Ultra-Rapid Warming Yields High Survival of Mouse Oocytes Cooled to \(-196^\circ\text{C}\) in Dilutions of a Standard Vitrification Solution

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Abstract

Intracellular ice is generally lethal. One way to avoid it is to vitrify cells; that is, to convert cell water to a glass rather than to ice. The belief has been that this requires both the cooling rate and the concentration of glass-inducing solutes be very high. But high solute concentrations can themselves be damaging. However, the findings we report here on the vitrification of mouse oocytes are not in accord with the first belief that cooling needs to be extremely rapid. The important requirement is that the warming rate be extremely high. We subjected mouse oocytes in the vitrification solution EAFS 10/10 to vitrification procedures using a broad range of cooling and warming rates. Morphological survivals exceeded 80\% when they were warmed at the highest rate (117,000 \(\mu\text{C/min}\)) even when the prior cooling rate was as low as 880 \(\mu\text{C/min}\). Functional survival was \(>81\%\) and 54\% with the highest warming rate after cooling at 69,000 and 880 \(\mu\text{C/min}\), respectively. Our findings are also contrary to the second belief. We show that a high percentage of mouse oocytes survive vitrification in media that contain only half the usual concentration of solutes, provided they are warmed extremely rapidly; that is, \(>100,000 \mu\text{C/min}\). Again, the cooling rate is of less consequence.

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Introduction

The ability to cryobiologically preserve mammalian sperm and preimplantation embryos has played a central role in assisted reproduction in women, in improving the genetic quality of livestock, and in the maintenance of mutant and transgenic lines of mice and other mammals [1]. The successful cryopreservation of mammalian embryos was first reported in 1972 for mice [2]. More recently, the cryopreservation of the human oocyte has become a matter of intense interest [3]. First, it would permit women to delay the onset of child bearing without adverse consequences. Second, it would permit women who face the daunting prospects of becoming sterile from chemotherapy and radiation therapy to subsequently give birth to children. Third, the preservation of unfertilized oocytes does not create the ethical and legal problems that can occur with frozen embryos. These are the chief reasons for the interest; but unfortunately, the results so far have not matched the interest. As of the end of 2008, only about 900 babies world-wide have been derived from cryopreserved oocytes as opposed to tens of thousands that have developed from frozen embryos [4], and the procedure is still considered experimental.

A major cause of lethal injury during cryopreservation is the formation of more than a trace amount of ice within a cell. (Karlsson et al. [5] have calculated that the limiting amount of internal ice compatible with viability in hepatocytes is 2 to 4\% of their water.) One route to avoid it is vitrification. In the vitrification approach, ice formation is avoided by suspending the cells in very high concentrations of solutes, including ones that permeate the cell, and cooling them at high rates to temperatures below \(-100^\circ\text{C}\). As a result, the water in the system is converted from a liquid to a glass with no ice formation. The approach also requires high warming rates to ensure that the system does not convert from glass to ice during warming.

There have been two firmly held premises in the vitrification approach. One is that avoiding ice formation in cells and obtaining high survivals demands the highest of cooling rates. Consequently, a series of devices have been developed over the past decade that achieve cooling rates of \(\geq 10,000 \mu\text{C/min}\) by permitting the manipulation of very small volumes of oocyte suspensions. These include electron microscope grids [6], nylon mesh [7], open-pulled straw (OPS) [8], cryoloop [9], microdrop method [10,11], and Cryotops [12,13].

Some authors have noted almost parenthetically that rapid warming is also necessary to prevent the vitreous water in cells from crystallizing during warming. But apart from a few scattered reports, only recently has it been experimentally demonstrated that the warming rate is much the more critical determinant of whether mouse oocytes survive vitrification procedures, and not the cooling rate [14].

