Milestones in assisted reproduction and related technologies

Part 2: Sperm, oocyte, and reproductive tissue embryo cryopreservation

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 human gamete and embryo cryopreservation technologies were developed in the last few decades but the science that led to its development commenced about five hundred years ago. The authors have chronicled these events and milestones as comprehensively as possible in chart form. The authors do not claim these charts to be complete but have recorded almost all the major milestones and events that led to the development of human gamete and embryo cryopreservation. We have described the beginnings of sperm preservation in 1322 in Arabia, and the attempt to freeze sperm in snow in 1776 by Lazzaro Spallanzani in Italy and then finally sperm was cryopreserved using the cryoprotectant glycerol in Poland (1937), Russia (1949) and the UK (1949). These successes in sperm cryopreservation stimulated research to cryopreserve the human embryo and oocyte. The human embryo was successfully cryopreserved with live birth in India by Mukerjee et al. in October 1978 while the human oocyte was first cryopreserved by Al-Hasani and coworkers in 1986 in Germany and Chen in 1988 in Australia but reproducibility in oocyte cryopreservation remained a challenge until Porcu et al. in 1997 in Italy described a method for the cryopreservation of the human oocyte that could be routinely reproduced. In 1995 and 1996 Ali et al. successfully cryopreserved the human embryo in Singapore with no loss of survival in vitro by using the ultra-rapid cryopreservation technique of vitrification called VS14 previously developed in 1992 by him at the Australian National University. He could not perform a clinical trial after his relocation elsewhere because he was not obtain ethical clearance but other workers elsewhere successfully cryopreserved both embryos of various developmental stages and oocytes of many speci4es including human with VS14/EG5.5 some with live young beginning from year 200 onwards. In 1998, Mukaida et al. in Japan reported the the first live-birth in the human from vitrified embryos in a clinical trial. In subsequent years vitrification replaced conventional slow controlled-rate freezing by the late 2000's.

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Correspondence: Jaffar Ali; email: jrbf.online@gmail.com cc: jaffarali.abdullah@gmail.com
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Introduction

The milestones that led to the development of the current state of the art of assisted reproduction technology (ART) including cryopreservation of the human gametes and embryos began about five hundred years ago. The science of cryopreservation of human gamete and embryo cryopreservation as we know it today only commenced in the last few decades. After years of research and experimentation, we appear to have succeeded in this quest.

In the present communication the authors have attempted to chronicle the development of the methodologies for the efficacious cryo-storage of the human sperm, embryo and oocyte cryopreservation as completely as possible in a concise manner in chart-form. The significant contributions in cryopreservation whether grandeur or miniscule is recorded as accurately as possible, irrespective of its

geographical origins. Furthermore, this endeavor also attempts to ameliorate subtle imprecisions which has created confusion on the origination of research findings and methodologies in cryopreservation so that the genuine contributions of some are not erroneously attributed to others.

This task of chronicling the milestones in the development and acquisition of knowledge in the gamete and embryo cryopreservation technologies will inadvertently leave out some contributions, in particular those not published in the English mainstream scientific literature.

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 felt this effort has recorded almost all the significant contributions in cryopreservation. It is anticipated this work will become a useful reference material to the profession and is 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) and individual journals.

Results

The Charts 4 to 6 below chronicles the milestones pertaining to spermatozoa, embryo, and oocyte cryopreservation in their chronological order of development. The milestones pertaining to preimplantation genetics, application of artificial intelligence in ART, in vitro maturation, micromanipulation and embryonic stem cell technology are given in Charts 7 onwards which will be published separately.

Discussion

We have chronicled the milestones in the development of the science and cryogenic methods of human gamete and embryo cryopreservation as accurately as possible given the resources available to us. We have also tried to present these milestones as fairly as possible without bias in acknowledging and recording important contributions.

Dean (2018) wrote in the World Intellectual Property Organization (WIPO) Magazine that, Quote: “The New Shorter Oxford English Dictionary defines the verb “plagiarize” as follows: “Take and use as one’s own (the thoughts, writings, inventions, etc., of another person); copy (literary work, ideas, etc.) improperly or without acknowledgement; pass off the thoughts, work, etc. of (another person) as one’s own.” “Plagiarism” is the noun form of this verb.” Unquote.

Some irregularities appear to occur in cryopreservation research. This suggests the peer review process undertaken by journals may have failed to detect discrepancies in manuscripts prior to publication, in particular, plagiarism of inventions. It also suggests that some peer reviewers are not thorough in their peer review process. It is also possible the peer review process only takes into consideration articles published in mainstream journals published in the English language. The existence of a large number of publications that addressed the matter of impropriety in science bear testimony to the occurrence of misconduct in research and publications (Guimarães 1998; Jia H. 2006; Lazarides et al., 2019 ; Goodman and Mallet. 2012; Lose G. 2011;Misra, Ravindran , 2020). It is also possible some acts of apparent misconduct may not be deliberate but due to lack of diligence in literature search.

The present work suggest that the significant and tangible advances made in the area of cryopreservation of gametes and embryos over the last five decades or more portend well for the future of cryopreservation in healthcare and in agriculture. One lingering problem is that of non-asepsis in handling transplantable tissues (embryos and gametes), a practice that is not in keeping with Good Laboratory Practice/Good Clinical Practice (GLP/GCP). This issue has received much attention and has been debated in the recent past (reviewed by Joaquim et al., 2017). Effort has been made to overcome this deficiency by some workers and suppliers of cryopreservation products. As a result, we now have some commercial methods of cryopreservation of gametes and embryos that are aseptic and appear compliant with GLP/GCP. Nevertheless, the practice of non-asepsis in cryopreservation appears to continue and persist to the present in some ART facilities. This has to be addressed to

ensure transplantable embryos and gametes in healthcare are handled in a manner that is GLP/GCP-compliant.

Furthermore, for reasons of safety it is important that vitrification formulations for human application in the future are products of proper systematic scientific inquiry. This is because whimsical formulations are potentially hazardous and could have long-term adverse consequences on the offspring. Regulatory authorities should be able to differentiate between whimsical formulations (sometimes referred to as “kitchen science”) with those that are products of proper systematic scientific inquiry that adheres to research methodology that is executed with proper experimental design.

Conclusion

In conclusion, the quest for the cryopreservation of gametes and embryos that commenced in the 1960’s appears largely resolved by the late 2010’s, however what is thought to be good at the present time will likely be proven inadequate in the future or even inappropriate. Therefore, there is scope for continuous improvement in cryopreservation methods. The fraternity must exercise caution with non-aseptic cryopreservation technology and when confronted with whimsically derived formulations of cryoprotectants for the cryopreservation of human gametes and embryos.

Reference

Dean O. Is plagiarism unlawful? WIPO Magazine. https://www.wipo.int/wipo_magazine/en/2018/si/article_0008.html Accessed 30 September 2021

Goodman SR, Mallet RT. Plagiarism. Exp Biol Med (Maywood). 2012;237(7) :739. doi: 10.1258/ebm.2012.012e02.

Guimarães S. A fraude científica. Um mal a ter em conta, também entre nós [Scientific fraud. A disease we find among ourselves]. Acta Med Port. 1998;11 (8-9):783-5. Portuguese, English abstract

Jia H. Frequent cases force China to face up to scientific fraud. Nat Med. 2006; 12(8):867. doi: 10.1038/nm0806-867a

Joaquim DC, Borges ED, Viana IGR, Navarro PA, Vireque AA. Risk of Contamination of Gametes and Embryos during Cryopreservation and Measures to Prevent Cross-Contamination. Biomed Res Int. 2017;2017:1840417

Lazarides MK, Gougoudi E, Papanas N. Pitfalls and Misconducts in Medical Writing. Int J Low Extrem Wounds. 2019;18(4):350-353. doi: 10.1177/1534734619870083.

Lose G. Plagiarism. Int Urogynecol J. 2011 Aug;22(8):903-4. doi: 10.1007/s00192-011-1437-9

Misra DP, Ravindran V. Publication misconducts related to copyright: tread carefully to avoid falling. J R Coll Physicians Edinb. 2020;50(1):3-5. doi: 10.4997/JRCPE.2020.101.

Chart 4. Milestones in Sperm and Testicular Tissue Cryopreservation

Year	Milestone/Event	Reference
1322	One of the earliest reports of an attempt at preservation of biological material related to a successful animal reproduction was that of the use of donor stallion sperm by the Arabs in 1322 . A spy of an Arab chieftain preserved horse semen using a concoction containing milk as a chemo- protectant and a sponge as the vehicle.	https://beva.onlinelibrary.wiley.com /doi/epdf/10.1111/j.2042-3306.1969.tb03355.x Accessed 11Apr2021 J.M. Bowen Artificial insemination in the horse. Equine Vet. J. 1969; 1(3):98-110. https://doi.org/10.1111/j.2042-3306.1969.tb03355.x Also cited in: H.I. Nielsen & J. Ali, Embryo culture media, culture techniques and embryo selection: A tribute to Wesley Kingston Whitten. J. Reprod Stem Cell Biotechnol 1(1):1-29, 2010

