell Dev. Biol. 2015;31:26.1–26.24 Robinson WH, Brown BV. 1953. Cited in: Manisha M. Ribosomes: Discovery, Occurrence and Functions. Ribosomes: Discovery, Occurrence and Functions (biologydiscussion.com) Accessed 11Sept2021
1955	The nutritional needs of animal cells in culture, which is still used in modern day, was systematically defined by Eagle.	Eagle H. Nutrition needs of mammalian cells in tissue culture. Science. 1955;122:501–514

1956	Arthur Kornberg and co-workers discovered DNA Polymerase in 1956.	Kornberg A, Lehman IR, Bessman M, Simms E. Enzymic synthesis of deoxyribonucleic acid. Biochim Biophys Acta. 1956; 21:197–198 Kornberg A, Lehman IR, Bessman MJ, Simms ES. Enzymic synthesis of deoxyribonucleic acid 1956. Biochim Biophys Acta. 1989;1000:57-58 Lehman IR. Historical perspective: Arthur Kornberg, a giant of 20th century biochemistry. Trends Biochem Sci. 2008; 33(6):291-296
1956	Rich and Davies discovered the double stranded RNA in 1956.	Varshavsky A. Discovering the RNA Double Helix and Hybridization. Cell. 2006; 127(7): 1295–1297.
1958/9	Discovery of tRNA by 3 groups independently. 1. Hoagland et al. 1956,1957, 1958, 2. Ogata and Nohara, 1957 3. Holley, 1957 This RNA was originally called sRNA by Zamecnik and his colleagues. This RNA is now known as tRNA.	Hoagland MB, Keller EB, Zamecnik PC. Enzymatic carboxyl activation of amino acids. J. Biol. Chem. 1956 ;218: 345–358 Hoagland MB, Zamecnik PC, Stephenson ML. Intermediate reactions in protein biosynthesis. Biochim. Biophys. Acta. 1957;24:215–216 Hoagland MB, Stephenson ML, Scott JF, Hecht LI, Zamecnik PC. A soluble ribonucleic acid intermediate in protein synthesis. J. Biol. Chem. 1958;231: 241–257 Ogata K and Nohara H The possible role of the ribonucleic acid (RNA) of the pH 5 enzyme in amino acid activation Biochim. Biophys. Acta.1957; 25: 659–660 Holley R An alanine-dependent, ribonuclease-inhibited conversion of AMP to ATP and its possible relationship to protein synthesis; J. Am. Chem. Soc. 1957;79: 658–661 Rajbhandary UL, Köhrer C. Early days of tRNA research: Discovery, function, purifi cation and sequence analysis. J Biosci;2006; 31(4):439–451,
1960/1	Messenger RNA (mRNA) was discovered and named by Monod in 1960.	Pardee, Jacob, Monod, Brenner, Watson, Meselson 1960/61 Cited In: Cobb M. Who discovered messenger RNA? Curr Biol. 2015;25, R523–R548
1960	RNA polymerase was discovered independently by Charles Loe, Audrey Stevens and Jared Hurwitz.	Stevens A. 1960 Incorporation of the adenine ribonucleotide into RNA by cell

		fractions from E. coli Biochem. and Biophys. Research Communs., 3, 92 (1960). Hurwitz J, Bresler AE. The incorporation of ribonucleotides into ribonucleic acid. J Biol Chem. 1961;236:542-548 Hurwitz J.The Discovery of RNAPolymerase J Biol Chem. 2005;280(52):42477–42485 Loe C, 1960; Cited In: Palanivelu P. Single Subunit RNA Polymerases: An Insight into their Active Sites and Catalytic Mechanism. Biotechnol J Internat. 2017; 20(3): 1-35
1961 1965	**Landmark development** Genetic code deciphered by the groups of MW Nirenberg and HG Khorana. Nirenberg's work solved the first part of the puzzle while Khorana's work completed the puzzle	Nirenberg MW, Matthaei JH. The dependence of cell-free protein synthesis in E. coli upon naturally occurring or synthetic polyribonucleotides. Proc Nat Acad Sci. 1961; 47(10):1588-1602 Söll D, Ohtsuka E, Jones DS, Lohrmann R, Hayatsu H, Nishimura S, Khorana HG. Studies on polynucleotides, XLIX. Stimulation of the binding of aminoacyl-sRNA's to ribosomes by ribotrinucleo- tides and a survey of codon assignments for 20 amino acids. Proc Nat Acad Sci.1965;54(5):1378-1385 Nirenberg M. Historical review: Deciphering the genetic code – a personal account. Trends Biochem Sci. 2004; 29(1):46-54 Fry M. *The Deciphering of the Genetic Code. Landmark Exp Mol Biol. 2016; 421–480.*
1965	A defined serum-free medium was introduced by Ham in 1965.	Ham RG. An improved nutrient solution for diploid Chinese hamster and human cell lines. Exp Cell Res. 1963; 29(3: 515–526.
1976	Sperm zona binding assay	Yanagimachi R, Yanagimachi H, Rogers BJ. The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa. *Biol Reprod.* 1976;15:471–476
1977	Calcium oscillations, the rise in free calcium in oocytes was discovered to be triggered by sperm penetration.	Ridgway EB, Gilkey JC, Jaffe LF. Free calcium increases explosively in activating medaka eggs. Proc Natl Acad Sci USA. 1977; 74:623–7. Steinhardt R, Zucker R, Schatten G. Intracellular calcium release at fertilization in the sea urchin egg. Dev Biol. 1977; 58:185–96.
July 1978	**Landmark development** First birth of human baby through in vitro fertilization and embryo transfer (UK).	Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. Lancet. 1978; 2(8085): 366.

Oct 1978	**Landmark development** First birth of human IVF baby from a cryopreserved embryo (India)	Mukherjee S, Mukherjee S, Bhattacharya SK. The feasibility of long term cryogenic storage of viable human embryos- a brief pilot study report. Indian J. Cryogenics 1978; 3:80 Also see: Anand Kumar TC. Architect of India's first test tube baby: Dr Subas Muckerji (16 January 1931–19 July 1981). Curr Sci 1997; 72: 526–531 Bharadwaj A. The Indian IVF saga: a contested history. Reprod Biomed Soc Online 2016; 2: 54–61. Yovich J. History of IVF from the Indian Perspective. 2020. (PDF) History of IVF from the Indian Perspective (researchgate.net) Accessed 14Sep2021
1980	Structure and function of the zona pellucida Structure and function of the zona pellucida was identified.	Bleil JD, Wassarman PM. Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. Dev Biol. 1980; 76(1): 185-202
1974	First transgenic mice were produced in 1974.	Jaenisch R, Mintz B. Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. Proc Natl Acad Sci U S A. 1974 Apr;71(4):1250-1254.
1984	Development of the hypo-osmotic sperm tail test . It is used for the identification of viable sperm in a specimen of immotile sperms.	Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJ. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil. 1984; 70(1): 219-228
1971 1983	**Landmark development** Polymerase chain reaction (PCR) was originally described by Kjell Kleppe in H Gobind Khorana's laboratory in 1971, followed by Panet et al., 1973 in the same laboratory but went unnoticed until Kary B. Mullis refined the technique in 1983.	Kleppe K, Ohtsuka E, Kleppe R, Molineux I, Khorana HG. Studies on polynucleotides. XCVI. Repair replications of short synthetic DNA's as catalyzed by DNA polymerases. J Mol Biol 1971 56 (2): 341–361. doi:10.1016/0022-2836(71)90469-4 Panet, A., van de Sande, J. H., Loewen, P. C., Khorana, H. G., Raae, A. J., Lillehaug, J. R., & Kleppe, K. (1973). Polynucleotides. CXXIII. Physical characterization and simultaneous purification of bacteriophage T4 induced polynucleotide kinase, polynucleotide ligase, and deoxyribonucleic acid