The second premise in the vitrification approach is that the vitrification solution in which the cells are suspended must have a very high concentration of a mixture non-electrolytic solutes. We have used EAFS 10/10, a solution developed by Pedro et al. [15],
where E, A, F, and S refer to ethylene glycol (EG), acetamide, Ficoll, and sucrose. The mass composition (Table 1, top row) is 3.23 molal EG, 3.27 mol acetamide, 0.72 mol sucrose, 0.15 mol salt, and 20.7 wt. % Ficoll (24% w/v). The total molality is 7.37 molal, of which 6.5 molal is permeating (EG and acetamide), and the remainder non-permeating. This composition is relatively typical of most vitrification solutions in containing mixtures of permeating and nonpermeating solutes. These very high concentrations can have serious consequences. First, they can be chemically toxic to the cells. Second, they produce major cell osmotic dehydration that may itself be damaging. In EAFS 10/10, for example, in approximately 2 min, the oocyte water contents will drop to near 0.3/7.4 or 5% of their isotonic value. However, as they approach equilibrium over the next ~20 min, their water volume will gradually increase to 28% as the permeating solutes enter (Table 1, column M) [16]). Ordinarily, if one attempts to reduce the concentration greatly increase the cooling rate required to achieve vitrification and greatly increase the warming rate needed to prevent devitrification [17].

Based on our previous demonstration that a very high warming rate and not a high cooling rate was the essential element to surviving vitrification procedures in full strength EAFS [16], we hypothesized for the present study that the use of very high warming rates might also permit the use of more dilute vitrification solutions. As we report, that has turned out to be the case.

Materials and Methods

Collection of Oocytes

Mature female ICR mice were induced to superovulate with intraperitoneal injections of 5 IU of equine chorionic gonadotropin (eCG) and 5 IU of human chorionic gonadotropin (hCG) (Sigma, St. Louis) given 48 h apart. Ovulated unfertilized oocytes were collected from the ampullar portion of the oviducts at 13 h after hCG injection and were freed from cumulus cells by suspending them in modified phosphate-buffered saline (PBS) containing 0.5 mg/ml hyaluronidase followed by washing with fresh PBS medium.

Table 1. Derivative solute concentrations in various dilutions of the EAFS10/10 vitrification solution.

| Relative concn. | Total mass (g) | Total volume (ml) | EG molality | Acetamide molality | Sucrose molality | NaCl molality | Molar Osmol | Rel. vol cell |
|-----------------|----------------|-------------------|-------------|--------------------|-----------------|---------------|--------------|--------------|
| EAFS 1×         | 11.52          | 10                | 3.23        | 3.27               | 0.72            | 0.15          | 1.345        | 0.292        | 0.062        | 0.996        | 0.277        |
| 0.875×          | 11.33          | 10                | 2.57        | 2.6                | 0.57            | 0.15          | 1.217        | 0.272        | 0.056        | 0.849        | 0.325        |
| 0.75×           | 11.14          | 10                | 2.01        | 2.04               | 0.45            | 0.15          | 1.079        | 0.241        | 0.051        | 0.726        | 0.38         |
| 0.5×            | 10.76          | 10                | 1.15        | 1.16               | 0.26            | 0.15          | 0.771        | 0.78         | 0.172        | 0.035        | 0.533        | 0.518        |
| 0.33×           | 10.51          | 10                | 0.7         | 0.71               | 0.16            | 0.15          | 0.538        | 0.544        | 0.12         | 0.024        | 0.436        | 0.633        |

Columns D-G. The molalities are calculated as Columns N-Q of Table 2/MW of the respective solute.

Columns H-K. The molalities are calculated as 100× Column B/W% in Column G-K of Table 2/MW of the solute.

Column L: The nonpermeating solutes are sucrose and PBS (we ignore the very small contribution of the Ficoll and BSA). Their combined osmolality is Column F-0.276. We assume the osmolality of PBS to be equal to that of the same molality of NaCl. The osmolality of NaCl = 2ωm where 2 is the number of species into which the molecule dissociates and ω is the osmotic coefficient.

Column M: The volume of water in the oocytes after equilibration with the external medium relative to the volume of water in an isotonic cell. It is 0.276/Column L.

Ethics Statement

All the procedures involving mice were carried out under the University of Tennessee Institutional Animal Care and Use Committee protocol 911-0710, approved 9 July 2010 and renewed 5 May 2011. It has been assigned a pain/distress category of C.
The temperature/time curve became very curvilinear above
11 published by Amersham Biosciences (Piscataway, NJ), the
manufacturer of Ficoll 70, shows that the osmolality of a
0.003 molal solution of Ficoll 70 in water is roughly four times
the osmolality. For the 0.006 molal in EAFS 10/10, the osmolality is
probably 0.06 osm, or 10-fold higher. But that is still too small to
contribute significantly to the osmotic equilibrium, and we have
ignored that contribution.

