—Mini Review—

Cryopreservation of Human Embryos and Oocytes

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Abstract: *A procedure to cryopreserve mammalian embryos resulting in the birth of offspring was first described almost forty years ago. This procedure resulting in the birth of a child was reported 26 years ago. Since then, the preservation of human oocytes and embryos by cooling them to low subzero temperatures has become an integral part of Assisted Reproductive Technologies (ART). Hundreds of thousands of children have now been born after having been cryopreserved as oocytes or embryos. These results owe as much to the fundamental understanding of cryobiology as to the application of reproductive medicine. This brief review summarizes the history of embryo cryobiology, and presents a synopsis of basic cryobiology as it applies to present methods to improve the cryopreservation of human oocytes.*

Key words: *Cryopreservation, Human, Embryo, Oocyte, Vitrification*

Introduction

In January 1977, Robert Edwards stated "The storage of human preimplantation embryos at low temperatures could be valuable in clinical practice for the cure of infertility and possibly to avert inherited defects in children" [1]. Edwards made this statement at a symposium in England ten months before the *in vitro* conception of Louise Brown, the first child derived from Assisted Reproductive Technology (ART) [2]. At that same time, Edwards also suggested that it might be desirable to cryopreserve oocytes that have not yet begun their final maturation.

The application of cryobiology to reproductive medicine has made Edwards' statement 33 years earlier truly prescient. The cryopreservation of human oocytes and embryos has become an integral procedure in ART, and the importance of cryopreservation today in the treatment of human infertility can hardly be overstated. For example, in 2001 in the United States more than 4,700 pregnancies were produced by transfer of cryopreserved human embryos [3]. In Europe during 2005, transfer of cryopreserved embryos resulted in more than 13,000 clinical pregnancies [4], and in Canada alone during that same year 464 babies were born from frozen embryos [5].

Brief History of Embryo Cryopreservation

Although application of the procedure began slowly, the impetus to cryopreserve human oocytes and embryos was the demonstration in 1972 that mouse embryos that had been frozen to –196º or even to –269ºC developed into normal live young when thawed and transferred into foster recipient mothers [6]. In that initial report, both dimethyl sulfoxide (DMSO) and glycerol were shown to act as cryoprotective additives (CPA), protecting the embryos against freezing damage. Some four years later, oocytes of the mouse were successfully cryopreserved and fertilized *in vitro*, resulting in the birth of live offspring [7, 8]. It was not until in 1983 that the first human pregnancy resulting from a cryopreserved embryo was reported, although the fetus aborted spontaneously late in gestation [9]. The following year, two full-term human pregnancies and birth were reported [10]. In 1986, a human pregnancy resulting from *in vitro* fertilization of a cryopreserved oocyte was reported [11]. In all of these studies, the cryopreservation procedure used, including the CPA itself, DMSO, was essentially the same one that had been used for the initial report with mouse embryos [6].

During the late 1970s to mid-1980s, prompted by efforts to cryopreserve domestic animal embryos,

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important fundamental and practical observations were made about the cryobiology of oocytes and embryos. First, it was shown that several low molecular weight compounds, such as ethylene glycol and propylene glycol, also acted as CPAs to protect embryos against freezing damage [12]. A major practical improvement, with important implications for basic cryobiology, was the demonstration that sheep and cattle embryos could be cryopreserved by use of a method referred to as "interrupted cooling" [13]. Embryos suspended in DMSO were cooled slowly but only to an intermediate subzero temperature of about –35ºC (instead of –80ºC as in the original method), and then were immersed directly into liquid nitrogen (LN2). A critical aspect of this method was that the frozen embryos had to be warmed relatively rapidly at about 360ºC/min if they were to survive, instead of at 25ºC/min as in the original embryo freezing method. It must be noted that the method described by Willadsen [13] has been used to cryopreserve literally millions of cattle embryos, as well as embryos of more than twenty other species [14]. At about this same time, the concept of "osmotic buffers" was described in which a non-permeating solute such as a sucrose was used to prevent osmotic shock of embryos when recovered from concentrated CPAs after cryopreservation [15]. Incorporating an osmotic buffer into the container in which the embryo was frozen permitted domestic animal embryos to be transferred directly into the uterus of a cow without the use of a microscope or other laboratory equipment [16, 17]. This method was also adapted for freezing embryos of laboratory animals [18].