1776	Lazzaro Spallanzani, in a landmark development demonstrated that sperm frozen in snow did not die but were merely temporarily inactivated under cold conditions and brought to life after the semen was thawed [8]. This was in 1776.	Spallanzani L. 1776. Opuscoli di Fisica Animale e vegetabile […] aggiuntevi alcune lettere relative ad essi opuscoli dal celebre Signor Bonnet di Ginevra e da altri scritte all'Autore. Modena. Cited In: Lazzaro Spallanzani: At the Roots of Modern Biology. J Exp Zoo.(Mol Dev Evol). 1999;285:178–196 Clarke GN. A.R.T. and history, 1678-1978. Hum Reprod 2006; 21(7):1645–1650 Puerta Suárez J, du Plessis SS, Cardona Maya WD. Spermatozoa: A Historical Perspective. *Int J Fertil Steril*. 2018;12(3):182-190. doi:10.22074/ijfs.2018.5316
1908	The beneficial effects of glycerol and non-permeable cryoprotectants, including sucrose, on plant cryostability were described as early as in 1908 (Maksimov, 1913).	Maksimov NA.On destroying by frost and cold-resistant of plants. Izvestija Sankt-Peterbusrgskogo Lesnogo Instituta. 1913;5:25–31 Cited in: Isachenko E, Isachenko V, Katkov II, Dessole S, Nawroth F. Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success. Reprod Biomed Online. 2003;6(2):191-200. doi: 10.1016/s1472-6483(10)61710-5.
1920	Use of semen extenders was described in the 1920's	Ivanov II, 1920. Cited in: Ombelet W, Van Robays J. Artificial insemination history: hurdles and milestones. Facts Views Vis Obgyn. 2015;7(2):137-143.
1930s	Cryobiology was investigated in detail by Luyet and coworkers beginning in the late 1930s till about the 1960s.	Schmidt PJ, Basile J (2006). Luyet and the beginnings of transfusion cryobiology. Transfus Med Rev 20(3):242–246 Cited in: H.I. Nielsen & J. Ali, Embryo culture media, culture techniques and embryo selection: A tribute to Wesley Kingston Whitten. J. Reprod Stem Cell Biotechnol 1(1):1-29, 2010
1937	Bernstein and Petropavlovski demonstra-ted in 1937 the cryoprotective effects of glycerol for the cryopreservation of sperm. Others that attempted to cryopreserve sperm include Jahnel 1938 and Parkes, 1945	Bernstein AD, Petropavlovski VV. Influence of nonelectrolytes on viability of spermatozoa. Buleten Experimentalnoi Biologii i Medicini 1937 III 21–25 Jahnel F. Über die Widerstandsfähigkeit von menschlichen Spermatozoonen gegenüber starker Kälte. Klinische Wochenschrift 1938 17:1273–1274 Parkes AS. Preservation of human spermatozoa at low temperatures. Br Med J 1945 2:212–213

		Cited In: Isachenko V, Isachenko E, Katkov II, Montag M, Dessole S, Nawroth F, Van Der Ven H. Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapor: effect on motility, DNA integrity, and fertilization ability. Biol Reprod. 2004 Oct;71(4):1167-1173. doi: 10.1095/biolreprod.104.028811.
1938	Successful vitrification of frog sperm by Luyet and Hodapp in 1938 but not rat sperm	Luyet BJ, Hoddap A. Revival of frog's spermatozoa vitrified in liquid air. Proceedings of the Meeting of Society for Experimental Biology . 1938; 39, 433–434. Cited In: Isachenko E, Isachenko V, Katkov II, Dessole S, Nawroth F. Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success. Reprod Biomed Online. 2003;6(2):191-200. doi: 10.1016/s1472-6483(10)61710-5.
1939	Use of egg yolk to protect sperm during cooling.	Phillips EJ, Lardy HA. A yolk-buffer pabulum for the preservation of bull semen. J Dairy Sci. 1940;23:399-404. Cited In: Ombelet W, Van Robays J. Artificial insemination history: hurdles and milestones. Facts Views Vis Obgyn. 2015;7(2):137-143.
1940	Effects of cold temperatures on cells have been investigated empirically (Luyet & Gehenio, 1940).	Luyet BJ, Gehenio, PM. The mechanism of injury and death by low temperature. A review. Biodynamica 1940; 3(60):33-99. The mechanism of injury and death by low temperature. A review. (cabdirect.org) Also Cited In: Ali J. Factors affecting the ultrarapid vitrification and cryopreservation of embryos. PhD Thesis. 1992. Australian National University, Canberra.
1940	Shettles (1940) in the course of a study of the physiology of human sperm had been able to vitrify and revive a few per cent of seminal human sperm immersed in capillary tubes in the liquified gases and later rapidly warmed.	Shettles L B. Am. J. Physiol. 1940;128:408 Cited In Hoagland H, Pincus G. Revival of mammalian sperm after immersion in liquid nitrogen. J Gen Physiol 1942;25:337–344
1941	Use of Sodium citrate in addition to egg yolk for preserving sperm under refrigeration for 3 days without loss of viability.	Salisbury GW, Fuller HK, Willett EL. Preservation of bovine spermatozoa in yolk-citrate diluents and field results from its use. J Dairy Sci. 1941;24:905-10
1942	Human sperm vitrified with good survival of 67% of original motile forms of the sperm (Hoagland and Pincus, 1942) but subsequent	Hoagland H, Pincus G. Revival of mammalian sperm after immersion in

	workers using contemporary vitrification protocols vitrified sperm unsuccessfully possibly due to intolerance of high cryoprotectant concentrations needed to achieve vitrification. This problem remained a technical challenge up to the early 2000s until this problem was revisited and resolved successfully but without the use of cryoprotectants by another group to be described later in this section (isachenko et al 2003.	liquid nitrogen. J Gen Physiol 1942;25:337–344 Isachenko E, Isachenko V, Katkov II, Dessole S, Nawroth F. Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success. Reprod Biomed Online. 2003;6(2):191-200. doi: 10.1016/s1472-6483(10)61710-5.
1949	Polge et al. 1949 have been credited with being the first to cryopreserve sperm in 1949 using the cryoprotectant glycerol although sperm cryopreservation was reported earlier by Bertstein and Petrppavlovski in 1937. Smirnov had also cryopreserved sperm in the same year as Polge in 1949 but his work was published in Russian.	Bernstein AD, Petropavlosky VV. The influence of nonelectrolytes on survival of spermatozoa (message III). Bull. Eksp. Biol. Med. 1937;3(1):41-43. (Russian) Cited In: I. I. Katkov II, V. F. Bolyukh VF, Sukhikh GT. KrioBlast™ as a New Technology of Hyper-fast Cryopreserva-tion of Cells and Tissues.Part I. Thermo-dynamic aspects and potential applications in reproductive and regenerative medicine. Cell Technol Biol Med. 2018;4:530-535 See entries in previous rows above for year 1937 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
1953	First pregnancy after insemination with frozen thawed semen	Sherman JK 1953. Cited In: Ombelet W, Van Robays J. Artificial insemination history: hurdles and milestones. Facts Views Vis Obgyn. 2015;7(2):137-143.
1955	The development of cryopreservation protocols for the bull to be used for AI in the dairy industry began in the 1950s. Bratton et al. (1955) demonstrated in field trials that bovine sperm frozen to −79°C and packed on dry ice could still yield high fertility.	Bratton RW, Foote RH and Cruthers JC 1955. Preliminary fertility results with frozen bovine spermatozoa. Journal of Dairy Science 38, 40–46. Cited In: Lonergan P. Review: Historical and futuristic developments in bovine semen technology. Animal. 2018 Jun;12(s1):s4-s18. doi: 10.1017/S175173111800071X.

1953	In 1953 Sherman and Bunge froze human sperm equilibrated with 10 percent glycerol on dry ice with a 67 percent survival rate. Shortly thereafter Sherman and Bunge (1953) reported three pregnancies with the use of AI in combination with frozen–thawed sperm.	Sherman and Bunge, 1953 Cited In:Watson PF. Chris Polge, CBE FRS (16 August 1926–17 August 2006): a personal appreciation. Cryo Letters 2008;29(1):1–6.
1957	Sherman continued to pursue the improvement of human sperm preservation by investigating various cryopreservation protocols along with freeze-drying techniques (Sherman, 1957).	Sherman JK. Freezing and freeze-drying of bull spermatozoa. Am. J. Physiol. 1957; 190(2): 281-286.
1975	It was found that lipids in egg yolk could protect bull sperm from “cold shock,” the sensitivity of cells to reduced temperatures	Watson, P.F., 1976. The protection of ram and bull spermatozoa by the low density lipoprotein fraction of egg yolk during storage at 5°C and deep freezing. J. Therm. Biol. 1, 137–141 Cited In: Holt WV. Basic aspects of frozen storage of semen. Anim Reprod Sci. 2000 Aug 18;62(1-3):3-22. doi: 10.1016/s0378-4320(00)00152-4.
1963	One of Sherman’s discoveries was that storing human sperm at liquid nitrogen (LN2) temperature (−196°C) was superior to storage at −75°C. In addition, no loss of motility was observed when the sperm were stored in LN2 for one year; however, there was a decline in motility after storage at −75°C (Sherman, 1963).	Sherman JK. Improved methods of preservation of human spermatozoa by freezing and freeze-drying. Fert. Steril.1963; 14:243. Cited In. Roussel JD, Austin CR. Preservation of primate spermatozoa by freezing. J Reprod Fertil. 1967; 13:333-335
1964	Prior to 1964 all pregnancies were produced from short-term storage of sperm; however, Perloff et al. (1964) reported pregnancies from insemination with frozen–thawed sperm stored for one to 5.5 months.	Perloff WH, Steinberger E, Sherman JK Conception with human spermatozoa frozen by nitrogen vapor technic. Fertil Steril. 1964; 15:501–5041964
1962/3	The biophysical principles of cryopreservat-ion including supercooling were explained by Mazur (1962/3).	Mazur P. (1962). [Review of Biological Effects of Freezing and Supercooling (Monograph No. 9 of the Physiological Society), by Smith AU]. Am Scientist 1962; 50(4):418A-418A. http://www.jstor.org/stable/27838552 Mazur P. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. J. Gen. Physiol. 1963; 47:347-369. Cited in: Mazur, P. (1996). Chapter 10 Principles of medical cryobiology: The freezing of living cells, tissues, and organs. Principles of Medical Biology,1996; 355–384. And
1973 1975	Lipids in egg yolk protected bull sperm from “cold shock”	Watson PF, Martin ICA. (1973). The response of ram spermatozoa to preparations of egg yolk in semen diluents