Description of the Cryotop
The Cryotop (Kitazato Co., Fuji, Japan) consists of a flat
rectangular leaf of polypropylene measuring 20 x 0.7 x 0.1 mm
that is attached to a thin handle some 5 cm long. A photograph is
shown in [18]. For a run, about 6 oocytes were transferred at 22
C to successive drops of the desired dilution of EAFS 10/10, and
then 0.1 milliliter of the last solution along with the six oocytes was
inserted into an outer insulating tube(s) just prior to cooling. The elapsed time between the initial
exposure of the oocytes to the EAFS and the initiation of cooling
was held close to two minutes.

Achieving Various Cooling and Warming Rates with Cryotops.

As indicated in Table 3, three of the seven cooling rates studied
used Cryotops cooled either in LN2 (−196°C) (Cooling Protocol 7) or
in LN2 vapor (−150°C) (Cooling Protocols 2 and 3). In two
cases (Protocols 2 and 5), the Cryotop was insulated. In one case
(Protocol 7) it was not. All samples on Cryotops were warmed at the highest rate of 117,500°C/min by abrupt immersion of the
naked Cryotop in 2 milliliters of 0.5 M sucrose at 23°C. (Sucrose is an
impermeant solute, the purpose of which is to provide osmotic
buffering when the oocytes are diluted out of the concentrated
EAFS 10/10). This means that the insulation surrounding the
Cryotops in cooling protocols 2 and 5 had to be removed under or
just above the LN2 prior to initiating warming. Cooling rates were
calculated from 20°C to −120°C; warming rates from −130°C to
−30°C. The upper limit of −30°C was chosen because the temperature/time curve became very curvilinear above −30°C as
a result of the progressive melting of ice in the concentrated
solution.

The cooling and warming rates of samples on Cryotops were
determined by cementing the junction of a 50 μm copper/constantan thermocouple to a Cryotop and overlaying it with 0.1 μl of EAFS [18]. The highest cooling and warming rates were
so high that they required a special, but inexpensive computerized
oscilloscope to record the temperature-time traces. An oscillo-
scope trace of one cooling and warming run involving an
uninsulated Cryotop is shown in [18].

Achieving Various Cooling and Warming Rate with ½ ml Straws

Lower cooling and warming rates were achieved by placing
samples in ½ ml insemination straws (I’Aigle, Normandy, France). For cooling (Table 3), the straws were placed in LN2 or
in LN2 vapor. Two of the samples in straws were cooled with
insulation; two of them were cooled without insulation. All four
were warmed without insulation in a water bath at 0°C or at 25°C
(Table 4).

The cooling and warming rates in straws were determined by
placing a 36 ga thermocouple in the column of medium in the straw [14,16].

Post-Thawing Procedures and the Determination of Survival

The “thawed” oocytes on Cryotops were collected from the
2 milliliters of 0.5 M sucrose/PB1 and were then pipetted into fresh
0.5 M sucrose/PB1 solution. Approximately 10 min later, they
were transferred to fresh PB1 medium lacking sucrose, and then
transferred to and cultured in modified M16 medium for 2 hrs.
(We simply refer to this as M16). Oocytes thawed in the straws
were expelled into a watch glass containing 2 milliliters of 0.5 M sucrose
in PB1 [19]. From there on, the procedure was the same as with
Cryotops.

Viability was initially assessed at three time points based on
osmotic responsiveness and morphological normality. First, the
oocytes were examined during the 10 min in sucrose/PB1. Membrane-intact oocytes were expected to shrink with time
because the sucrose is hypertonic. Second and third, they were
examined after being placed in M16 and after two hours incubation. They fall into two binary groups: Degenerate oocytes are clearly non-viable. The others are indistinguishable from fresh oocytes, and we know from past experiments that the plasma membranes in this latter group are intact and function normally with respect to their osmotic response to hypertonic and hypotonic media, and with respect to their ability to remain supercooled in the presence of external ice. The ability to manifest these characteristics after two hours in M16 is considered a rather stringent test of viability. Still other criteria of normality are given in [19, pp. 48–49]. They mostly deal with measures of the integrity of the plasma membrane.