		polymerase. Biochemistry, 12(25), 5045–5050. Kary B. Mullis; Henry A. Erlich; Norman Arnheim; Glenn T. Horn; Randall K. Saiki; Stephen J. Schar. United States Patent no. 4,683,195. US Patent No. 4,683,202 Inducted in 1998. Process for amplifying, detecting, and/or-cloning nucleic acid sequences. 28 July 1987 Bartlett, J. M. S., & Stirling, D. (n.d.). A Short History of the Polymerase Chain Reaction. PCR Protocols. Methods Mol Biol. 2003;226:3-6. Kary B Mullis. In: https://www.nobelprize.org/prizes/chemistry/1993/mullis/facts/ (Accessed 23July2021)
1981	**Landmark development** Development of the scanning tunnelling microscope by Gerd Binnig and Heinrich Rohrer	Cited In: Robinson AL. Electron Microscope Inventors Share Nobel Physics Prize: Ernst Ruska built the first electron microscope in 1931; Gerd Binnig and Heinrich Rohrer developed the scanning tunneling microscope 50 years later. Science. 1986;14;234(4778):821-822.
1982	Ooplasmic cytoplasm transfer in the mouse	Muggleton-Harris A, Whittingham D, Wilson L. Cytoplasmic control of preimplantation development in vitro in the mouse. Nature. 1982;299(5882):460–462
1990	Ooplasmic cytoplasm transfer in the monkey with live birth	Flood JT, Chillik CF, van Uem JF, Iritani A, Hodgen GD. Ooplasmic transfusion: prophase germinal vesicle oocytes made developmentally competent by microinjection of metaphase II egg cytoplasm. Fertil Steril. 1990;53(6):1049–1054.
1992	Observation that sperm quality diminished with increased age in humans.	Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during past 50 years. BMJ. 1992; Spermatozoa: A Historical PerspectiveInt J Fertil Steril, Vol 12, No 3, Oct-Dec 2018 190 305(6854): 609-613
1992/3	**Landmark development** Venki Ramakrishnan described the structure of the ribosome, going on to win the Nobel Prize in 2009.	Ramakrishnan V, White SW. The structure of ribosomal protein S5 reveals sites of interaction with 16S rRNA. Nature. 1992;358:768–771. Ramakrishnan V, Finch JT, Graziano V, Lee PL, Sweet RM. Crystal structure of globular domain of histone H5 and its implications for nucleosome binding. Nature. 1993;362:219–223.

1994	Fertilization, embryo development, and birth of live and normal young was achieved after electrofusion of mouse oocytes with spermatids	Ogura A, Matsuda J, Yanagimachi R. Birth of normal young after electrofusion of mouse oocytes with round spermatids. Proc Natl Acad Sci USA. 1994; 91(16): 7460-7462.
1998	**Landmark development** Mice are cloned from somatic cells Wakayama et al. (reported in July 1998), and Kato et al., 1998 (reported in December 1998) independently	Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature. 1998 Jul 23;394(6691):369-374. Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H, Tsunoda Y. Eight calves cloned from somatic cells of a single adult. Science. 1998 Dec 11;282(5396):2095-2098.
1999	Discovery of the (gene silencing) SiRNA	Hamilton A.J. and Baulcombe,D.C. (1999) A novel species of small antisense RNA in post-transcriptional gene silencing. Science, 286, 950–952
2006	Presence of gamete receptors reported	Shur BD. Reassessing the role of protein-carbohydrate complementarity during sperm-egg interactions in the mouse. Int J Dev Biol. 2008; 52(5-6): 703-715.
2017	Functional human oocytes were generated by transfer of polar body genomes	Ma H, O'Neil RC, Marti Gutierrez N, Hariharan M, Zhang ZZ, He Y, Cinnioglu C, Kayali R, Kang E, Lee Y, Hayama T, Koski A, Nery J, Castanon R, Tippner-Hedges R, Ahmed R, Van Dyken C, Li Y, Olson S, Battaglia D, Lee DM, Wu DH, Amato P, Wolf DP, Ecker JR, Mitalipov S. Functional Human Oocytes Generated by Transfer of Polar Body Genomes. Cell Stem Cell. 2017; 5;20(1):112-119

Chart 2: Milestones in ART- Embryo Culture and Transfer

Year	**Milestone/Event**	**Reference**
1880	Schenck attempted to culture the embryo in vitro but failed.	Schenck S L. Das Siiugethierei kiinstlick befruchtet ausserha!b des mutterthieres. Mitt. Embr. Inst. K. K. Univ. Wien. 1880; 1, 107. Cited in: Brinster RL. In vitro culture of mammalian embryos. J. Anim Sci. 1968; 27:1-14

1882	**Landmark development** Development of a physiological saline solution, the Ringer solution, by Sidney Ringer in London. It is the precursor of all subsequent 'physiological salines' and related media that have enabled normal cell function to be sustained and studied in vitro, and in later years for the culture of mammalian embryos	Ringer S. Concerning the Influence exerted by each of the Constituents of the Blood on the Contraction of the Ventricle. J Physiol. 1882; 3(5-6): 380–393. Ringer S. Regarding the Action of Lime Potassium and Sodium Salts on Skeletal Muscle. J Physiol. 1887; 8:20-24 Also see: Moore B. In Memory of Sidney Ringer [1835-1910]: Some account of the Fundamental Discoveries of the Great Pioneer of the Bio-Chemistry of Crystallo-colloids in Living Cells. Biochem J. 1911; 5(6-7): i.b3–xix. Miller DJ. Sydney Ringer; physiological saline, calcium and the contraction of the heart. J Physiol. 2004;16; 555(Pt 3): 585–587
1891 /7	**Landmark development** First successful transfer in the mammal (rabbit) reported by Walter Heape. *Of considerable interest is that in the 1897 experiment Heape concluded that the surrogate had no influence on the genetics of the donor embryos it carried to term. While this was considered true in the late 1890's we now know this conclusion is no longer tenable at present times. We now have evidence to show the surrogate can transfer her genes (a tiny proportion) to the donor embryos. The genetic material in the uterine fluid of the surrogate can be taken up by and expressed in the donor embryo (see Villella et al., 2015)*	Heape, W Preliminary note on the transplantation and growth of mammalian ova within a uterine foster-mother. Proc. Roy. Soc. Lond. B Biol. Sci. 1891; 48:457-458. Heape W. Further note on the transplantation and growth of mammalian ova within a uterine foster-mother. Proc. Roy. Soc. Lond. B Biol. Sci. 1897; 62:178-83. Vilella F, Moreno-Moya JM, Balaguer N, Grasso A, Herrero M, Martínez S, Marcilla A, Simón C. Hsa-miR-30d, secreted by the human endometrium, is taken up by the pre-implantation embryo and might modify its transcriptome. Development. 2015. 15;142(18):3210-21
1912 a 1912 b 1925 1929 1930	Other that attempted to culture the embryo in vitro include: • Mark and Long, 1912 (Univ of California; they used blood plasma as the medium) • Brachet, 1912-13, (Univ. of Brussels; used blood plasma) • Maximov, 1925 (Leningrad/later Chicago Univ ; used plasma clots) • Lewis and Gregory 1929/30 (Harvard Univ; used blood plasma) • Pincus 1930 (Cambridge Univ.; used plasma and chick embryo extract – in the form of a clot) But were not successful because they used biological fluids	Mark FL, Long JA. Studies on the early stages of development in the rat and mice. Univ Cal Zool Pub.1912a; 9,105. Cited In: Biggers, JD. Pioneering mammalian embryo culture. In Mammalian Preimplantation Embryo. Eds. Bavister BD, New York, USA:Plenum 1987;1-22 Bracket A. Development in vitro de blastodermes et de jeunes embryons de mammiferes, CR Hebd. Seances Acad Sci. 1912b; 155,1191-1193. In: Hans Ingolf Nielsen HI, Ali J. Embryo Culture Media, Culture Techniques and Embryo Selection: A Tribute to Wesley Kingston Whitten. J Reprod Stem Cell Biotechnol.2010; 1(1): 1-29.