		during storage at 5°C and -196°C. Aust J Biol Sci. 1973; 26: 927-35. Watson PF, Martin ICA. the influence of some fractions of egg yolk on the survival of ram spermatozoa at 5°C. Aust J Biol Sci. 1975; 28(2):145-152
1973 1987	Sherman, 1973 and Critser et al. (1987a, 1987b) showed loss of motility in sperm is due to addition and removal of cryoprotectants which caused shrinkage or swelling of sperm which is due to their "osmotic tolerance levels"	Sherman JK . 1973 . Synopsis of the use of frozen human semen since 1964: State of the art of human semen banking . Fertil Steril 24 : 397 – 416 Critser JK Arneson BW Aaker DV Ball GD. 1987 . Cryopreservation of human spermatozoa, n. Post-thaw chronology of motility and of zonafree hamster ova penetration . Fertil Steril 47 : 980 – 984 . Critser JK Huse-Benda AR Aaker DV Arneson BW Ball OD. . 1987 . Cryopreservation of human spermatozoa. I. Effects of holding procedure and seeding on motility, fertilizability, and acrosome reaction . Fertil Steril, 1987; 47: 656 – 663 .
1976 1977 1979	Slow-freeze cooling-warming relationships-Development of the rapid freeze-rapid thaw theory for slow freezing embryos At the Cambridge University/Huntington Rd - Steen Willadsen, DVM modified conventional "slow freezing" to -78C before rapid cooling (i.e., "plunge") in LN2 by instead plunging at -38C (defined as "rapid freezing") in his domestic sheep model. In doing so, he concluded that rapid warming at 37C was required to optimize survival. Subsequently, Whittingham and coworkers verified the dynamic relationship between cooling and warming (Slow freeze-Slow warm, Rapid freeze-rapid warm) in the SF Mouse model. *(Contributed by Dr Mitchel C. Schiewe, 2024, and in part by Dr. James J. Stachecki, 2024 independently)*	Willadsen SM, Polge C, Rowson LEA, Moor RM. Deep freezing of sheep embryos. J Reprod Fert 1976; 46:151-4. Willadsen SM. Factors affecting the survival of sheep embryos during deep-freezing and thawing. In: The Freezing of Mammalian Embryos, edited by K. Elliott and J Whelan. Amsterdam: Elsevier Excerpta Medica, North Holland. Pp 175-89,1977. Willadsen SM. Factors affecting the survival of sheep embryos during-freezing and thawing. Ciba Found Symp., 1977 (52):175-201. Whittingham DG, Wood M, Farrant J, Lee H, Halsey JA. Survival of frozen mouse embryos after rapid thawing from -196 degrees C. J Reprod Fertil 1979;56:11-21.

1983 1984	Direct Sucrose Dilution – In situ using 0.25 ml straws Stanley Leibo definitively proved the usefulness of a 0.75-1.0M Sucrose solution to elude the CPA of mouse and bovine embryos in a single step using a 0.25ml Plastic straw (in situ dilution post-warming directly in the straw). This technique was subsequently adopted by WF Rall and colleagues in early ambient vitrification experiments (se below). *(Contributed by Dr Mitchel C. Schiewe, 2024)*	Leibo SP. A one-step in situ dilution method for frozen-thawed bovine embryos. Cryo-Letters 1983;4:387-400. Leibo SP. A one-step method for direct nonsurgical transfer of frozen-thawed bovine embryos. Theriogenology 1984;21:767-790.
1985 1986 1987 1989 1991	Early Embryo Vitrification Embryo vitrification under ambient conditions by Rall et al, 1987 involved the use of his 3rd generation solution (VS3a: 6.5M glycerol with high BSA content). Note that VS2 was a PPG based solution that proved embryo toxic. It should also be noted it utilized a modified one-step straw device system with in situ dilution of vitrified-embryos into a larger column of 0.75M sucrose (approx. 4:1 dilution). At the same period of time, Schiewe applied the technology to sheep blastocysts concluding his dissertation studies at NIH (Poolesville, MD), while Leibo and Rall were studying cattle blastocysts at Rio Vista (San Antonio, TX). Healthy live lambs and calves were produced in 1987/1988. It is worth recognizing the Belgian Lab of Drs. A. Massip, P. Vanderzwalmen, B. Scheffen and F. Ectors were reporting successful vitrification in 1986 in both the mouse and cow. However, the lower molar EG solutions in a plastic straw device suggest that ultra rapid freezing of the solution was likely occurring.	Scheffen B, Vanderzwalmen P, Massip A. A simple and efficient procedure for preservation of mouse embryos by vitrification. Cryo Lett. 1986; 7:260-69. Massip A, Vanderzwalmen P, Scheffen B, Ectors F. Pregnancies following transfer of cattle embryos preserved by vitrification. Cryo Lett. 1986; 7:270-73. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at 196 degrees C by vitrification. Nature. 1985; 313 (6003):573–575 Rall WF, Wood MJ, Kirby C, Whittingham DG. Develop ment of mouse embryos cryopreserved by vitri-fication. J Reprod Fertil 1987; 80(2):499–504 Schiewe MC. Comparative estrous synchronization, ovarian stimulation, luteal function and embryo cryopreservation of domestic sheep and non-domestic species. Dissertation 1989, Uniformed Serv Univ Heatlh Sciences (USUHS), Pg. 1-238. Schiewe MC, Rall WF, Stuart LD, Wildt DE. Ovine embryo cryopreservation: analysis of cryoprotectant, cooling rate and in situ straw dilution using conventional freezing or vitrification. Theriogenology 1991;36:279-293.
1996	The sperm's osmotic tolerance limit was described by Willoughby et al., 1996	Willoughby CE, Mazur P, Peter AT, Critser JK. Osmotic tolerance limits and properties of murine spermatozoa. Biol. Reprod. 1996;55:715–727
1996	First pregnancy after ICSI with cryopreserved testicular sperm, 1996	Gil-Salom M, Romero J, Minguez Y, Rubio C, De los Santos MJ, Remohí J, Pellicer A. Pregnancies after intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. Hum Reprod. 1996;11(6):1309-13

2002	Successful vitrification of human sperm without cryoprotectant	Nawroth F, Isachenko V, Dessole S, Rahimi G, Farina M, Vargiu N, Mallmann P, Dattena M, Capobianco G, Peters D, Orth I, Isachenko E. Vitrification of Human Spermatozoa Without Cryoprotectants. Cryolett. 2002; 23(2):93-102
2004	Szell et al., 2013 have reported the live-births with sperm that had been cryopreserved for 40 years	Szell AZ, Bierbaum RC, Hazelrigg WB & Chetkowski RJ. Live births from frozen human semen stored for 40 years. J Assist Reprod Genet. 2013; 30:743–744
2009 2015 2017	Synthetic protein-free culture and cryoprotectant media are equal in efficacy to protein-containing media in culture and cryopreservation of sperm	Ali J. live birth following cryopreservation of testicular spermatozoa in a synthetic spermatozoa cryopreservation medium. Unpublished observations. KFMC, Riyadh.2009 Ali J .Synthetic Biology in Healthcare and Conservation: II. Successful Formulation of a Synthetic Spermatozoa Cryopreservation Medium. International Conference for Innovation in Biomedical Engineering and Life Sciences. ICIBEL2015, 6–8 December 2015, Putrajaya, Malaysia Putraja, Malaysia. Springer Publishers, pg. 172-174 Ata'Allah, G. A., Adenan, N. A. M., Razali, N., Palaniappan, K., Saad, R., Idris, S. K., Kanniah, K., & Ali, J. (2017). Comparative characteristics of spermatozoa harvested and cryopreserved in culture and cryoprotectant media with or without donor serum proteins. Reproductive Biology, 17(2), 172–179.

Chart 5. Milestones in Embryo Cryopreservation

Year	Milestone/Event	Reference
476-529 AD	Aryabhata, an Indian Astronomer described for the first time the number zero "0" and calculated the value of "pi" at 3.1416 which hundreds of years later enabled mathematical calculations that permitted the determination of the physico-chemical factors that impacted successful cryopreservation of cells.	Aryabhata. In: Ali J. History of Embryo and Oocyte Cryopreservation. Proceedings of VIRTUAL FERTIVISION, 16th Annual Conference, 4-6 December 2020, India
1930-1960s	Early attempts at cryopreservation by vitrification but was abandoned in the 1960's due to the toxicity of very high concentrations of cryoprotectants needed to achieve vitrification.	Luyet BJ and coworkers. In: Schmidt PJ. Basile J Luyet and the beginnings of transfusion cryobiology. Transfus Med Rev 2006; 20(3):242–246
1958	Ferdows attempted to cryopreserve rabbit embryos with little success. Others were likewise not successful until the 1970s	Ferdows 1958. In: H.I. In: Ali J. Factors affecting the ultrarapid vitrification and cryopreservation of embryos. PhD Thesis. 1992. Australian National University, Canberra.