Functional Assay: In vitro Fertilization and Development to 2-Cell Embryos

In later experiments, we subjected a subset of samples of oocytes to a functional assay of viability; namely, oocytes that had yielded high survival based on the morphological/osmotic criteria. The functional assay consisted of carrying out in vitro fertilization (IVF) of the oocytes and determining the percentage that developed to the 2-cell stage. Sperm from male ICR mice were collected from the epididymides, transferred to M16 medium, incubated in a 5% CO2/95% air for 1 hr, and then transferred to fresh M16 medium lacking sperm, and allowed to incubate for 1 day. At this point, the percentage that had developed to the 2-cell stage was determined.

Statistics

Error figures in tables and error bars in graphs are standard errors (SEM, standard deviations of the mean). Tests of significance were carried out by 1-way ANOVA using Graphpad Software’s Instat, V. 3.02 followed by the Turkey-Kramer Multiple Comparison Test.

Results

Figure 1 plots survival as a function of cooling rate and warming rate for oocytes suspended in 1 x EAFS 10/10 (top panel) and in three dilutions of EAFS (0.875 x, 0.75 x, and 0.5 x). For each dilution, the oocytes were cooled at 3 or 4 different rates ranging from a low of 37 °C/min to a high of 69,250 °C/min, and for each cooling rate they were warmed at three rates; namely, 2,170 °C/min, 2,950 °C/min, and 117,500 °C/min. The survivals in this plot are based on the morphological normality, membrane intactness, and osmotic responsiveness of the oocytes after the post thawing protocol.

Table 3. Protocol of each cooling procedure.

| Protocol No. | Device | The device was covered with | Cooled by | Cooling rate ± SE (°C/min) |
|--------------|--------|-----------------------------|-----------|---------------------------|
| 1            | Straw  | 1/2-ml straw + double glass* | LN2 vapor | 37 ± 0°                  |
| 2            | Cryotop| Cryotop-cap-straw + small glass† | LN2 vapor | 95 ± 4°                  |
| 3            | Straw  | None                         | LN2 vapor | 187 ± 6°                 |
| 4            | Straw  | 1/2-ml straw                 | LN2       | 522 ± 54°                |
| 5            | Cryotop| Cryotop-cap-straw            | LN2 vapor | 876 ± 11°                |
| 6            | Straw  | None                         | LN2       | 1,827 ± 214°             |
| 7            | Cryotop| None                         | LN2       | 69,250 ± 4,285°          |

*1/4 ml sample straw was covered with a 1/2 ml straw, a 7 mm OD ×90 mm glass tube, and a 10 mm OD ×90 mm glass tube.
†The Cryotop was covered with the Cryotop-cap-straw and a 7 mm OD ×90 mm glass tube.
*The straw or Cryotop were placed horizontally on a Styrofoam disk (14 cm diameter, 1.5 cm thick) floated on the surface of LN2 in a 41 Dewar flask for 5 min before being immersed in LN2.
†The cooling rates were determined in [14].

Table 4. Protocol of each warming procedure.

| Protocol No. | Device | Air at 23–25 °C | Covered by | Warmed with | Stirring* | Warming rate ± SE (°C/min) |
|--------------|--------|----------------|------------|-------------|-----------|--------------------------|
| 1            | Straw  | 10 – Stirring* | None       | Water at 0°C | +         | 2,170 ± 114°             |
| 2            | Straw  | 10 –                | None       | Water at 25°C | +        | 2,950 ± 119°             |
| 3            | Cryotop| 0 –                | None       | Sucrose solution at 25°C | –     | 117,500 ± 10,632°       |

*+, yes; –, no.
†The warming rates were calculated between −70 and −35 °C in [16].
‡The warming rate was determined in [18].

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treatment. Details are given in [19]. Table 5 gives details on the dilution of EAFS and the cooling and warming rate to and from $-196^\circ C$. The dilutions were 1.0, 0.875, 0.75, or 0.5. The cooling rates ranged from 37°C/min to 69,250°C/min; the warming rates (ºC/min) were 117,500 ($\Delta$), 2,950 (○), and 2,170 (■). The protocols are given in Tables 3 and 4. Survivals are based on morphological appearance and osmotic behavior after warming and after 1-2 hr incubation in M16 medium.