The significance of this latter observation is that in 1985 Renard also modified the same procedure for use with human embryos [19]. This new procedure was to suspend human embryos in 1.5 M 1,2-propanediol (= propylene glycol) supplemented with 0.1 M sucrose within a plastic straw, cool the sample at 0.3ºC/min from about –7º to –35ºC, plunge it into LN2 for storage, and warm the frozen sample at about 300ºC/min. This method yielded significantly higher morphological survival of embryos than the original method in which embryos were frozen in DMSO; most importantly, it produced substantial improvement in clinical pregnancies [20]. Therefore, it was rather quickly adopted by many clinics performing human ART. Remarkably, even twenty-five years later, this is the most common method used to cryopreserve human embryos all over the world. It is commonly referred to as the "slow freezing" or "slow cooling" method to cryopreserve human embryos. The significance of this fact will be discussed below.

That same year, 1985, a two-page experimental report titled "Ice-free cryopreservation of mouse embryos at –196ºC by vitrification" was published in the journal, Nature [21]. The report described a method to preserve mouse embryos by suspending them in a concentrated solution of several CPAs contained in a plastic straw, cooling the embryos rapidly by direct immersion of the straw in LN2, and after a brief time at –196ºC, warming the straw at 300ºC/min or 2,500ºC/min. This method is commonly referred to as "vitrification" or "non-equilibrium cooling". Within five years after the Nature article was published, offspring had been produced from vitrified embryos of mice [22], rabbits [23], sheep [24], and cattle [25]. Since then, various improvements have been made to vitrification methods for embryos of cattle [26, 27], and have also been applied to embryos of other domestic species, such as those of pigs [28, 29] and horses [30]. Given the fact that this method was first described twenty-five years ago, it does not seem appropriate to refer to it as "recent" or "novel", yet even in 2009 and 2010 several authors have described it that way.

Cryopreservation of Human Embryos by Freezing

In the meantime, however, following the publication of the article by Lassalle *et al.* [19], numerous articles describing the cryopreservation of human embryos by that slow freezing method were published. Many reviews that summarize both the cryobiological aspects of the procedure as well as the clinical and physiological variables have been published [31–38]. Furthermore, by this time, sufficient numbers of children had been born of women impregnated with cryopreserved embryos so that analyses of these children have also been published [39–41]. Among the variables studied were the embryonic stage of development that was frozen, the concentration of sucrose in the cryoprotectant solution, the type and concentration of the macromolecule in the medium, whether the embryo that was frozen resulted from intracytoplasmic sperm injection or from standard *in vitro* fertilization, whether the zona pellucida was damaged during freezing or not, and the effect of culture on thawed embryos before they were transferred. Additional investigations have examined the influence of the hormone regimen used to induce oocyte maturation, as well as that used to prepare the uterine endometrium for implantation.

Yet, despite the many studies that have been

performed, the following freezing procedure was virtually the same in almost all of those investigations.

Standard Human Embryo Freezing Procedure

(1) Embryos incubated for 10 min in 1.5 M propanediol supplemented with 0.1, 0.2 or 0.3 M sucrose as a cryoprotectant.
(2) Embryos placed into a plastic straw, cooled at 2ºC/min to –7°C and held for several minutes to equilibrate at that temperature.
(3) Sample seeded to induce ice formation and held for an additional 10 min.
(4) Straw cooled at 0.3ºC/min to –30ºC.
(5) Straw then cooled at 50ºC/min to –150ºC, and finally placed into LN2 for storage.
(6) Frozen straws thawed by being held in air for 30 sec, and then in a water bath for >30 sec.
(7) Cryoprotectant removed either by stepwise-dilution of CPA or incubated in hypertonic sucrose for several minutes.