		In: Nielsen & J. Ali, Embryo culture media, culture techniques and embryo selection: A tribute to Wesley Kingston Whitten. J. Reprod Stem Cell Biotechnol 1(1):1-29, 2010
1960-1970s	Mathematical calculations enabled determination of physico-chemical factors impacting cryopreser- vation of cells. Foremost were the contributions of Mazur and Leibo and coworkers	Mazur P. Kinetics of Water Loss from Cells at Subzero Temperatures and the Likelihood of Intracellular Freezing J Gen Physiol. 1963 Nov 1; 47(2): 347–369.
1971/2	Successful cryopreservation of Mouse Embryo by Whittingham and coworkers and Wilmut independently.	Whittingham DG (1971) Survival of mouse embryos after freezing and thawing. Nature 233(5315):125–126 Whittingham DG, Leibo SP, Mazur P (1972) Survival of mouse embryos frozen to 196 degrees and 269 degrees C. Science 178 (4059):411–414 Wilmut I (1972) The low temperature preservation of mammalian embryos. J Reprod Fertil 31(3):513–514
1974 1981 1982 1983	Sucrose was used to achieve partial dehydration initially by Mazur, Miller, and Leibo in 1974 for bovine blood cells. This concept was later used for cryopreservation of cells, gametes and embryos.	Mazur P, Miller RH, Leibo SP. Survival of frozen-thawed bovine red cells as a function of the permeat-ion of glycerol and sucrose. J Membr Biol. 1974;15(2):137–158 Smorag Z,Katska L, Wierzchos E. Some factors affecting the viability of mouse and cattle embryos frozen to -40°C before transfer in liquid nitrogen. Anim. Reprod. Sci.,1981;4:65-72. Massip A, Van der Zwalmen P. In vitro survival of mouse embryos frozen in glycerol or glycerol sucrose. Cryo-letters, 1982; 3:326, Abstr. Renard JP, Heyman Y, Oz UJP. Congélation de l'embryon bovin: une nouvelle méthode de décongélation pour le transfert cervical d'embryons conditionnés une seule fois en paillettes. Annls Méd Vét. 1982; 126;23-32. Bui-Xuan N, Renard JP, Garnier V. Rapid freezing and thawing of 2 cell stage rabbit embryos after partial dehydration at room temperature. Cryobiology, 1983; 20:742, Abstr.

1976 1977 1979	Slow-freeze cooling-warming relationships-Development of the rapid freeze-rapid thaw theory for slow freezing embryos At the Cambridge University/Huntington Rd -Steen Willadsen, DVM modified conventional "slow freezing" to -78C before rapid cooling (i.e., "plunge") in LN2 by instead plunging at -38C (defined as "rapid freezing") in his domestic sheep model. In doing so, he concluded that rapid warming at 37C was required to optimize survival. Subsequently, Whittingham and coworkers verified the dynamic relationship between cooling and warming (Slow freeze-Slow warm, Rapid freeze-rapid warm) in the SF Mouse model. *(Contributed by Dr Mitchel C. Schiewe, 2024, and in part by Dr. James J. Stachecki, 2024 independently)*	Willadsen SM, Polge C, Rowson LEA, Moor RM. Deep freezing of sheep embryos. J Reprod Fert 1976; 46:151-4. Willadsen SM. Factors affecting the survival of sheep embryos during deep-freezing and thawing. In: The Freezing of Mammalian Embryos, edited by K. Elliott and J Whelan. Amsterdam: Elsevier Excerpta Medica, North Holland. Pp 175-89,1977. Willadsen SM. Factors affecting the survival of sheep embryos during-freezing and thawing. Ciba Found Symp., 1977 (52):175-201. Whittingham DG, Wood M, Farrant J, Lee H, Halsey JA. Survival of frozen mouse embryos after rapid thawing from -196 degrees C. J Reprod Fertil 1979;56:11-21.
1978	***Landmark development*** First report of an IVF live-birth from human frozen-thawed embryo, Oct 1978. This claim was not acknowledged by the scientific community until much later. 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 a stepwise exposure from low to high concentration of the cryoprotectant dimethyl sulphoxide before freezing the embryo. These workers used gonadotrophin stimulation to retrieve more than one oocyte, follicle development was monitored hormonal and 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***	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

	from 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 "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. See Anand-Kumar, 1981).	
1980	A two-step freezing protocol was proposed to avoid freezing injury and was found to improve survival following cryopreservation of embryos in which a lower concentration of cryoprotectant are used before exposure to the actual freezing concentration of the cryoprotectant in conventional freezing methods. Wood and Farrant were probably oblivious of the work of Mukherjee et al (1978) published in the Indian J cryogenics.	Wood MJ. Farrant J. Preservation of mouse embryos by two-step freezing. Cryobiology 1980; 17:178-180
1982 1984	The use of sucrose for partial dehydration of in cryopreservation of embryos by conventional freezing techniques was first attempted with success by Renard and coworkers in 1984. Sucrose was shown to be non-toxic when exposed to embryos for 30 minutes (1982). It is noteworthy that it was Mazur et al., 1974 used sucrose for the first time for partial dehydration in the cryopreservation of bovine blood cells. This has been mentioned in his chart previously.	Renard J-P, Heyman Y, Gil J-P. Congelation de l'embryon bovin : une nouvelle methode de d&congelation pour le transfert cervical d'embryons conditionnes une seule fois en paillettes. Ann. Med. Vet. 1982;126:23-32 (French, cited in publication below). Renard J-P, Bui-Xuan-Nguyen N, Garnier V. Two-step freezing of two cell stage rabbit embryos after partial dehydration at room temperature. J. Reprod. Fertil. 1984;71:573 -580
1983	First IVF pregnancy/birth from human frozen-thawed embryo, 1983 but this was a still-birth. This report was/is generally acknowledged by mainstream scientific literature as the first report of a human live birth from frozen-thawed embryo. These workers used a 4-stepwise protocol for gradual exposure of the embryos to increasing concentrations of the cryoprotectant.	Trounson A, Mohr L. Human pregnancy following ryopreservation, thawing and transfer of an eight-cell embryo. Nature. 1983;305(5936):707–709
1983 1984	**Direct Sucrose Dilution – In situ using 0.25 ml straws** Stanley Leibo definitively proved the usefulness of a 0.75-1.0M Sucrose	Leibo SP. A one-step in situ dilution method for frozen-thawed bovine embryos. Cryo-Letters 1983;4:387-400.

	solution to elude the CPA of mouse and bovine embryos in a single step using a 0.25ml Plastic straw (in situ dilution post-warming directly in the straw). This technique was subsequently adopted by WF Rall and colleagues in early ambient vitrification experiments (se below). *(Contributed by Dr Mitchel C. Schiewe, 2024)*	Leibo SP. A one-step method for direct nonsurgical transfer of frozen-thawed bovine embryos. Theriogenology 1984;21:767-90.
1985 1986 1987 1989 1991	**Early Embryo Vitrification** Embryo vitrification under ambient conditions by Rall et al, 1987 involved the use of his 3rd generation solution (VS3a: 6.5M glycerol with high BSA content). Note that VS2 was a PPG based solution that proved embryo toxic. It utilized a modified one-step straw device system with in situ dilution of vitrified-embryos into a larger column of 0.75M sucrose (approx. 4:1 dilution). At the same period of time, Schiewe applied the technology to sheep blastocysts concluding his dissertation studies at NIH (Poolesville, MD), while Leibo and Rall were studying cattle blastocysts at Rio Vista (San Antonio, TX). Healthy live lambs and calves were produced in 1987/1988. The Belgian Lab of Drs. A. Massip, P. Vanderzwalmen, B. Scheffen and F. Ectors were reporting successful vitrification in 1986 in both the mouse and cow. However, the lower molar EG solutions in a plastic straw device suggest that ultra rapid freezing of the solution was likely occurring. *(Contributed by Dr Mitchel C. Schiewe, 2024)*	Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at 196 degrees C by vitrification. Nature. 1985; 313 (6003):573–575 Scheffen B, Vanderzwalmen P, Massip A. A simple and efficient procedure for preservation of mouse embryos by vitrification. Cryo Lett. 1986; 7:260-69. Massip A, Vanderzwalmen P, Scheffen B, Ectors F. Pregnancies following transfer of cattle embryos preserved by vitrification. Cryo Lett. 1986; 7:270-73. Rall WF, Wood MJ, Kirby C, Whittingham DG. Develop ment of mouse embryos cryopreserved by vitri-fication. J Reprod Fertil 1987; 80(2):499–504 Schiewe MC. Comparative estrous synchronization, ovarian stimulation, luteal function and embryo cryopreservation of domestic sheep and non-domestic species. Dissertation 1989, Uniformed Serv Univ Heatlh Sciences (USUHS), Pg. 1-238. Schiewe MC, Rall WF, Stuart LD, Wildt DE. Ovine embryo cryopreservation: analysis of cryoprotectant, cooling rate and in situ straw dilution using conventional freezing or vitrification. Theriogenology 1991;36:279-93.
1980	A two-step freezing protocol was proposed to avoid freezing injury and was found to improve survival following cryopreservation of embryos in which a lower concentration of cryoprotectant are used before exposure to the actual freezing concentration of the cryoprotectant in conventional freezing methods. Wood and Farrant were probably oblivious of the work of Mukherjee et al (1978) published in the Indian J cryogenics.	Wood MJ. Farrant J. Preservation of mouse embryos by two-step freezing. Cryobiology 1980; 17:178-180