		Maximov, A. Tissue cultures of young mammalian embryos. Contr. Embryol. Carneg. Instn, 1925, 16:47-113. Cited In: Kirby DRS.The development of mouse blastocysts transplanted to the scrotal and cryptorchid testis. J Anal(Lond). 1963; 97(1):119-30 Lewis WH, Gregory PW (1929) Cinematographs of living developing rabbit-eggs. Science 69:226–229 Pincus G. Observations on the living eggs of the rabbits. Proc Roy Soc Lond. B Biol.Sci. 1930; 107:132-67.
1922	Current form of artificial insemination	Ivanov II. 1922. Cited In: Cited in: Ombelet W, Van Robays J. Artificial insemination history: hurdles and milestones. Facts Views Vis Obgyn. 2015;7(2):137-43.
1934	Live birth following transfer of embryo after in vitro fertilization of rabbit oocytes. (this work could not be repeated by both the authors and other workers until many years later)	Pincus, G., Enzmann, E.V., 1934. Can mammalian eggs undergo normal development in vitro. Proc. Natl. Acad. Sci. USA 20,121–122.
1937 1949 a 1949 b	**Landmark development** Cryopreservation of sperm although credited to Polge and co-workers (1949a) but others have reported the same independently at least 2 years previously, viz: Bernstein and Petropavlovski in 1937; Also Smirnov cryopreserved sperm in 1949b in the same year as Polge but Smirnov published his report in the Russian language consequently did not come to the attention of vast majority of workers ***(Milestones in sperm cryopreservation are given elsewhere in this manuscript)***	Bernstein AD, Petropavlosky VV. The influence of nonelectrolytes on survival of spermatozoa (message III). Byull. Eksp. Biol. Med. 1937;3(1):41-43.Russian In: I. I. Katkov II, V. F. Bolyukh VF, Sukhikh GT. KrioBlast™ as a New Technology of Hyper-fast Cryopreservation of Cells and Tissues. Part I. Thermodynamic Aspects and Potential Applications in Reproductive and Regenerative Medicine. Cell Technol Biol Med. 2018;4:530-535 Polge C, Smith AU, Parkes AS (1949) Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 164 (4172):666 Smirnov IV. Preservation of domestic animal semen by deep cooling. Sovetskaja Zootechnia 1949; 4:63–65 In: Isachenko V, Isachenko E, Katkov II, Montag M, Dessole S, Nawroth F, Van Der Ven H (2004) Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapor: effect on motility, DNA integrity, and fertilization ability. Biol Reprod 71(4):1167–1173 Also cryopreserved sperm.

		Cited in: Katkov Polge et al. 1949
1938/ 1964	Invention of artificial vagina for the cattle	Milovanov VK. Artificial Insemination of Livestock in the U.S.S.R. Trans. By Birron A and Cole ZS. 1964; S Monson, Jerusalem Tech. Services, US Dept Commerce, Washington, DC. Cited in: Ombelet W, Van Robays J. Artificial insemination history: hurdles and milestones. Facts Views Vis Obgyn. 2015;7(2):137-43.
1942	Viability Stain for sperm	Lasley JF, Easley GT, McKenzie FF. A staining method for the differentiation of live and dead spermatozoa. I. Applicability to the staining of ram spermatozoa. The Anatomical Record. 1942; 82(2): 167-174.
1943	First suggestion for the oxidative stress affecting sperm function	MacLeod J. The role of oxygen in the metabolism and motility of human spermatozoa. American Journal of Physiology-Legacy Content. 1943; 138(3): 512-518
1944	Rock and Menkin claimed to have fertilized the human egg. Only 3 of 800 eggs cleaved. By the mid 1950s Rock stopped his work on IVF probably because of poor outcome.	Rock J, Menkin MF. In vitro fertilization and cleavage of human ovarian eggs. Science. 1944 Aug 4;100(2588):105-107.
1949	John Hammond Sr, occasionally managed to grow 8-cell mouse embryos to the blastocyst stage in a partially defined growth solution. Hammond could not get 2-cell mouse embryos to cleave in his growth solution (Hammond, 1949).	Hammond J Sr. Recovery and culture of tubal mouse ova. Nature (London). 1949; 163:28-29
1950	Use of antibiotics in culture medium.	Foote RH, Bratton RW. The fertility of bovine semen in extenders containing sulfanilamide, penicillin, streptomycin, and polymyxin. J Dairy Sci. 1950;33:544-547.
1954 a 1954 b	Thibault and his group and Moricard in France reported fertilization and early development of rabbit ova More than 50 claims of successful fertilizations have been have reported (Austin, 1961) but only a few of these reports have been acknowledged by the scientific community which are chronicled herein	Moricard, R., 1954b. Observation of in vitro fertilization in the rabbit. Nature 173, 1140. Thibault C, Dauzier L, Wintenberger S. [Cytological study of fecundation in vitro of rabbit ovum]. C R Seances Soc Biol Fil. 1954a;148(9-10):789-790. Cited In: Biggers JD. IVF and embryo transfer: historical origin and development. Reprod Biomed Online. 2012 Aug;25(2):118-27 Austin, C.R., 1961. The Mammalian Egg. Blackwell, Oxford. Cited in: *Ibid*

1955/6	***Landmark Development in ART & Preimplantation Embryology** 1. First report of successful embryo culture in a nutrient solution in vitro in 1955. Published in 1956 by Wesley K Whitten, while working at the John Curtin School of Medical Research (JCSMR) of the Australian National University (ANU) successfully used an adapted/modified Ringer's Bicarbonate solution containing glucose, penicillin, streptomycin and 1% fresh hen's thin egg white to culture mouse embryos from the 8-cell in vivo generated mouse embryos to the blastocyst stage in vitro on regular basis but could not get his embryos to go past the 2-cell stage (2-cell block). Whitten replaced hen egg white with bovine serum albumin. 2. Later Whitten (1957) supplemented his medium (the Whitten's medium) with Calcium lactate following which he was able to overcome the 2-cell block in the mouse and could regularly obtain hatching and hatched blastocysts. 3. He was perhaps the first to witness embryo hatching in vitro. *It was only after this development research into preimplantation embryology took off on an exponential rate.*	1. Whitten WK (1956). Culture of Tubal Mouse Ova. Nature. 1956; 177(4498), 96–96. 2. Whitten WK: Culture of tubal ova. Nature 1957; 179:1081-1082 3. Whitten WK. Narrated by Whitten to his student J. Ali in person in 1989/90. In: Nielsen HI, Ali J. Embryo culture media, culture techniques and embryo selection: A Tribute to Wesley Kingston Whitten. J Reprod Biotechnol Fertil. 2010; 1(1):1-29. *One of us (JA) was mentored by WK Whitten (co-supervisor), and JN Shelton (supervisor) Dec1989-July1992.*
1956 1957	Embryo transfer (ET) technique in the mouse developed by Adams 1956, and McLaren and Michie (1956). McLaren and Biggers showed that mouse embryos that developed in vitro by Whitten's technique could develop into viable fetuses using the ET technique developed by Adam 1956, and McLaren and Michie (1956).	Adams, C. E., *"Artificial Insemination"*, 5, Third International Congress on Animal Reproduction, Cambridge (1956).pp5-6 McLaren A. Michie D. Studies on the transfer of fertilized mouse eggs to uterine foster mothers. J Exp Biol. 1956; 33:394-416. Cited In: Spinks N. R., Ryan J. P. O'Neill C. Antagonists of embryo-derived platelet-activating factor act by inhibiting the ability of the mouse embryo to implant. J reprod Fert. 1990; 88:241-248 McLaren A. Biggers JD. Successful development and birth of mice cultivated in vitro as early embryos. Nature. 1958; 182, 877-878. Cited In: Lopata A. History of the egg in embryology. J Mamm Ova Res. 2009; 26:2-9
1959	**Landmark development**	Chang, M.C.,. Fertilization of rabbit ova in vitro. Nature 1959;184:466–467.

	First report of successful in vitro fertilization, generation of embryos in vitro followed by successful embryo transfer and live births in the mammal – rabbit (by MC Chang of the Worcester Foundation)	
1962	Embryo co-culture with cells of the ampullary region of the mouse Fallopian tube overcame the 2-cell block. This became the forerunner of the co-culture system developed by a number of workers. However the use of hetegeneous cell in coculture of human was short-lived.	Biggers JD , Gwatkin RB, Brinster RL. Development of mouse embryos in organ cultures of fallopian tubes on a chemically defined medium. Nature. 1962; 26(194): 747-749 Mansour RT, Aboulghar MA, Serour GI, Abbass AM. Co-culture of human pronucleate oocytes with their cumulus cells. Hum Reprod. 1994 Sep;9(9):1727-1729
1963	**Landmark development** Droplet cultures under oil was applied for embryo culture in 1963 A method of embryo culture, the microdroplet culture technique based on the work of Gwatkin et al., (1963) for studying the effect of viruses on ova which was later adapted and applied by Brinster for embryo culture (1963). This offered better control over the culture conditions and is the mainstay in human ART for culture of embryos. It was originally adapted from Lwoff et al., (1955) who used the technique to study the release of poliomyelitis virus from cells (cited in Biggers, 1987).	Gwatkin RB (1963) Effect of viruses on early mammalian development. I. Action of Mengo encephalitis virus on mouse ova cultivated in vitro. Proc Natl Acad Sci USA. 1963; 50:576–581 Brinster RL A method for in vitro cultivation of mouse ova from two-cell blastocyst.Exp Cell Res. 1963; 32:205–208 Lwoff, A., Dulbecco R, Vogt M, Lwoff M. Kinetics of the release of poliomyelitis virus from single cells. Virology. 1955; 1:128-139. Also see: Biggers JD Pioneering mammalian embryo culture. In: Bavister BD (ed) The mammalian preimplantation embryo: regulation of growth and differentiation in vitro.1987; Plenum Press, New York/London, pp 1–22
1965 1966 1969	In vitro maturation of human oocytes by Robert Edwards.	Edwards RG. Maturation in vitro of human ovarian oöcytes. Lancet 1965;286:926-9. Edwards RG, Donahue RP, Baramki TA, Jones HW Jr. Preliminary attempts to fertilize human oocytes matured in vitro. Am J Obstet Gynecol 1966;96:192-200. Edwards RG, Bavister BD, Steptoe PC. Early stages of fertilization in vitro of human oocytes matured in vitro. Nature 1969;221:632-5. Cited in: Hatırnaz S, Ata B, Hatırnaz ES, Dahan MH, Tannus S, Tan J, Seang Lin Tan SL. Oocyte in vitro maturation: A sytematic