With only a very few exceptions, alternative compounds have not been tested as possible CPAs for human embryos subjected to slow freezing, yet it is well established with embryos of laboratory, domestic and wild species that various solutions may be used and may be more effective to protect against freezing damage. For the seeding process, there is no evidence that slow cooling to the seeding temperature or holding at –7ºC improves embryo survival. In fact, those steps simply add about 25 min to the freezing procedure. Most importantly, to our knowledge there are no data in the literature at all regarding the effect of cooling rate from –7º to –30ºC on survival of human embryos. This is a striking omission since it has been known for 38 years that one of the principal variables that determine the survival of embryos is the rate at which they are cooled to subzero temperatures [6]. It is as if the procedure to freeze human embryos was to apply the rationale: “Slow cooling is good. Slower cooling must be better”. Finally, the method used to recover the thawed embryos from the CPA solution is not very efficient and risks damaging them by osmotic shock. Methods used in the cattle industry to recover cryopreserved embryos, permitting the thawed embryos to be transferred directly into recipient cows, are substantially more efficacious [42–44]. The point is that the most common method used to cryopreserve human embryos in 2010 is virtually the same as the one first published in 1985 [19].

The Role of Cooling and Warming Rates in Cryopreservation

In their original report describing the cryopreservation of embryos by vitrification, Rall and Fahy [21] emphasized the fact that “successful vitrification” requires the use of highly concentrated solutions of cryoprotectants. They also emphasized that, to vitrify, a solution must be cooled at high rates so that ice crystals do not form; rather, at high cooling rates the solution forms a viscous, non-crystalline solid at low subzero temperatures. The original application of vitrification to cryopreserve embryos was based on detailed studies by Fahy of complex solutions subjected to subzero temperatures [45, 46]. Since the report by Rall and Fahy, considerable effort has been expended to derive solutions (and devices to hold them) that vitrify when cooled at high rates to low temperatures. In general, the consensus of most authors in the literature is that a vitrification procedure is one that, when used to cool a sample to temperatures below –120ºC, yields a clear, transparent medium rather than an opaque, milky solid, which is the appearance of ice. A related question is what happens to a vitrified medium when it is heated. If the medium becomes milky during warming, that is interpreted to mean that the vitrified medium has crystallized or has “frozen” during warming. Most often in studies of oocyte and embryo cryopreservation, explicit evidence of vitrification is not presented. Rather, if the medium consists of high CPA concentrations, if the specimen is cooled at a high rate, e.g. >1,000ºC/min, and if the medium appears transparent when viewed while submerged in LN2, then it is assumed to have vitrified. We suggest that it would be helpful if the words “freezing”, “frozen”, “thawing” and “melting” be used to refer to the formation and dissolution if ice crystals, but the words “vitrification” and “liquefaction” be used to refer to media that form transparent solids when cooled at high rates, and that become fluid without forming ice during warming.

Using the criteria listed above, some investigators have attempted to identify the conditions under which various solutions do vitrify. For example, depending on the concentration of each compound, 20 μl droplets of mixtures of propylene glycol and glycerol were observed to vitrify when cooled rapidly [47]. But some mixtures that vitrified during cooling did become opaque during warming, presumably due to ice formation. A much more comprehensive study of the behavior of ten cryoprotectants mixed in various combinations has also been made [48]. In this latter study, the biological

Table 1. Data of Rall and Fahy [21] showing the effect of warming rate on the survival of eight-cell mouse embryos cooled to –196ºC.