1982 1984	The use of sucrose for partial dehydration of in cryopreservation of embryos by conventional freezing techniques was first attempted with success by Renard and coworkers in 1984. Sucrose was shown to be non-toxic when exposed to embryos for 30 minutes (1982). It is noteworthy that it was Mazur et al., 1974 used sucrose for the first time for partial dehydration in the cryopreservation of bovine blood cells. This has been mentioned in this chart previously. The use of sucrose is now the mainstay in VSs, and also spawned research into the use of other non-permeating cryoprotectants.	Renard J-P, Heyman Y, Gil J-P. Congelation de l'embryon bovin : une nouvelle methode de d&congelation pour le transfert cervical d'embryons conditionnes une seule fois en paillettes. Ann. Med. Vet. 1982;126:23-32 (French, cited in publication below). Renard J-P, Bui-Xuan-Nguyen N, Garnier V. Two-step freezing of two cell stage rabbit embryos after partial dehydration at room temperature. J. Reprod. Fertil. 1984;71:573 -580
1983	First IVF pregnancy/birth from human frozen-thawed embryo, 1983 but this was a still-birth. This report was/is generally acknowledged by mainstream scientific literature as the first report of a human live birth from frozen-thawed embryo. These workers used a 4-stepwise protocol for gradual exposure of the embryos to increasing concentrations of the cryoprotectant .	Trounson A, Mohr L. Human pregnancy following ryopreservation, thawing and transfer of an eight-cell embryo. Nature. 1983;305(5936):707–709
1983 1984	**Direct Sucrose Dilution – In situ using 0.25 ml straws** Stanley Leibo definitively proved the usefulness of a 0.75-1.0M Sucrose solution to elude the CPA of mouse and bovine embryos in a single step using a 0.25ml Plastic straw (in situ dilution post-warming directly in the straw). This technique was subsequently adopted by WF Rall and colleagues in early ambient vitrification experiments (se below). *(Contributed by Dr Mitchel C. Schiewe, 2024)*	Leibo SP. A one-step in situ dilution method for frozen-thawed bovine embryos. Cryo-Letters 1983;4:387-400. Leibo SP. A one-step method for direct nonsurgical transfer of frozen-thawed bovine embryos. Theriogenology 1984;21:767-790.
1984	The revival of vitrification was suggested as a strategy for cryopreservation.	G M Fahy, D R MacFarlane, C A Angell, H T Meryman Vitrification as an approach to cryopreser-vation. Cryobiology. 1984; 21(4):407-26.
1984	Live-births from human frozen-thawed embryos. This report is considered the first live-birth by most commentators as they were oblivious of the work of Mukherjee and coworkers (1978).	Zeilmaker GH, Alberda AT, van Gent I, Rijkmans CM, Drogendijk AC. Two pregnancies following transfer of intact frozen– thawed embryos. Fertil Steril. 1984;42:293–296
1985 1986 1987 1989 1991	**Early Embryo Vitrification** Embryo vitrification under ambient conditions by Rall et al, 1987 involved the use of his 3rd generation solution	Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at 196 degrees C by vitrification. Nature. 1985; 313 (6003):573–575

	(VS3a: 6.5M glycerol with high BSA content). Note that VS2 was a PPG based solution that proved embryo toxic. It should also be noted it utilized a modified one-step straw device system with in situ dilution of vitrified-embryos into a larger column of 0.75M sucrose (approx. 4:1 dilution). At the same period of time, Schiewe applied the technology to sheep blastocysts concluding his dissertation studies at NIH (Poolesville, MD), while Leibo and Rall were studying cattle blastocysts at Rio Vista (San Antonio, TX). Healthy live lambs and calves were produced in 1987/1988. It is worth recognizing the Belgian Lab of Drs. A. Massip, P. Vanderzwalmen, B. Scheffen and F. Ectors were reporting successful vitrification in 1986 in both the mouse and cow. However, the lower molar EG solutions in a plastic straw device suggest that ultra rapid freezing of the solution was likely occurring. *(Contributed by Dr Mitchel C. Schiewe, 2024)*	Scheffen B, Vanderzwalmen P, Massip A. A simple and efficient procedure for preservation of mouse embryos by vitrification. Cryo Lett. 1986; 7:260-69. Massip A, Vanderzwalmen P, Scheffen B, Ectors F. Pregnancies following transfer of cattle embryos preserved by vitrification. Cryo Lett. 1986; 7:270-73. Rall WF, Wood MJ, Kirby C, Whittingham DG. Develop ment of mouse embryos cryopreserved by vitri-fication. J Reprod Fertil 1987; 80(2):499–504 Schiewe MC. Comparative estrous synchronization, ovarian stimulation, luteal function and embryo cryopreservation of domestic sheep and non-domestic species. Dissertation 1989, Uniformed Serv Univ Heatlh Sciences (USUHS), Pg. 1-238. Schiewe MC, Rall WF, Stuart LD, Wildt DE. Ovine embryo cryopreservation: analysis of cryoprotectant, cooling rate and in situ straw dilution using conventional freezing or vitrification. Theriogenology 1991;36:279-293.
1985/7	First pregnancy and live births after vitrification of mouse embryos, 1987. The vitrification solution (VS) consisted of DMSO, propylene glycol, Acetamide and the non-permeating polyethylene glycol (called VS1). Acetamide is known to be embryotoxic. The concern for embryotoxicity of the VS solution led to a search for less or least toxic VSs.	Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at 196 degrees C by vitrification. Nature. 1985; 313 (6003):573–575 Rall WF, Wood MJ, Kirby C, Whittingham DG. Develop ment of mouse embryos cryopreserved by vitri-fication. J Reprod Fertil 1987; 80(2):499–504
1986	Szell and Shelton established that 1.0 mol/L (1M) sucrose is not toxic to embryos.	Szell A and Shelton JN. Sucrose dilution of glycerol from mouse embryos frozen rapidly in liquid nitrogen vapour. J Reprod Fertil. 1986; 76: 401–408.
1988	In view of the detrimental effects of high concentrations of cryoprotectant required to achieve vitrification, K. Papis, a Polish cryobiologist, then (1988) working in Japan, attempted to reduce the concentration of permeating cryoprotectants by replacing them in part with a non-permeating cryoprotectant , 1.0 M sucrose. This effort to reduce toxicity of VSs led to a	Papis , 1988. Personal communication with one of us (JA). Papis K, Kojima T, Oguri N. Study of vitrification of rabbit embryos - an effect of sucrose addition. Cryobiology 1991;28:71. Papis K, Fujikawa S, Kojima T, Oguri N. Effect of the composition of

	measure of success. His publication of this work was however delayed until 1991.	vitrification media on survival of rabbit embryos. Cryobiology, 1993;30:98-105.
1990	The first successful human cleavage-stage embryo by ultrarapid cryopreservation followed by a successful delivery was achieved by Gordts et al. 1990. However ultrarapid cryopreservation did not take off probably because of the shift towards vitrification.	Gordts S, Roziers P, Campo R, Noto V. Survival and pregnancy outcome after ultrarapid freezing of human embryos. Fertil Steril 1990;53:469-472
1990 1991	Day 4 mouse morulae were vitrified with no appreciable loss of viability using an ethylene glycol and sucrose-based VS. He demonstrated the critical need of incorporating sucrose in the VS to achieve excellent survival in mouse embryos. The VS consisted of Ethylene glycol, Ficoll and Sucrose (EFS). This was a major development in vitrification. K. Papis independently also demonstrated the importance of incorporating sucrose in the VS. See entry for 1991/3 below	Kasai M, Komi JH, Takakamo A, Tsudera H, Sakurai T, Machida T. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, with no appreciable loss of viability. J Reprod Fertil. 1990:89(1):91–97
1991 1993	Krzysztof Papis made substantial contributions to the development of embryo vitrification methods, in particular on the critical role of sucrose in the VS media and on the time needed for pre-equilibration and vitrification solutions. See entry for 1995,1999 and 2000 under “Oocyte Cryopreservation” in this report. *(Contributed by K. Papis, 2024)*	Papis K., Kojima T., Oguri N., Study of vitrification of rabbit embryos - an effect of sucrose addition. Cryobiology 1991, 28, 71. Papis K., Fujikawa S., Kojima T., Oguri N. Effect of the composition of vitrification media on survival of rabbit embryos. Cryobiology, 1993, 30, 98-105.
1991	A strategy to increase survival of embryos after vitrification was the method of “Direct Contact” with liquid nitrogen. This involved dropping a micro-drop of the VS containing the embryo directly into liquid nitrogen. This report spawned considerable innovation in vitrification that led to the utilization of micro-carriers in which asepsis could not be ascertained. The methods that employed micro-carriers as vehicle for the cryopreservation by vitrification of embryos and oocytes invariably exposed these transplantable embryos and oocytes to the non-aseptic environment. This technique (referred to as the Open technique) in subsequent years was adopted for human embryo and oocyte vitrification in spite of concerns of contamination and infection due to non-asepsis.	Riha J, Landa V, Kneissl J, Matus J, Jindra J, Kloucek Z (1991): Vitrification of cattle embryos by direct dropping into liquid nitrogen and embryo survival after nonsurgical transfer. Zivoc Vir 36:113–120