		review Turk J Obstet Gynecol. 2018 Jun; 15(2): 112–125
1971	The “sperm rise” or the swim-up technique of sperm preparation was described in 1971 ***(Milestones in sperm preparation are given elsewhere in this manuscript)***	Drevius LO. The 'sperm-rise' test. J Reprod Fertil. 1971;24(3):427-429.
1973	First IVF pregnancy in the human was reported in Australia	De Kretzer D, Dennis P, Hudson B, Leeton J, Lopata A, Outch K, Talbot J, Wood C. Transfer of a human zygote. Lancet, 1973 29;2:728-9)
July 1978	**Landmark Development in ART:** First live birth from IVF in the human was reported in England. These workers used laparoscopic recovery for the retrieval of a single oocyte obtained during natural cycle **(Noble Prize, 2010)**	Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. Lancet, 1978 ; 12;2:366
Oct 1978	**Landmark Development in ART** First live birth from cryopreserved IVF embryo in the human was reported in India. However this claim was not acknowledged until recently in 2002 by the Indian Council for Medical Research (narrated at the 23rd Conference of the Indian Society for Assisted Reproduction, 19-22 April 2018, Kolkata). These workers used gonadotrophin stimulation to retrieve more than one oocyte, follicle development was monitored hormonally and by ultrasound, ovulation was triggered by hCG administration, oocyte retrieval was performed by transvaginal aspiration. They preferred to cryopreserve the embryos and organize the transfer at a later point post-thaw. This is the modality currently adopted worldwide. ***This was / is a rare milestone of considerable import notable for the unfair chastisement this work and its authors received for assumed fraud from the authorities. This situation was further aggravated by the ridicule or scepticism expressed by peers, both local and international. These unfortunate circumstances supposedly compounded by poor health ultimately led to the untimely and unnatural demise of the team leader (SM; the first author). His last written message to his wife was***	Mukherjee S, Mukherjee S, Bhattacharya SK. The feasibility of long term cryogenic storage of viable human embryos- a brief pilot study report. Indian J. Cryogenics 1978; 3:80 Also see: Anand Kumar TC. Architect of India’s first test tube baby: Dr Subas Muckerji (16 January 1931–19 July 1981). Curr Sci 1997; 72: 526–531 Bharadwaj A. The Indian IVF saga: a contested history. Reprod Biomed Soc Online 2016; 2: 54–61. Yovich J. History of IVF from the Indian Perspective. 2020. (PDF) History of IVF from the Indian Perspective (researchgate.net) Accessed 14Sep2021 Beautiful Mind | Kolkata News - Times of India (indiatimes.com) Accessed 14Sep2021

	"I can't wait every day for a heart attack to kill me." Subsequent to the demise of SM the remaining members of the group assumed a low profile. The authenticity of this work came to the fore decades later). ***(Milestones in cryopreservation is chronicled in greater detail by the authors in the subsequent Chart or manuscript in this series)***	
1981	**Landmark Development in ART and Stem Cell Technology:** The isolation of embryonic stem cells (Evans and Kaufman, 1981 and Martin, 1981) heralded a new era in stem cell technology. ESCs are pluripotent and can differentiate into over 200 specialized cell types or most if not all cells of the whole animal or organism. ***(Noble Prize, 2007, awarded jointly to Sir Martin J. Evans, Mario R. Capecchi and Oliver Smithies)***	Evans MJ, Kaufman MH. Establishment in culture of pluripotent cells from mouse embryos. Nature. 1981 Jul 9;292(5819):154–156. G R Martin. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A. 1981 Dec; 78(12): 7634–7638
1984, 1985, 1986	Media for human IVF fertilization and embryo culture was developed by three groups of workers independently 1. Menezo's medium 2. Quinn's Human Tubal Fluid (HTF) 3. Mortimer's Synthetic Tubal Fluid (STF) Medium, 1986 Prior to this media previously used for other mammalian embryos and tissue culture was used for human IVF	Menezo Y, Testart J, Perrone D (1984) Serum is not necessary in human in vitro fertilization, early embryo culture, and transfer. Fertil Steril. 1984; 42: 750-755. Quinn P, Kerin JF, Warnes GM (1985) Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. Fertil Steril 44, 493-498 Mortimer D. Elaboration of a new culture medium for physiological studies on human sperm motility and capacitation. Human Reprod. 1986; 1: 247-250. Also see: Mortimer D. Human blastocyst development media. Human Reprod. 2001; 16: 2725
1986	**Landmark development** Live-birth from frozen-thawed human eggs, 1986 by Chen at the Queen Elizabeth Hospital, Adelaide, Australia ***(Milestones in oocyte cryopreservation are given elsewhere in this manuscript)***	Chen C. Pregnancy after human oocyte cryopreservation. Lancet 1986, 1,884 – 886
1987	Fertilization of human oocytes by microinjection of a single sperm under the zona pellucida	Laws-King A, Trounson A, Sathananthan H, Kola I. Fertilization of human oocytes by microinjection of a single spermatozoon under

		the zona pellucida. Fertil Steril. 1987;48:637-642.
1988	Live birth using subzonal sperm injection at National University of Singapore by SC Ng.	Ng SC, Bongso A, Ratnam SS, Sathananthan H, Chan CL, Wong PC, Hagglund L, Anandakumar C, Wong YC, Goh VH. Pregnancy after transfer of sperm under zona. Lancet. 1988; 1;2:790.
1988	Two babies born after epididymal sperm aspiration for men with congenital absence of the vas deferens and naming of the technique MESA	Patrizio P, Silber S, Ord T, Balmaceda JP, Asch RH. Two births after microsurgical sperm aspiration in congenital absence of vas deferens. Lancet. 1988, 10;2(8624):1364.
1989	First report on biopsy of human preimplantation embryos and sexing by DNA amplification A. Handyside.	Handyside AH, Pattinson JK, Penketh RJ, Delhanty JD, Winston RM, Tuddenham EG. Biopsy of human preimplantation embryos and sexing by DNA amplification. Lancet. 1989; 18;1:347-9).
1989	Report on the use of laser techniques in the field of assisted reproduction	Tadir Y, Wright WH, Vafa O, Ord T, Asch RH, Berns MW. Micromanipulation of sperm by a laser generated optical trap. Fertil Steril. 1989 Nov;52(5):870-873.
1990	Report of pregnancy after assisted hatching in human embryos.	Cohen J, Elsner C, Kort H, Malter H, Massey J, Mayer MP, Wiemer K. Impairment of the hatching process following IVF in the human and improvement of implantation by assisting hatching using micromanipulation. Hum Reprod. 1990;5(1):7-13.
1990	First “MIMIC” or now called “ICSI” was attempted by SC Ng at the National University of Singapore in 1990. Ng called his technique “MIMIC” (Micro-Insemination Micro-Injection into Cytoplasm) but this technique was not practiced in most countries due to regulation until it was revisited a few years later by Palermo (Palermo et al., 1992). It is noteworthy Ng was also the pioneer of the SUZI technique (1988) with first live-birth.	Ng SC, Bongso TA, Sathananthan AH, Ratnam SS. Micro-manipulation: its relevance to human IVF. Fertil.Steril. 1990; 53: 203-219 Ng SC, Bongso TA, Sathananthan AH, Ratnam SS. Microinjection of human oocytes: a technique for severe oligoasthenoteratozoospermia. Fertil Steril. 1991; 56(6):1117-11123
1992	**Landmark development** Report of pregnancy after intracytoplasmic sperm injection (ICSI) by G Palermo in Brussels	Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet. 1992;4;340:17-18
1994	Use of autologous cumulus cells for culture of human embryos in human IVF improved pregnancy rate with live births. It was felt to be safer than the use of heterogeneous cells.	Mansour RT, Aboulghar MA, Serour GI, Abbass AM. Co-culture of human pronucleate oocytes with their cumulus cells. Hum Reprod. 1994;9(9):1727-1729
1995	Elongated spermatids used to achieve pregnancy by S Fishel. But this work could not be reproduced.	Fishel S, Green S, Bishop M, Thornton S, Hunter A, Fleming S, al-Hassan S. Pregnancy after intracytoplasmic injection of spermatid. Lancet. 1995, 24;345(8965):1641-1642.