Total No. Embryos	Cooling Rate (ºC/min)	Warming Rate (ºC/min)	Survival ± SEM (%)
343	2,500	2,500	87.8 ± 1.9
110	20	2,500	83.6 ± 3.9
171	500	300	80.7 ± 4.0
63	2,500	10	0.0

consequences of exposing mouse embryos to these solutions were also determined. Based on that analysis of the solutions, Ali and Shelton derived efficient procedures to cryopreserve embryos of mice [49] and sheep [50]. The macroscopic appearance of plastic straws filled with various mixtures of ethylene glycol plus polyvinyl pyrrolidone have been published, illustrating the difference between vitrified and frozen samples at –196ºC [51]. In addition to the solutions themselves used for vitrification of embryos, considerable effort and ingenuity has been used to design and fabricate various devices to hold or support oocytes and embryos that are to be vitrified. These include an electron microscope grid [52], an open pulled straw [53], a cryo-loop [54], and a Cryo-Top [55], as well as several other devices.

All of these instruments were either selected, in the case of existing material, or were designed to yield very high cooling rates so that the CPA solution and the embryos suspended in it would vitrify when cooled. But a fundamental question is whether it is the cooling rate or the warming rate that is the critical determinant of oocyte and embryo survival. It is therefore useful to consider some old and some new observations regarding vitrification. Table 1 shows some of the original observations of Rall and Fahy as to the effect of cooling and warming rates on embryo survival [21]. When embryos were cooled to –196ºC at three rates (20ºC, 500ºC, 2,500ºC), survival was >80% if the embryos were warmed at 300 or 2,500ºC/min. But even if the embryos were cooled rapidly at 2,500ºC/min, none survived if they were warmed slowly at 10ºC/min.

Let us now consider some recent observations of Seki and Mazur [56]. Their data have been redrawn and are shown in Figs. 1 and 2. The data in Fig. 1 show survival of mouse oocytes as a function of cooling rate when cryopreserved samples are warmed at two high rates of 1,220 or 3,000ºC/min. In all cases, oocyte survival is high except for the one sample that had been cooled at about 35ºC/min. The data in Fig. 2 show the effect of warming rate on oocyte survival. Even if oocytes were cooled at only 190ºC/min (not a rapid cooling rate), 65% or more of them survive, If they are warmed at >1,000ºC/min. Of even more significance is the fact that survival of oocytes that had been cooled at 1,830ºC/min (a rapid cooling rate) is highly dependent on warming rate. The data of Seki and Mazur are completely consistent with the data of Rall and Fahy. High cooling rates yield high survival of oocytes and embryos ONLY IF the cryopreserved specimens are warmed at high rates. In other words, warming rate is the critical parameter that determines the survival of vitrified oocytes and embryos.

Cryopreservation of Human Oocytes

Human oocytes were first cryopreserved by "standard" methods of freezing in the late 1980s [11, 57]. However, the results were highly variable, because it was discovered that oocytes are far more sensitive to deleterious effects of low temperatures, even near 0ºC, than zygotes or cleavage-stage embryos. Therefore, for many years there was a "moratorium" on the cryopreservation of human oocytes. However, in the late 1990s, substantial progress had been made in cryopreserving animal oocytes by use of very high cooling rates [52–54]. This was achieved by the introduction of novel devices to hold or contain extremely small volumes of concentrated medium that almost certainly vitrified when immersed directly into LN2. Shortly after that, yet another device was derived to hold even small volumes of medium, and this new instrument was used to cryopreserve human oocytes, resulting in high survival and pregnancy after fertilization and transfer of the resultant zygote [58]. In the meantime, progress had also been made by investigators using more common methods of cryopreservation [59]. In the past decade, many modifications and improvements have been made to procedures to cryopreserve human oocytes by