1992-2001	One major concern in the late 1980's was the toxicity of vitrification solution due to the use very high concentrations of cryoprotectants to achieve vitrification. In an effort to reduce the toxicity of vitrification solutions Ali and Shelton, (from December 1989 through July 1992), undertook an exhaustive and systematic investigation on over 3,044 cryopro-tectant solutions involving 6,088 permutations. This effort paid dividends as Ali and Shelton success-fully identified one vitrification solution consisting of 5.5M Ethylene Glycol and 1.0M sucrose (called VS14 or EG5.5) which was least toxic and most efficacious of the thousands of cryoprotectant solutions tested. For the first time, vitrification (using VS14 or EG5.5) allowed all developmental stages of the mouse and day 6 sheep embryos to be vitrified/ cryopreserved with no loss of viability in vitro and in vivo (with live births). This study was a doctoral (PhD) research program of one of us (JA) undertaken at the Australian National University. (It is noteworthy EG5.5 is assumed to be the alternate name given to VS14 by Martino [Martino et al., 1996]. The research design and experiments to formulate and/or the origination of VS14 were not indicated or mentioned by Martino. Since EG5.5 is identical in composition to VS14 previously developed by Ali and Shelton [1992, 1993a-c] it is assumed to be identical. The use of the name "EG5.5" for VS14 led to considerable confusion on the origination of this formulation in later years prompting Ali [2001a,b] to take remedial steps in 2001).	Ali J. Factors affecting the ultrarapid vitrification and cryopreservation of embryos. PhD Thesis. 1992. Australian National University, Canberra. Ali J. Shelton, JN. Vitrification of sheep embryos. Presented at the Annual Conference of the Austra-lian Society for Reproduct-ive Biology, September 1992 Ali J. Shelton JN. Vitrif-ication of preimplantation stages of mouse embryos J. Reprod. Fertil. 1993a; 98:459-465 Ali J. Shelton JN. Success-ful vitrification of sheep day-6 embryos J. Reprod. Fertil. 1993b; 99:65-70 Ali J. Shelton .N. Design of vitrifcation solutions for the cryopre-servation of embryos J. Reprod. Fertil. 1993c; 99:471-477 Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultrarapid cooling.Biol Reprod. 1996; 54:1059–1069 Ali J. Source of vitrification solution. Fertil Steril 2001a; 76:214–215 Ali J. Vitrification of embryos and oocytes with 5.5 mol/l ethylene glycol and 1.0 mol/l sucrose. Hum Reprod. 2001b;16:1777– 1778
1995-2000	Krzysztof Papis, while working in Denmark and Japan, noted that pre-equilibration in equilibration solution and equilibration in VS beyond 10-15 minutes and 30 seconds respectively in vitrification procedures was detrimental for the viability of the oocytes. This finding led to an appreciation of the need for caution and prudence during	Papis K., Avery B., Holm., Callesen H., Greve T. The effect of vitrification solution, equilibration time and direct dilution method on survivability of equilibrated or vitrified bovine in vitro matured oocytes. Theriogenology, 1995, 43, 293. Papis, K., Shimizu, M., Izaike, Y. The effect of gentle pre-equilibration on

	equilibration of oocytes and to avoid excessive exposure to equilibration solutions in vitrification procedures.	survival and development rates of bovine in vitro matured oocytes vitrified in droplets. Theriogenology, 1999, 51, 173. Papis K., Shimizu, M., Izaike, Y. Factors affecting the survivability of bovine oocytes vitrified in droplets. Theriogenology, 2000, 54, 651-658.
1995/ 1996	**Landmark development** First report of successful vitrification of the human embryo (day 2 cleavage stage) was reported by Ali in 1995/6 at the National University of Singapore (NUS) with 100% in vitro survival and viability (developing to blastocyst stage in cleavage medium - blastocyst medium was not developed until much later). The vitrification solution used was VS14 or EG5.5) which Ali and Shelton have developed in 1992/ 1993a-c. In 1996 Ali moved from the NUS to a Gulf Arab country where he failed to convince the IRB Committee which denied him approval for a human trial on the VS14 vitrification technology insisting that they would wait until advanced nations can prove the efficacy of vitrification. This decision effectively put an end to Ali's work on vitrification. Since then, Ali could not pursue any further research on vitrification. Furthermore, the IRB Committee's decision ensured their country lost its place in history because a short time later other workers else-where in various parts of the world (see Ali and Shelton, 2006; and Ali et al., 2017) successfully performed vitrification of all developmental stages of the human and animal embryos of all preimplantation development stages, and oocytes with live births from year 2000 onwards using the VS14/EG5.5 (5.5M ethylene glycol and 1M sucrose) vitrification technology developed by Ali and Shelton (1992/1993a-c). The said IRB Committee's lack of confidence, which did not appear motivated by sound scientific judgement or rationale in Ali's vitrification technology effectively led their nation to lose the opportunity to become the first, historically, to report pregnancies/ livebirths from vitrified human embryos.	Ali J, Bongso A, Ratnam SS. Chromo-somal analysis of day-2 human embryos vitrified with VS14. Med. Sci. Res.(UK). 1995; 23:539 -540. Ali J. Developmental competence of unipronuclear and triploid day-2 human embryos after vitrification with VS14. Med. Sci. Res. (UK). 1996; 24:377-378 (Journal defunct; Lippincott Plsrs). Ali J, Shelton JN. Vitrification of preimplantation stages of mouse embryos. J. Reprod. Fertil. 1993a; 98:459-465 Ali J. Shelton JN. Successful vitrification of sheep day-6 embryos. J. Reprod. Fertil. 1993b; 99:65-70 Ali J. Shelton .N. Design of vitrifcation solutions for the cryopreservation of embryos J. Reprod. Fertil. 1993c; 99:471-477 Ali J and Shelton JN. Vitrification of sheep embryos Conference of the Australian Society for Reproductive Biology, September 1992 Ali J. Factors affecting the ultrarapid vitrification and cryopreservation of embryos. PhD Thesis. 1992. Australian National University, Canberra. Ali J. Shelton JN. Development of vitrification solutions. In: Vitrification in Assisted Reproduction. A User's Manual and Trouble-shooting Guide. M.Tucker & J. Liebermann, Eds. Informa Healthcare Medical Books, UK,2006; p45-63 32 Ali J, Al Harbi NH, Ali N. Historical Background on Gamete and Embryo Cryopreservation. In: Cryopreservation of Mamm. Gametes & Embryos. Nagy, et al. (Eds.). Springer International Publishing, Humana Press.2017; p.3-20. 10.1007/978-1-4939-6828-2

1998	**Landmark development** The race to report the first pregnancy/ livebirth from vitrified embryos was won by Kasai's group in Japan. They reported the first human live birth following vitrification. One twin pregnancy occurred of 18 transfers resulting in live birth of twins from vitrified day 2/3 human embryos. The VS solution used contained ethylene glycol, ficoll and sucrose (EFS).	Mukaida T, Wada S, Takahashi K, Pedro PB, An, TZ, Kasai M. (1998). Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos. Human Reprod. 1998; 13(10), 2874–2879.
2000	Live-births from vitrified human blastocysts using VS14 (developed by Ali and Shelton, 1992, 1993a-c).	Choi DH, Chung HM, Lim JM et al. Pregnancy and delivery of healthy infantsdeveloped from vitrified blastocysts in an IVFET program. Fertil Steril. 2000; 74:838–839.
2001	Live-birth from 8-cell human embryos vitrified using a VS consisting of 40% Ethylene glycol (equivalent to 5.5 - 6.0M Ethylene glycol) and 0.6M sucrose (which is curiously very similar to the VS reported by Hotamisligil et al. 1996, that is, 6.0M Ethylene glycol +0.5M sucrose to that of Ali and Shelton's, 1993 report (VS14; 5.5M +1.0M sucrose). El-Danasouri and Selman (2001/2) have not indicated how they formulated their VS.	El-Danasouri I, Selman HA (2001) Successful pregnancies and deliveries after a simple vitrification protocol for day 3 human embryos. Fertil Steril 76:400–402 Hotamisligil S, Toner M, Powers RD. Changes in Membrane Integrity, Cytoskeletal Structure, and Developmental Potential of Murine Oocytes after Vitrification in Ethylene Glycol. Biol Reprod, 1996; 55: 161-168 Ali, J. and Shelton, J.N. Design of vitrifcation solutions for the cryopre-servation of embryos J. Reprod. Fertil. 1993c; 99:471-477
2001	Birth of an infant from cryopreserved embryos (zygotes) produced by IVM oocytes derived from an unstimulated patient with PCOS.	Chian RC, Gülekli B, Buckett WM, Tan SL.Pregnancy and delivery after cryopreservation of zygotes produced by in-vitro matured oocytes retrieved from a woman with polycystic ovarian syndrome. Hum Reprod. 2001;16(8):1700-1702.
2002	Live-births from human zygotes vitrified using a VS consisting of 40% Ethylene glycol (equivalent to 5.5-6.0M Ethylene glycol) and 0.6M sucrose.	Selman HA, El-Danasouri I Pregnancies derived from vitrified human zygotes. Fertil Steril. 2002; 77:422–423
2021-2024	Rapid 1-step 1-minute warming and rehydration in the vitrification embryo cryopreservation protocol.	Manns JN, Katz S III, Whelan J, Patrick JL, Holt T, Merline AM, Taylor TH. Validation of a new, ultra-fast blastocyst warming technique reduces warming times to 1 minute and yields similar survival and re-expansion compared to blastocysts warmed using a standard method ASRM Scientific Congress Expo; Oct 17-20, Baltimore, MD (2021)

		Fertil Steril 116, Issue 3, Supplement E165 Hrvojevic K, Uhler ML, Hirshfeld-Cytron J, Brohammer R, Wagner Y, Susralski A, Jasulaitis S, Liebermann J. Fast and furious: pregnancy outcome with a rapid warming protocol. Fertility and Sterility. 2023 Oct 1;120(4):e59. Liebermann J, Hrvojevic K, Hirshfeld-Cytron J, Brohammer R, Wagner Y, Susralski A, Jasulaitis S, Chan S, Takhsh E, Uhler M. Fast and furious: pregnancy outcome with one-step rehydration in the warming protocol for human blastocysts. Reproductive BioMedicine Online. 2024 Apr 1;48(4):103731.