1995, 1997	Sequential medium G1 and G2 formulation for the development of human blastocyst in culture with live birth. The G1 and G2 media were developed by Gardner and Lane (1993)	Barnes, F.L., Crombie, A., Gardner, D.K. et al. (1995) Blastocyst development and pregnancy after in vitro maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. Hum. Reprod., 10, 3243–3247. Also see: Gardner DK, Lane M (1993) Amino acids and ammonium production regulate mouse embryo development in culture. Biol Reprod 48, 377-385 Gardner DK, Lane M. Culture and selection of viable blastocysts: a feasible proposition for human IVF? Human Reprod. Update. 1997; 3: 367-382
1995	**Landmark development** The isolation of primate embryonic stem cells by Jamie Thomson.	J A Thomson, J Kalishman, T G Golos, M Durning, C P Harris, R A Becker, J P Hearn Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci U S A. 1995 Aug 15; 92(17): 7844–7848
1995 1996	**Landmark development** The first successful vitrification of Day 2 human embryos with no loss of survival with cleavage of all embryos to day 3 and subsequent stages of embryo development was reported by Jaffar Ali in 1995/6. This was the first report of the successful vitrification of the human embryo. Ali moved elsewhere after this accomplishment where he was not permitted to undertake a clinical trial in the human using his method of vitrification. ***(Milestones in vitrification of gametes and embryo are given elsewhere in this manuscript)***	Ali J (1996) Developmental competence of unipronuclear and triploid day-2 human embryos after vitrification with VS14. Med Sci Res 24:377–378 Ali J, Bongso A, Ratnam SS (1995) Chromosomal analysis of day-2 human embryos vitrified with VS14. Med Sci Res 23:539–540 Also see: Ali J (2001) Source of vitrification solution. Fertil Steril 76:214–215 Ali J (2001) Vitrification of embryos and oocytes with 5.5 mol/l ethylene glycol and 1.0 mol/l sucrose. Hum Reprod 16:1777–1778
1997 1998 1999 a 1999 b 1999c	**Landmark development** Live-births from cryopreserved (slow cooling method) human oocyte could be obtained on regular basis using an efficacious controlled-rate freezing technique that utilized propanediol. The problem of oocyte cryopreservation by conventional controlled cooling was finally put to rest.	Porcu E, Fabbri R, Seracchioli R, Ciotti PM, Magrini O, Flamigni C. Birth of a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. Fertil Steril . 1997;68(4):724–726 Fabbri R, Porcu E, Marsella T, Primavera MR, Seracchioli R, Ciotti PM, Magrini O, Venturoli

	It is noteworthy that Dr Gook collaborated with the group of Porcu et al. – to establish their oocyte freezing program and achieve healthy babies after human oocyte slow freezing. (*The entry above was contributed by Dr Mitchel C Schiewe, 2024; corroborated in person by Dr Debra Gook during the ESHRE2026 conference in London*)	S, Flamigni C) Oocyte cryopreservation. Hum Reprod 1998;13(Suppl 4):98–108 Porcu E (1999a) Freezing of oocytes. Curr Opin Obstet Gynecol . 1999a;11(3):297–300 Review Porcu E, Fabbri R, Ciotti PM, Petracchi S,Seracchioli R, Flamigni C.(Ongoing pregnancy after intracytoplasmic sperm injection of epididymal spermatozoa into cryopreserved human oocytes. J Assist Reprod Genet 1999b; 16(5):283– 285 Porcu E, Fabbri R, Petracchi S, Ciotti PM, Flamigni C. Ongoing pregnancy after intracytoplasmic injection of testicular spermatozoa into cryopreserved human oocytes. Am J Obstet Gynecol 1999c; 180 (4): 1044–1045
1997, 2000	**Significant development** First pregnancies and livebirths from embryos generated in a synthetic Protein-Free Medium (SPFM) for Human IVF with live births. The SPFM utilized, for the first time, a completely chemically defined media devoid of added proteins of biological origin beginning from oocyte aspiration, sperm preparation, fertilization and the culture of resultant embryos all the way to blastocyst stages.	Ali J. Formulation of a protein-free culture system for the culture of human embryos: preliminary findings and pregnancies. Proc. XVI Annual Sci Meeting Fertil Soc Australia, 2-4 Nov, 1997, Adelaide, Australia, p.34 Ali J, AlNatsha SD, Shahata MAM. Formulation of a protein-free medium for human assisted reproduction. Human Reprod. 2000; 15(1): 145-156 Ali J. Investigation into the nutrient requirement of the human embryo: Successful formulation and clinical trial of a novel protein-free embryo culture medium. Emirates Med. J. 2001;18:195-202 Ali J. Generation of viable human embryos in a protein-free embryo culture (art-7b) medium enhances clinical pregnancy rate and prevents disease transmission in assisted reproduction. MEFS J. 2004;9:118-127
1997	Cytoplasmic transfer from donor to recipient oocyte in the human with live-birth	Cohen J, Scott R, Schimmel T, Levron J, Willadsen S. Birth of infant after transfer of anucleate donor oocyte cytoplasm into recipient eggs. Lancet. 1997;350(9072):186–187
1998	**<u>Landmark Development in ART & Stem Cell Technology:</u>** The isolation of human embryonic stem cells by Jamie Thomson.	Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145–1147

2002	Development of a single non-renewable medium for human embryo blastocyst culture. Actual work on this medium commenced in 1991 or earlier.	Biggers JD, Racowsky C. The development of fertilized human ova to the blastocyst stage in KSOM(AA) medium: is a two-step protocol necessary? Reprod Biomed Online. 2002;5(2):133-40 Also see: Lawitts JA, Biggers JD 1991 Optimization of mouse embryo culture media using simplex methods. Journal of Reproduction and Fertility 91, 543–556
2003 2004 2006	Report of derivation of male germ cell from mouse embryonic stem cell (Toyooka et al., 2003; Geijsen et al., 2004; Nayernia et al., 2006). **Landmark Developments in ART & Stem Cell Technology :** Nayernia's group (2006; Newcastle University, UK) reported live births in the mouse following injection of sperm produced from mouse ES cells into mature oocytes.	Toyooka Y, Tsunekawa N, Akasu R, Noce T. Embryonic stem cells can form germ cells in vitro. Proc Natl Acad Sci USA. 2003; 100:11457–11462. Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K, Daley GQ. Derivation of embryonic germ cells and male gametes from embryonic stem cells. Nature 2004; 427: 148–154. Nayernia K, Nolte J, Michelmann HW, Lee JH, Rathsack K, Drusenheimer N, Dev A, Wulf G, Ehrmann IE, Elliott DJ, Okpanyi V, Zechner U, Haaf T, Meinhardt A, Engel W. In vitro-differentiated embryonic stem cells give rise to male gametes that can generate offspring mice. Dev Cell. 2006; 11:125–132
2003	1. Report of oocyte production in vitro from mouse embryonic stem cells. These oocytes can be activated to undergo parthenogenesis and develop to blastocysts in vitro. 2. Offspring were born in mice from oocytes derived from female Embryonic Stem Cells and iPSCs induced to differentiate to Primordial Germ Cell-like Cells that formed aggregates in culture. These were grafted under the ovary bursa which formed mature artificial oocytes. The oocytes were fertilized and 2-cell embryos were transferred to surrogates which later delivered fertile normal pups.***(Milestones in embryonic stem cell technology may be published elsewhere)***	Hubner K, Fuhrmann G, Christenson LK, Kehler J, Reinbold R, De La Fuente R, Wood J, Strauss JF III, Boiani M, Scholer HR. Derivation of oocytes from mouse embryonic stem cells. Science. 2003 300:1251–1256. Hayashi K, Ogushi S, Kurimoto K, Shimamoto S, Ohta H, Saitou M. Offspring from oocytes derived from in vitro primordial germ cell-like cells in mice. Science 2012; 338:971–975.
2013	In vitro follicle activation with live-birth	Kawamura K, Cheng Y, Suzuki N, Deguchi M, Sato Y, Takae S, Ho CH, Kawamura N, Tamura M, Hashimoto S et al. . Hippo