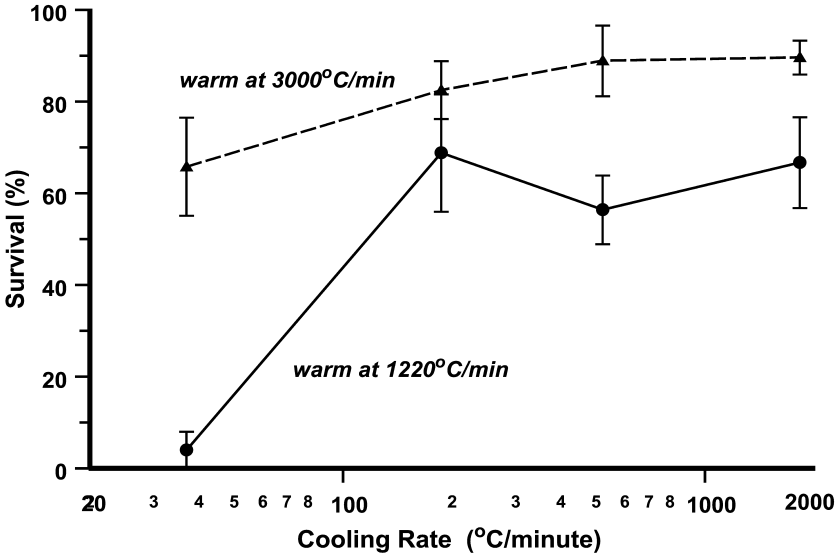

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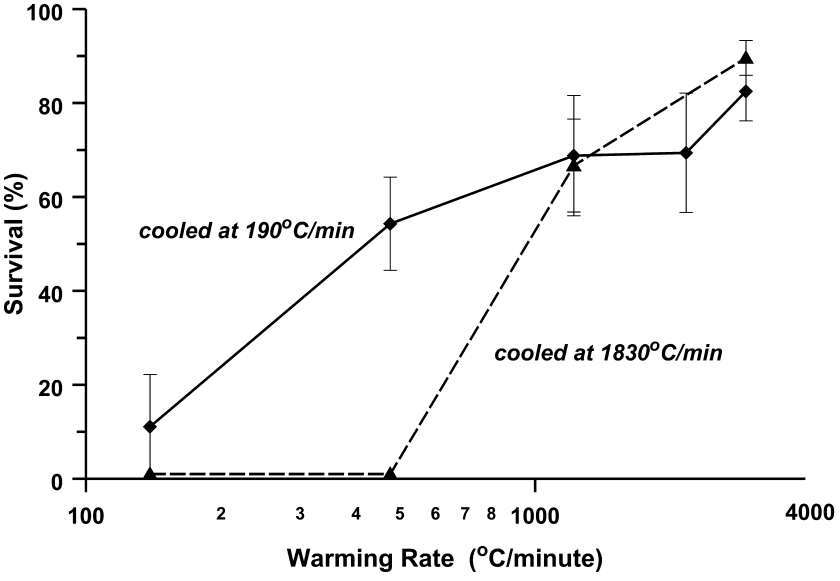
Fig. 1. Survival of mouse oocytes vitrified in EFS



Seki and Mazur 2009 Cryobiology 59:75-82

Fig. 2. Survival of mouse oocytes vitrified in EFS

numerous groups of investigators. Many reviews describing these improvements, as well as the clinical results have been published [55, 60–68]. By this time, it is difficult to document all of the clinical cases in which human oocytes have been successfully cryopreserved, have been fertilized and resultant zygotes or embryos have been transferred. But a conservative estimate is that thousands of children have now been born after having developed from cryopreserved oocytes.

Conclusion

As noted in the Introduction, the procedure of cryopreservation has become an integral part of clinical methods of human assisted reproductive techniques (ART). Many of the alternative methods of treating human infertility by the powerful techniques of ART (hormonal stimulation of women's ovaries, oocyte maturation, fertilization by intracytoplasmic sperm injection, genetic analysis of embryos by molecular techniques, single embryo transfer) have all been rendered more efficient and easier to perform because human oocytes and embryos can now be cryopreserved very successfully. This very brief review has touched on a very few of the highlights that have led to the current results.

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