6. Milestones in Oocyte and Ovarian Tissue Cryopreservation

Year	Milestone/Event	Reference
1893	First attempt to cryopreserve oocytes by Picket in 1893 .	Cited In: Hafez ES. Superovulation and preservation of mammalian egg. Acta Endocrinol Suppl 1969;140:5–44
1953	First attempt to cryopreserve ovarian tissue	Parkes AS. Factors affecting the viability of frozen ovarian tissue. J. Endo-crinol. 1958;**17:** 337-343.
1953	First pregnancy from frozen sperm in human.	Bunge RG, Sherman JK. Fertilizing capacity of frozen human sperma-tozoa. Nature. 1953 Oct 24;172 (4382):767-8.Cited in: Betsy J, Kumar S. Cryopreservation: History and development. InCryopreservation of fish gametes 2020 Jun 28 (pp. 135-149). Singapore: Springer Singapore.
1958/ 1959	First attempt to cryopreserve mouse oocytes using glycerol failed. Later it was shown glycerol though useful for sperm cryopreservation.	Sherman JK, Lin TP. Survival of unfertilized mouse eggs during freezing and thawing. Proc. SOC. Exp. Biol. Med.1958; 98: 902-905. Sherman JK, Lin TP. Tempe-rature shock and cold-storage of unfertilized mouse eggs. Fertil Steril 1959;10(4):384–396
1953	First attempt to cryopreserve ovarian tissue	Parkes AS. Factors affecting the viability of frozen ovarian tissue. J. Endo-crinol. 1958;**17:** 337-343.
1960	First live birth of normal offspring obtained from cryopreserved mouse orthotopic ovarian tissue grafts	Parrott DMW. The fertility of mice with orthoptopic ovarian grafts derived from frozen tissue. J. Reprod. Fertil.1960; **1:** 230-241.
1958/ 1959	First attempt to cryopreserve mouse oocytes using glycerol failed. Later it was	Sherman JK, Lin TP. Survival of unfertilized mouse eggs during

	shown glycerol though useful for sperm cryopreservation but is comparatively toxic to embryos (Ali and Shelton 1993c) and thus to oocytes as well.	freezing and thawing. Proc. SOC. Exp. Biol. Med.1958; 98: 902-905. Sherman JK, Lin TP. Tempe-rature shock and cold-storage of unfertilized mouse eggs. Fertil Steril 1959;10(4):384–396
1959	First attempt to cryopreserve sheep oocytes using glycerol failed.	Avrill RLW, Rowson LES (1959) Attempts at storage of sheep ova at low temperatures. J Agri Sci 52:392–395
1977	Live-birth from cryopreserved mouse oocytes	Whittingham DG. Fertilization in vitro and development to term of unfertiliz-ed mouse oocytes previously stored at --196 degrees C. J Reprod Fertil. 1977;49(1):89-94.
1979	Successful fertilization in vitro of cryopreserved rat oocytes	Kasai M, Iritani A, Chang MC. Fertilization in vitro of rat ovarian oocytes after freezing and thawing. Biol Reprod. 1979 Nov;21(4):839-44.
1980	Conventional controlled rate cooling of mouse oocytes led to disruption of the meiotic spindle.	Magistrini M, Szo¨llo¨si D (1980) Effects of cold and of isopropyl-N-phenylcarbamate on the second meiotic spindle of mouse oocytes. Eur J Cell Biol 22(2):699–707
1986 April	First pregnancy and llve-birth from controlled rate freezing and rapid thawing by Chen at the Queen Elizabeth Hospital, Adelaide, Australia	Chen C, Pregnancy after human oocyte cryopreservation. Lancet 1986; 1,884 – 886 Chen.C. Pregnancies after human oocyte cryopreservation. Fifth World Congress on In Vitro Fertilization and Embryo Transfer. Norfolk, USA, abstr. 1987; PS 51, 19.
1986 Sept	Second human pregnancy from oocytes cryopreserved by controlled–rate freezing.	Al-Hasani S, Diedrich K, van der Ven H, Krebs D. [Initial results of the cryopreser-vation of human oocytes]. Geburtshilfe Frauenheilkd. 1986;46(9):643–644.
1987	Human oocytes held for 24hrs post retrieval do not tolerate cryopreservation by controlled-rate freezing	Al-Hasani S, Diedrich K, van der Ven H,Reinecke A, Hartje M, Krebs D Cryopreservation of human oocytes. Hum Reprod 1987;2(8):695–700 100.
1987	Second live birth in the human from cryopreserved unfertilized oocytes .	van Uem JF, Siebzehnrubl ER, Schuh B, Koch R, Trotnow S, Lang N Birth after cryopreser-vation of unfertilized oocytes. Lancet 1. 1987; (8535): 752–753
1987	Conventional controlled rate cooling of mouse oocytes led to disruption of the meiotic spindle which reconstituted itself in an abnormal configuration with the chromatids strewn in the ooplasm in a haphazard manner at 37°C	Pickering SJ, Johnson MH (1987) The influence of cooling on the organizat-ion of the meiotic spindle of the mouse oocyte. Hum Reprod 2(3): 207–216
1988	Vitrification of mouse oocytes using VS1 (Rall and Fahy 1985) consisting of DMSO, 15.5% acetamide, 10% 172-propanediol, 6% polyethylene glycol led	Kola I, Kirby C, Shaw J, Davey A, Trounson A. Vitrification of mouse oocytes results in aneuploid zygotes

	to aneuploidy zygotes and malformed foetuses. This is probably related to the toxicity of acetamide.	and malformed fetuses. Teratology 1988;38(5):467–474
1988	Three live-births (1 singleton and 1 twin) from frozen-thawed human oocytes were reported.	Chen C. Pregnancies after human oocyte cryopreservation. Ann N Y Acad Sci. 1988;541:541–549
In spite of the sporadic successes in human oocyte cryopreservation however successful cryopreservation of human oocytes on routine basis remained largely unattainable. And it was clear that more work is needed to make human oocyte cryopreservation efficacious.		
1989	First successful vitrification of mature mouse oocytes which subsequently fertilized in vitro and after transfer to surrogates resulted in the birth of live young. The viability rate was good (87% survived; 82 % of survived oocytes fertilized in vitro and of these 46% became live young.	Nakagata N. High survival rate of unfertilized mouse oocytes after vitrification. J Reprod Fertil. 1989; 87(2):479–483
1989	Successful vitrification of germinal vesicle (GV) stage mouse oocytes. The VS used was similar to VS1 which consisted of: 20.5% w/v dimethyl sulphoxide (DMSO), 15.5% w/v acetamide, 10% w/v propylene glycol and 6% polyethylene glycol. Vitrified GV stage oocytes resumed meiosis but vitrification appears associated with chromosomal and cellular disorders. This may be related to the use of toxic acetamide in the VS.	Van Blerkom J (1989) Maturation at high frequency of germinal-vesicle-stage mouse oocytes after cryo-preservation: alterations in cyto-plasmic, nuclear, nucleolar and chromo-somal structure and organi-zation associated with vitrif-ication. Hum Reprod 1989;4(8):883–898
1992	Cooling injury in oocytes following conventional controlled rate cryopreservation. Difficulties with oocyte freezing by conventional slow freezing persisted.	Vincent C, Johnson MH (1992) Cooling, cryoprotectants, and the cytoskeleton of the mammalian oocyte. Oxf Rev Reprod Biol 14:73–100
1993 1994	Human oocytes were cryopreserved using propanediol. The cryopreserved oocytes could be fertilized in vitro and showed no chromosomal damage. Subsequent to this report of Gook and her coworkers, and other workers, have used propanediol to successfully cryopreserve human oocytes with live births by conventional controlled-rate cooling (given below).	Gook DA, Osborn SM, Johnston WI. Cryopreser-vation of mouse and human oocytes using 1,2-propanediol and the configuration of the meiotic spindle. Hum Reprod. 1993;8(7):1101-1109. Gook DA, Osborn SM, Bourne H, Johnston WI (1994) Fertilization of human oocytes following cryopre-servation; normal karyotypes and absence of stray chromosomes. Hum Reprod 9(4):684–691

1995	**Landmark development** Human oocyte slow freezing advancement required Dr. Debra Gook leaving Australia where experimentation was limited to 20 hours after fertilization. In 1994, she collaborated with M. Schiewe and UCI (USA) investigators to fertilize mature-slow frozen eggs using ICSI and the assessment of blastocyst development. The elevated abnormal fertilization rate was due to the IVM status of the experimental group, but reliable fertilization by ICSI was verified and in vitro blastocyst development (25%) confirmed (considering the suboptimal ECM pre-1995 was good). (See entry for year 1997 in this chart on the successful birth of a healthy human baby following slow freezing of the human oocyte. Dr. Debra Gook collaborated with the group of Porcu et al. on site at the latter's laboratory on human oocyte slow freezing. Dr Gook made significant contribution to human oocyte cryopreservation work performed at Porcu's laboratory. Later Porcu and coworkers reported the successful cryopreservation of human oocytes reported in 1997; Porcu et al., 1997). (*The above entry was contributed by Dr Mitchel C. Schiewe, 2024; corroborated in person by Dr Debra Gook during the ESHRE2026 conference in London*)	Gook D, Schiewe MC, Osborn SM, Asch RH , Jansen RPS, Johnston WI. Intracytoplasmic sperm injection and embryo development of human oocytes cryopreserved using 1,2-propanediol. Hum Reprod 1995; 10:2637-2641.
1996	Alex Martino then working in the laboratory of the late Stanley Leibo at the University of Guelph in Canada demonstrated the successful cryopreservation of bovine oocytes held in electronic microscope grids. The use of electron microscope grid with extreme low volume (<1uL) of VS was a strategy to prevent cooling injury in oocytes. Martino used a vitrification solution consisting of 5.5M ethylene glycol and 1.0M sucrose which appear identical to VS14 formulated by Ali and Shelton [1993a-c). Martino called the same VS "EG5.5" which led to confusion as to its origins which led one of the originators of VS14 (JA) to publish letters in two journals to	Martino A, Songsasen N, Leibo SP (1996). Development into blastocysts of bovine oocytes cryopreserved by ultrarapid cooling.Biol Reprod 1996; 54:1059–1069 Ali J (2001a) Source of vitrification solution. Fertil Steril. 2001;76:214–215 Ali J (2001b) Vitrification of embryos and oocytes with 5.5 mol/l ethylene glycol and 1.0 mol/l sucrose. Hum Reprod.2001;16:1777– 1778