		signaling disruption and Akt stimulation of ovarian follicles for infertility treatment. Proc Natl Acad Sci USA 2013;110:17474–17479
2013	**Minimally invasive technique for assessment of embryo competence and viability** The identification and quantification of embryonic proteins present in extreme low concentrations in the blastocoel was described by Poli et al., 2013. This technique is less risky compared to invasive forms of PGT	Poli M, Ori A, Child T, Jaroudi S, Spath K, Beck M, Wells D. Characterizat -ion and quantification of proteins secreted by single human embryos prior to implantation. EMBO Mol Med. 2015;7(11):1465-1479.
2013	**Minimally invasive technique for PGT.** A procedure to source for embryonic DNA for Minimally invasive PGT for preimplantation genetic assessment from blastocoel fluid was reported. It is an injection and aspiration procedure which avoids serious risks to the embryo (Palini et al., 2013; Perloe et al, 2013; Poli et al, 2013; Gianaroli et al, 2014).	Palini S., Galluzzi L., De Stefani S., Bianchi M., Wells D., Magnani M., Bulletti C. Genomic DNA in human blastocoele fluid. Reprod. Biomed. Online. 2013;26:603–610 Poli M, Jaroudi S, Sarasa J, Spath K, Child T, Wells D. The blastocoel fluid as a source of DNA for preimplantation genetic diagnosis and screening. Fertil Steril. 2013;100:S37 Perloe M, Welch C, Morton P, Venier W, Wells D, Palini S. Validation of blastocoele fluid aspirat-ion for preimplantation genetic screening using array comparative geno-mic hybridization (aCGH) Fertil Steril. 2013; 100: S208. Gianaroli L, Magli MC, Pomante A, Crivello AM, Cafueri G, Valerio M, Ferraretti AP. Blastocentesis: a source of DNA for preimplantation genetic testing. Results from a pilot study. Fertil Steril. 2014;102:1692–1699
2020	Assisted fertilization by sperm zona fusion by pressing sperm to oolemma using injection pipette.	Hatakeyama S, Araki Y, Araki Y, Ohgi S, Yanaihara A. First birth following assisted sperm fusion insemination using sperm bound to zona pellucida. J Assist Reprod Genet. 2020;37(8):1849-1851.
2022	**Landmark development** Report of synthetic mouse fetus that developed to day 8.5. The fetus had forebrain and midbrain regions and the beginnings of a beating heart-like structure, a trunk comprising a neural tube and somites, a tail bud containing neuromesodermal progen- itors, a gut tube, and primordial germ cells.	Amadei G, Handford CE, Qiu C, De Jonghe J, Greenfeld H, Tran M, Martin BK, Chen DY, Aguilera-Castrejon A, Hanna JH, Elowitz MB, Hollfelder F, Shendure J, Glover DM, Zernicka-Goetz M. Embryo model completes gastrulation to neurulation and organogenesis. Nature 2022; 610:143–153 (2022)
2023	**Landmark development** Report of a synthetic human fetus from stem cells in the human. The fetus is devoid of beating heart and brain cells.	Communicated by Magdalena Zernicka-Goetz from Cambridge University and the Technological Institute of California at the year 2023 Annual Meeting organized by International Society for Stem Cell Research

		(ISSCR), June 14 - 17, 2023, Boston Convention and Exhibition Center, Boston, Massachusetts, United States of America. In: https://www.msn.com/en-us/news/technology/scientists-successfully-create-synthetic-human-fetus/ar-AA1cAWcz?ocid=msedgntp&cvid=02bb99f9367449dd9b66a60e124695c4&ei=18 . Accessed on 16 June 2023
2025	**Landmark development** The extra copy of chromosome 21 that cause Down syndrome can be removed. The work of Hashizume and coworkers successfully demonstrated the efficient elimination of excess chromosomes using multiple allele-specific targeting. This finding can be used to rescue somatic cells with trisomy eventhough the preferred method is the Non- chromosome-breaking elimination method.	Hashizume R, Wakita S, Sawada H, Takebayashi SI, Kitabatake Y, Miyagawa Y, Hirokawa YS, Imai H, Kurahashi H. Trisomic rescue via allele-specific multiple chromosome cleavage using CRISPR-Cas9 in trisomy 21 cells. PNAS nexus. 2025 Feb;4(2):pgaf022.

Chart 3. Milestones in ART - Development of techniques of sperm preparation

A. Preliminary research on sperm and semen		
Year	**Milestone/Event**	**Reference**
1929	Sperm count	Macomber D, Sanders MB. The spermatozoa count. N Eng J Med. 1929; 200(19): 981-984
1934	First attempt at sperm preparation	Milovanov VK (1934 a,b). Cited In: CW Emmens, GI Swyer. Observations on the motility of rabbit sperm in dilute suspension. J Gen Physiol 1948; 32(1):121–138
1936	Seminal plasma contains muscle stimulating substances (von Euler et al., 1936), later called prostaglandins by Bergström, (1967) that could cause uterine contractions (Karim et al., 1969; cited in Hinman, 1970).	Von Euler, 1936; Bergström, (1967); (Karim et al., 1969; cited in Hinman, 1970).
1942/6	Dilution of semen affected fertilization. The fertilising ability of the sperm appeared to be inversely related to the volume of the diluent. The fertilisation rate was significantly higher when the volume of diluent was low (0.1ml) compared to a larger volume (1.0ml) of diluents.	Chang MC. PhD Thesis, Physiology of ram sperm, Cambridge University, 1942. Cited In: Cited In: CW Emmens, GI Swyer. Observations on the motility of rabbit sperm in dilute suspension. J Gen Physiol. 1948;32(1):121-138 Chang MC. Science.1946;104: 361. Cited In: CW Emmens, GI Swyer. Observations on the

		motility of rabbit sperm in dilute suspension. J Gen Physiol. 1948;32(1):121-138
1943	Propensity for sperm to undergo oxidative damage. It was noted that incubation of sperm under conditions of high oxygen tension led to a rapid loss sperm viability.	MacLeod J. The role of oxygen in the metabolism and motility of humanspermatozoa. Am J Physiol 1943;138:512– 518; cited in Storey BT (1997) Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. Mol Hum Reprod. ; 3(3):203-213.
1945/6/8	Fructose detected in semen. Fructolysis is a major source of energy for sperm motility	Mann, T., Nature, 156, 80 (1945) In: Mann, T. Fructose, a Constituent of Semen. Nature 157, 79 (1946); Mann T. Fructose and fructolysis in semen in relation to fertility. Lancet. 1948;1(6499):446-448.
1951 1957	Demonstration of chemotaxis in spermatozoa	Moricard R, Bossu J. Arrival of fertilyzing sperm at the follicular cell of the secondary oocyte. Fert Steril 1951; 2: 260–266. Schwartz R, Brooks W, Zinsser HH. Evidence of chemotaxis as a factor in sperm motility. Fertil Steril 1957; 9: 300–308.
1951, 1952	Capacitation was independently discovered by Austin in Australia and Chang in the USA in 1951. Chang called it “maturation” but later Austin changed the term to “capacitation” to avoid confusion with the lengthy sperm maturation process. Capacitation is one of the final steps in the sperm maturation process	Austin CR. Observations on the penetration of sperm into the mammalian egg. Aust J Sci Res (Series B) 1951; 4:581-596. Austin CR. The ‘Capacitation’ of mammalian sperm. Nature 1952; 170:326. Chang MC. Fertilizing capacity of sperm deposited into the Fallopian tubes. Nature 1951; 168:697-698
1959	ATP reactivated motility of immobilised sperm (Bishop & Hoffmann-Berling, 1959).	Bishop DW, Hoffmann-Berling H. Extracted mammalian sperm models. I. Preparation and reactivation with adenosine triphosphate. J Cell Comp Physiol. 1959; 53:445-466.
1962	Some factors that prevented capacitation were found in seminal plasma. These were called decapacitation factors. These factors were secreted by the epididymis and accessory organs into the seminal plasma.	Bedford JM & Chang MC. Removal of decapacitation factor from seminal plasma by high-speed centrifugation. Am J Physiol. 1962; 202 (1):179-181
1965a,b	Sperm-toxic component detected in seminal plasma	Shannon P (1965a) Contribution of seminal plasma, sperm numbers, and gas phase to dilution effects of bovine sperm. J.Dairy Sci. ; 48:1357; Shannon P (1965b) Presence of a heat-labile toxic protein in bovine seminal plasma. J.Dairy Sci.; 48:1362-1365.
1968	Prolonged exposure of the sperm to autologous seminal plasma has adverse effects on sperm function that affects its ability to penetrate cervical mucus.	Kremer J. The in vitro spermatozoal penetration test in fertility investigations. PhD dissertation. University of Groningen, The Netherlands;