	explain the origins of VS14 or EG5.5 (Ali, 2001a,b).	
1996 1998	Stachecki demonstrated the detrimental effects of sodium on oocyte freezing. He successfully cryopreserved mouse oocytes using propanediol at room temperature by using choline as a substitute for sodium to avoid sodium-loading during cooling resulting in 93% survival rates and subsequently documented live births with the same technique in the mouse and human. (Contributed by JJ Stachecki, 2024)	Stachecki JJ, Cohen J, Willadsen SM. Cryopreservation of unfertilized mouse oocytes: the effect of replacing sodium with choline in the freezing medium. Cryobiology. 1998;37(4): 346 -354. Stachecki JJ, Cohen J, Willadsen S. Detrimental effects of sodium during mouse oocyte cryopreservation. Biol Reprod. 1998;59(2):395-400.
1997 1998 1999a 1999b 1999c	**Landmark development** Live-births from cryopreserved 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 has finally been put to rest. (*The entry below was contributed by Dr Mitchel C Schiewe, 2024; corroborated by Dr Debra Gook in person at the ESHRE2026, London*) It is noteworthy that Dr Gook collaborated on site with the group of Porcu et al. – to establish their oocyte freezing program and achieve healthy babies following human oocyte slow freezing. Human oocyte slow freezing advancement required Dr. Debra Gook leaving Australia where experimentation was limited to 20 hours after fertilization to collaborate with the groups of Schiewe (USA) and Porcu (Italy).	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 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
1998 1999	The technique of oocyte cryopreser-vation using propanediol could now be reproduced successfully for human oocyte cryopreservation with live births in other laboratories.	Nawroth F, Kissing K Pregnancy after intracytoplasmatic sperm injection (ICSI) of cryopreserved human oocytes. Acta Obstet Gynecol Scand. 1998; 77(4):462–463 Polak de Fried E, Notrica J, Rubinstein M,Marazzi A, Go´mez GM

		(1998) Pregnancy after human donor oocyte cryopreservation and thawing in association with intracytoplasmic sperm injection in a patient with ovarian failure. Fertil Steril. 1998; 69(3):555–557 Young E, Kenny A, Puigdomenech E, Van Thillo G, Tivero´n M, Piazza A. Triplet pregnancy after intracyto-plasmic sperm injection of cryopre-served oocytes: case report. Fertil Steril 1998; 70(2): 360–361 Wurfel W, Schleyer M, Krusmann G, Hertwig IV, Fiedler K (1999) Fertilization of cryopreser- ved and thawed human oocytes (Cryo-Oo) by injection of spermatozoa (ICSI)– medical management of sterility and case report of a twin pregnancy. Zentralbl Gynakol . 1999; 121(9):444–448
1998 1999	Vajta et al. reported the use of the open-pulled straw method for the successful cryopreservation by vitrification of bovine oocytes and embryos with high survival rates. *(Above contributed by JJ Stahecki, 2024)* The above micro device technique prevented cryoinjuries to the oocyte similar to another micro device demonstrated much earlier in 1991 by Riha et al., 1991 (see entry in this article) and by others (Lane et al., 1999) heralded the use of micro devices in cryopreservation.	Open Pulled Straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H. Mol Reprod Dev. 1998;51(1):53-58. Lane M, Schoolcraft WB, Gardner DK, Phil D. Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. Fertil Steril. 1999;72(6):1073-1078.
1998 1999 2000 2003 2005 2006 2010 2011	Successful vitrification of human oocytes with pregnancies and live-births using VS14 consisting of 5.5M ethylene glycol and 1M sucrose or with a similar formulation. Beginning from year 2000 onwards, it became apparent vitrification will become the technique of choice for the cryopreservation of both human oocytes as well as embryos. Controlled-rate freezing was slowly phased out in most laboratories.	Cha KY, Hong SW, Chung HM, Choi DH, Ko JJ, Yoon TK. Pregnancy and implantation from vitrified oocytes following In vitro fertilization (IVF) and In Vitro Culture (IVC). Proceedings of the 54th Annual Meeting of the American Society for Reproductive Medicine, Toronto, CA, September 25–30, 1999 Kuleshova L, Gianaroli L, Magli C, Ferraretti A, Trounson A (1999) Birth following vitrification of a small number of human oocytes: case report. Hum Reprod. 1999; 14(12): 3077–3079 Cha KY, Hong SW, Chung HM, Choi DH,Ko JJ, Yoon TK. Pregnancy and

		implantation from vitrified oocytes following In vitro Fertilizat- ion (IVF) and In Vitro Culture (IVC). Proceedings of the 55th Annual Meeting of the American Society for Reproductive Medicine, Toronto, CA, September 25–30, 1999. Fertil Steril. 1999, p.S2 Yoon TK, Chung HM, Lim JM et al. Pregnancy and delivery of healthy infants developed from vitrified oocytes in a stimulated in vitro fertilization-embryo transfer program. Fertil Steril.2000;74:180–181 Park SE, Chung HM, Cha KY et al Cryopreservation of ICR mouse oocytes:improved post-thawed preimplantation development after vitrification using Taxol, a cytoskele-ton stabilizer. Fertil Steril 2001; 75:1177–1184 Yin H, Kim SS, Fisher J et al. Invest-igation of optimal conditions for equilibrating ovarian tissue with ethylene glycol prior to vitrification. Fertil Steril. 2001;76(Suppl 1):S101 (abstr) Yoon TK, Kim TJ, Park SE et al. Live births after vitrification of oocytes in a stimulated in vitro fertilization-embryo transfer program. Fertil Steril. 2003;79:1323–1326 Kim TJ, Hong SW, Park SE et al Pregnancy after vitrification of human oocytes and blastocysts using same cryoprotectant solution, ethylene glycol, and sucrose. Fertil Steril. 2003; 80(Suppl 3):143 abstr. Kim T, Hong S, Cha K. Pregnancies from cryopreserved oocytes using vitrification protocol. Fertil Steril. 2005;84(Suppl. 1):S179 (abstr) Kim TJ, Hong SW, Chung HM et al Pregnancy and delivery after vitrification of human oocytes. Fertil Steril 2005; 83(Suppl. 5): S13 (abstr) Kim SH, Ku SY, Sung KC et al. Simplified EM grid vitrification is a

		convenient and efficient method for mouse mature oocyte cryopreservation. Yonsei Med J; 2006;47:399–404 Kim TJ, Laufer LR, Hong SW.) Vitrification of oocytes produces high pregnancy rates when carried out in fertile women. Fertil Steril 2010; 93:467–474 Kim TJ, Hong SW. Successful live birth from vitrified oocytes after 5 years of cryopre- servation. J Assist Reprod Genet . 2011;28(1):73–76 Choi DH, Chung HM, Lim JM et al. Pregnancy and delivery of healthy infants developed from vitrified blastocysts in an IVFET program. Fertil Steril. 2000;74:838–839 Chung HM, Seung WH, Hong MS et al. In vitro blastocyst formation of human oocytes obtained from unstimulated and stimulated cycles after vitrification at various maturational stages. Fertil Steril. 2000; 73:545–551 Chung HM, Hong SW, Lim JM, Lee SH, Cha WT, Ko JJ, Han SY, Choi DH, Cha KY. In vitro blastocyst formation of human oocytes obtained from unstimulated and stimulated cycles after vitrification at various maturat-ional stages. Fertil Steril . 2000; 73(3) :545–551 Hong S, Kim T, Lee S et al. Cryopreserved blastocysts using vitrification protocol give excellent pregnancy and implantation rates after thawing. Fertil Steril2005;84 (Suppl.1):S178–S179 (abstr)
2000	**Landmark development** First report of transplant of frozen-thawed ovarian tissue, 2000	Oktay K, Karlikaya G. Ovarian function after transplantation of frozen, banked autologous ovarian tissue. N Engl J Med. 2000 Jun 22;342(25):1919
2006	**Landmark development** First report of cryopreservation of intact human ovary with its vascular pedicle	Bedaiwy MA, Hussein MR, Biscotti C, Falcone T. Cryopreservation of intact human ovary with its vascular pedicle. Hum. Reprod. 2006 21: 3258-3269
2012	**Landmark development**	Müller A, Keller K, Wacker J, Dittrich R, Keck G, Montag M, Van der Ven

	First livebirth from cryopreserved ovarian tissue in the human.	H, Wachter D, Beckmann MW, Distler W. Retransplantation of cryopreserve-ed ovarian tissue: the first live birth in Germany. Dtsch Arztebl Int. 2012 Jan;109(1-2):8-13.
2022	**Landmark development** First livebirth from ovarian tissue cryopreserved by vitrification in the human.	Strypstein L, Van Moer E, Nekkebroeck J, Segers I, Tournaye H, Demeestere I, Dolmans MM, Verpoest W, De Vos M. First live birth after fertility preservation using vitrification of oocytes in a woman with mosaic Turner syndrome. J Assist Reprod Genet. 2022 Feb;39(2):543-549