		1968. Cited in Mortimer D. Sperm preparation methods. J Androl. 2000;21(3):357-366
1969	Respiration as another source of energy, in addition to fructose for sperm	Rikmenspoel R, Sinton S, Janick JJ. Energy conversion in bull sperm flagella. J Gen Physiol. 1969;54(6):782-805
1967	Prostaglandins in seminal plasma. Seminal plasma contains muscle stimulating substances (von Euler et al., 1936), later called prostaglandins by Bergström, (1967) that could cause uterine contractions	Bergström S. Prostaglandins: members of a new hormonal system. These physiologically very potent compounds of ubiquitous occurrence are formed from essential fatty acids. Science. 1967;157(3787):382–391. Cited In: Hinman JW. Prostaglandins: a report on early clinical studies. Postgrad Med J. 1970;46(539):562-575. Karim SM, R R Trussell RR , Hillier K, Patel RC. Induction of labour with prostaglandin F2a J Obstet Gynaecol Br Commonw. 1969;76(9):769-782.
1974	More than one factor in seminal plasma suspected to be toxic to sperm – related to 5 above	Dott HM. The effects of bovine seminal plasma on the impedance change frequency and glycolysis of bovine epididymal sperm. J Reprod Fert. 1974; 38:147-156
1976	It was reported that the natural substrates for peroxidation in sperm are unsaturated plasmalogens	Jones R, Mann T. Lipid peroxidation in sperm; formation, role of plasmalogen, and physiological significance. Proc R Soc. 1976;193, 317- 333.
1976	The presence of minute amounts of seminal plasma in prepared sperm suspension induces the acrosome reaction.	Oliphant G. Removal of sperm-bound seminal plasma components as a prerequisite to induction of the rabbit acrosome reaction. Fertil Steril. 1976;27(1):28-38.
1977	Glass wool filtration was used to separate motile sperm from seminal cellular debris and dead sperm.	Paulson JD, Polakoski KL: A glass wool column procedure for removing extraneous material from the human ejaculate. Fertil Steril. 1977, 28: 178-181.
1979	The presence of minute contaminants of seminal plasma in prepared sperm reduced or completely inhibited the fertilising capacity of the sperm.	Kanwar KC, Yanagimachi R, Lopata A. Effects of human seminal plasma on fertilizing capacity of human spermatozoa.Fertil Steril. 1979; 31(3): 321-327.
1979	Removal of decapacitation factors occurs during sperm preparation in vitro in medium containing albumin. These events help to strip-off the decapacitation factors from the surface of the sperm so that fertilization is not impeded.	Davis BK, Byrne R, Hungund B. Studies on the mechanism of capacitation. II. Evidence for lipid transfer between plasma membrane of rat sperm and serum albumin during capacitation in vitro. Biochim Biophys Acta. 1979;558(3):257-266; Choi YH, Landim-Alvarenga FC, Seidel GE Jr, Squires EL. Effect of capacitation of stallion sperm with polyvinylalcohol or bovine serum albumin on penetration of bovine

		zona-free or partially zona-removed equine oocytes. J Anim Sci. 2003;81(8):2080-2087.; Bedu-Addo K, Lefièvre L, Moseley FL, Barratt CL, Publicover SJ. Bicarbonate and bovine serum albumin reversibly 'switch' capacitation-induced events in human sperm. Mol Hum Reprod. 2005;11(9):683-691
1982	A heat-labile factor, called antifertility factor AF-1, purified from seminal plasma prevented fertilization by capacitated sperm but acrosome reaction was not impeded.	Reddy JM, Audhya TK, Goodpasture JC, Zaneveld LJ. Properties of a highly purified antifertility factor from human seminal plasma. Biol Reprod . 1982;27(5): 1076-1083.
1983	Prolonged exposure to seminal plasma of more than 30 minutes resulted in a reduction in the fertilisation rate	Rogers BJ, Perreault SD, Bentwood BJ, McCarville C, Hale RW, Soderdahl DW. Variability in the human-hamster in vitro assay for fertility evaluation. Fertil Steril. 1983; 39:204–211
1984	Decapacitation factors that prevented capacitation were inhibitory molecules that coated the surfaces of the sperm.	Fraser LR. Mouse sperm capacitation in vitro involves loss of a surface-associated inhibitory component. J Reprod Fertil 1984; 72: 373–384.
1986	Removal of decapacitation factors occurs during sperm preparation in vitro in medium containing and following incubation with bicarbonate (Lee and Storey, 1986; Neill and Olds-Clarke, 1987; Harrison, 1996) These events help to strip-off the decapacitation factors from the surface of the sperm so that fertilization is not impeded.	Lee MA, Storey BT.Bicarbonate is essential for fertilization of mouse eggs: mouse sperm require it to undergo the acrosome reaction. Biol Reprod. 1986;34(2):349-356; Neill JM, Olds-Clarke P. A computer-assisted assay for mouse sperm hyperactivation demonstrates that bicarbonate but not bovine serum albumin is required. Gamete Res. 1987;18(2):121-140; Harrison RA. A highly efficient method for washing mammalian spermatozoa. J Reprod Fertil. 1976;48(2):347-353.
1988	Centrifugation of unselected human sperm that resulted in pellet formation could result in the generation of free radical or reactive oxygen species (ROS) within the sperm pellet that could adversely affect sperm function in vitro	Aitken RJ, Clarkson JS. Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. J Androl. 1988;9:367–376
1989	Demonstration of photoactivation of and negative phototaxis in the human sperm which was applied to enhance the swim-up technique, especially in semen of poor quality.	Ali, J., Lim-Howe,D. and Halim,A.J.(1989). Improved harvest of motile sperm cells by photoactivation in the swim-up technique. Malaysian J. Reprod. Health. 7(2):111-114. Ali, J. (1993) A simple technique for harvesting sufficient amount of motile spermatozoa for GIFT and IVF procedures by multi-tube direct layering and photoactivation of semen of oligospermic men. Malaysian J. Reprod. Health 11:25-28

		Ali, J. (1994) Negative phototaxis in human spermatozoa. Assisted Reprod. Technol. Androl. 6:121-122
1991	Other filtration techniques for separation of motile sperm have been described, viz: Sephadex beads and L4 membrane	Drobnis EZ, Zhong CQ, Overstreet JW: Separation of cryopreserved human semen using Sephadex columns, washing or Percoll gradients. J Androl. 1991, 12: 201-208. Agarwal A, Manglona A, Loughlin KR: Filtration of spermatozoa through L4 membrane: a new method. Fertil Steril. 1991, 56: 1162-1165.
B. **Sperm separation: Development of the Swim-Up technique**		
1971	The “sperm rise” or the swim-up technique of sperm preparation was described in 1971	Drevius LO. The 'sperm-rise' test. J Reprod Fertil. 1971;24(3):427-9. Emmens CW, Swyer GI. Observations on the motility of rabbit sperm in dilute suspension. J Gen Physiol. 1948;32(1):121-138.
1978	Successful fertilization of human preovulatory oocytes was achieved using sperm obtained following the swim-up method by Lopata and colleagues at the Monash University, Australia in 1978	Lopata A, Brown JB, Leeton JF et al. (1978) In vitro fertilization of preovulatory oocytes and embryo transfer in infertile patients treated with clomiphene and human chorionic gonadotropin. Fertil Steril.; 30: 27–35.
1981-3a	The swim-up technique was standardized at the Monash University, Australia, by a PhD student, Mahadevan, that resulted in the classical swim-up technique that is still in use today	Mahadevan MM, Trounson AO, Leeton JF. The relationship of tubal blockage, infertility of unknown cause, suspected male infertility, and endometriosis to success of in vitro fertilization and embryo transfer. Fertil Steril. 1983a;40(6):755-762. Henkel RR, Schill WB. Sperm preparation for ART. Reprod Biol Endocrinol. 2003; 1:108; Henkel R. Sperm preparation: state-of-the-art--physiological aspects and application of advanced sperm preparation methods. Asian J Androl. 2012;14(2):260-269

1982/3	Centrifugal force of 200g is the highest the human sperm can endure without damage.	Mahadevan, MM. Personal Communication, 2012. In: Ali, J. Sperm preparation: swim-up and density gradient centrifugation. In: Male Infertility and Treatment. B. Woodward and J. Mehta, Ed. JP Publishers, UK. 2014.
1982	Conflicting report: Sperm can tolerate centrifugation of up to 800g.	Jeulin C, Serres C, Jouannet P. The effects of centrifugation, various synthetic media and temperature on the motility and vitality of human spermatozoa. Reprod Nutr Dev. 1982; 22:81–91.
1990	Modifications to the swim-up technique that utilized hyaluronate in the medium enhanced treatment outcome. Hyaluronate was both expensive and promoted the acrosome reaction.	Huszar G, Willetts M, Corrales M: Hyaluronic acid (Sperm Select) improves retention of sperm motility and velocity in normozoospermic and oligozoospermic specimens. Fertil Steril. 1990, 54: 1127-1134. Wikland M, Wik O, Steen Y, Qvist K, Söderlund B, Janson PO: A self-migration method for preparation of sperm for in vitro fertilization,. Hum Reprod. 1987, 2: 191-195.
1990/3	Use of multitubes in harvesting sperm in the swim-up technique was independently investigated and reported by two workers. Sufficient amounts of motile sperm could be obtained and successfully used in ART treatment of oligospermic men by the combined use of multi-tube direct layering and photoactivation or negative phototaxis of human semen in the swim-up technique.	Mortimer D: Semen analysis and sperm washing techniques. In: Control of Sperm Motility: Biological Clinical Aspects. Edited by: Gagnon C. 1990, CRC Press, Boca Raton, F1, 263-284. Ali, J. (1993) A simple technique for harvesting sufficient amount of motile spermatozoa for GIFT and IVF procedures by multi-tube direct layering and photoactivation of semen of oligospermic men. Malaysian J. Reprod. Health 11:25-28
1995	A mini swim technique was devised to harvest sufficient spermatozoa for ICSI in severe oligozoospermia	Al Hasani S, Küpker W, Baschat AA, Sturm R, Bauer O, Diedrich C, Diedrich K. Mini-swim-up: a new technique of sperm preparation for intracytoplasmic sperm injection. J Assist Reprod Genet. 1995 Aug;12(7):428-33.
2000	Studies have noted that centrifugation pelleting of sperm is harmful and should be used prudently to ensure that the g-force does not exceed 800g	Mortimer D. Sperm preparation methods. J Androl. 2000;21(3):357-366
C. **Sperm separation: Development of the Density Gradient technique**		
1958/1962	Separation of X-and Y-chromosome bearing sperm using high viscosity medium composed of egg yolk and	Bhattacharya, BC. Sex control in mammals. Z Tierzuecht Zuechtungsbiol 1958; 72:250–254;

	glycine. A success rate of …"77.4% of male offspring using the upper fraction of 'lighter' sperm and 73.7% female offspring using the lower fraction 'heavier' sperm in the rabbit.." was reported. However overall fertility rate with this technique was low (23.3% with an average litter size of 3). Interestingly however Bhattacharya as well as others could not repeat the successes this original experiment (Schilling, 1966). Bhattacharya (1962) noted sperm viability decreased as the length of the sperm preparation procedure increased which called for more efficient density gradient protocol.	Bhattacharya BC. Different sedimentation rates of X- and Y- sperm and the question of arbitrary sex determination. Zwiss Zool 1962; 166:203–250. Schilling E. Experiments in sedimentation and centrifugation of bull spermatozoa and the sex ratio of born calves. J Reprod Fertil 1966; 11:469–472
1964	Sucrose gradients were used to separate ram and rat sperm from their respective epididymal tissues and blood cells. These workers noted the formation of three distinct layers in the gradients: the uppermost contained epididymal tissue and blood cells; the mid-layer consisted of both epididymal tissue and sperm, whereas the lowermost layer was entirely of sperm, however some sperm had been decapitated.	Dawson RM, Scott TW. Phospholipid composition of epididymal spermatozoa prepared by density gradient centrifugation. Nature 1964; 202:292–293.
1964	Colloidal silica particles were used to determine the specific gravity of sperm.	Beatty RA. Density gradient media for mammalian spermatozoa. V Congresso internazionale per la Riproduzione animale e la Fecondzione artificiale, Trento, 1964:276. Cited In: Ali, J. Sperm preparation: swim-up and density gradient centrifugation. In: Male Infertility. Sperm Diagnosis, Management and Delivery. B. Woodward and J. Mehta, Eds. JP Publishers, UK (2014
1974	Subsequently Prakash reported his work on the use of discontinuous density gradients of colloidal silica particles to obtain a clean specimen of motile cattle sperm.	Prakash, C. Séparation des spermatozoïdes mobiles des autres cellules et des débris cellulaires présents dans le sperme bovin. An Biol Anim Biochim Biophys 1974; 14:363–369. Cited In: Harrison RA. A highly efficient method for washing mammalian spermatozoa. J Reprod Fertil 1976; 48(2):347–353.
1976	Ficoll gradient could also be used to separate ram sperm from seminal plasma and other seminal debris.	Harrison RA. A highly efficient method for washing mammalian spermatozoa. J Reprod Fertil 1976; 48(2):347–353.
1978	Colloidal silica particles coated with polyvinylpyrrolidone (PVP) was used for cell separation. This separation media	Pertoft H, Laurent TC, Låås T, Kågedal L. Density gradients prepared from colloidal silica particles coated by polyvinylpyrrolidone (Percoll). Anal Biochem 1978; 88:271–282

	was marketed as Percoll (which was derived from: Pertoft's colloids).	
1981	Gorus and Pipeleers (1981) used Percoll to separate motile sperm from seminal plasma, dead sperm and other seminal cellular debris.	Gorus FK, Pipeleers DG. A rapid method for the fractionation of human spermatozoa according to their progressive motility. Fertil Steril 1981; 35:662–665.
1984	Percoll was used to harvest human motile sperm which had no deleterious effects on the fertilizing capacity. Percoll was later thought to be inflammatory, had high endotoxin content and was found to have a deleterious effect on sperm membrane. Its use was discontinued and replaced with other commercial colloidal preparations such as Nycondenz.	Bolton VN, Braude PR. Preparation of human spermatozoa for in vitro fertilization by isopycnic centrifugation on self-generating density gradients. Arch Androl 1984; 13:167–176.
1988	Nycodenz was shown to be superior to Percoll when used in density gradients for sperm separation.	Gellert-Mortimer ST, Clarke GN, Baker HWG, Hyne RV, Johnston WIH: Evaluation of Nycodenz and Percoll density gradients for the selection of motile human spermatozoa. Fertil Steril. 1988, 49: 335-341.
2011	The beads of colloidal particles used in density gradients strip-off decapacitation factors from the surface of the sperm so that fertilization is not impeded.	Leahy T, Gadella BM. Sperm surface changes and physiological consequences induced by sperm handling and storage. Reproduction. 2011;142(6):759-778.
2025	It is critical to equilibrate HEPES buffered medium in 5-6%CO_2, identical to embryo culture medium, to maintain pH at the optimal level because commercial HEPES buffered medium contain amounts of sodium bicarbonate that appear to cause a drift towards alkaline phase that could be harmful to gametes and embryos.	Sharif Hussain SI, Alwuhoush AYA, Naif Alhathal N, Ali J. Impact of temperature and incubation in 6%CO2 in air on testicular sperm motility. Part 1: TESA sperm. Abst #314 and Impact of temperature and incubation in 6%CO2 in air on testicular sperm motility. Part 2: micro TESA sperm Abst #331 ESA-SRB Conference 2025, Perth Australia. Sharif Hussain SI, Alwuhoush AYA, Naif Alhathal N. Impact of temperature and incubation in 6%CO_2 in air on testicular sperm motility. Part 1: TESA sperm. J Reprod Biotechnol Biomed Sci.14:9-13 Sharif Hussain SI, Alwuhoush AYA, Naif Alhathal N. Impact of temperature and incubation in 6%CO2 in air on testicular sperm motility. Part 2: microTESA sperm. J Reprod Biotechnol Biomed Sci.14:19-23