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Figure 1. Survival of mouse oocytes as a function of the dilution of EAFS and the cooling and warming rate to and from $-196^\circ C$. The dilutions were 1.0, 0.875, 0.75, or 0.5. The cooling rates ranged from 37°C/min to 69,250°C/min; the warming rates (ºC/min) were 117,500 ($\Delta$), 2,950 (○), and 2,170 (■). The protocols are given in Tables 3 and 4. Survivals are based on morphological appearance and osmotic behavior after warming and after 1-2 hr incubation in M16 medium.

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Figure 2 shows morphological/osmotic survival as a function of the relative concentration of EAFS and includes the results for decreasing the concentration of EAFS from 1/2 to 1/3. In that last case, we see that even with the highest warming rate of 117,500°C/min (open triangles), survival drops from ~90% to 5%. Decreasing the concentration of solutes in the EAFS means decreasing the concentration of the non-permeating sucrose and Ficoll in the solutions and that in turn means that the oocytes are more hydrated in 1/2× EAFS than in full strength EAFS 10/10, and are more hydrated in 1/3× EAFS than in 1/2× EAFS. In the case of those in 1/3× EAFS, we presume that the high water content leads to the formation of enough intracellular ice during the subsequent cooling to kill them at that point. In the case of those cooled in concentrations of EAFS ≥0.5× the internal crystals that form during cooling can be prevented from recrystallizing to a lethal size by warming very rapidly.

Recrystallization is the conversion of a population of small ice crystals to a fewer number of larger crystals, a conversion that is driven by the former having a higher surface free energy than the latter. This implies that if warming rates higher than the 117,500°C/min used here were attainable, it might be possible to achieve reasonably high survivals of mouse oocytes that are subjected to vitrification procedures in even more dilute solutions.

As indicated, the survivals in Figures 1 and 2 are based on morphological and osmotic normality. We have also obtained a measure of functional survival after vitrification in terms of the percentages of the oocytes that undergo fertilization and development to the 2-cell stage. Those percentages are shown in Table 6 where they are compared with morphological/osmotic survivals. In all these cases, the oocytes were warmed at the highest rate attainable (117,500°C/min). Several conclusions can be drawn with respect to morphological/osmotic survivals: [1] The average morphological survival (Column 3) is 89.3% [2]. Cooling rates ranging from 95°C/min to 69,000°C/min are essentially without effect [3]. Relative EAFS concentrations ranging from 0.5× to 1× are almost without effect, and [4] these conclusions hold only when oocytes are warmed at the highest achievable rate.

The picture changes somewhat when we compare functional survivals (columns 5 and 6). When the highest cooling rate and highest warming rate are combined, functional survivals range from 81% to 67% as the EAFS concentration is reduced from 1× to 0.5×. A cooling rate of 880°C/min is still relatively benign, but now a cooling rate of 95°C/min is decidedly damaging. The most likely explanation is that when oocytes are cooled at 95°C/min, the crystals that form intracellularly are large enough to be immediately lethal.

**Discussion**

Subsequent to our initial finding published in 1972 [2], our laboratory has found that if mouse oocytes or preimplantation embryos suspended in a cryoprotectant like 1 M ethylene glycol are cooled at rates exceeding 2°C/min, they undergo lethal intracellular ice formation near $-40^\circ C$, the homogeneous nucleation temperature of water [19]. As stated in the Introduction, one route to avoiding lethal IIF, and one being increasingly favored, is to suspend cells in much higher concentrations of cryoprotectants and cool them at much higher rates. Under such conditions, intracellular water can be converted into an innocuous vitreous or glassy state. Although the oocytes/embryos are cooled too rapidly in this procedure to undergo any osmotic dehydration during cooling, the nonpermeating solutes in vitrification solutions cause appreciable osmotic dehydration before cooling begins, and that dehydration in turn enhances the probability of vitrification.

The next step in the conceptual picture was our finding that if the water content of oocytes was reduced to between 40 and 23% of normal, only 12% and 0%, respectively, underwent visible IIF.
during cooling. For the others, the manifestations of internal ice appeared during warming [20]. We concluded that what we were seeing in these others during warming was the recrystallization or growth of small innocuous ice crystals that had formed during cooling to a lethal size during warming. We further found that the shorter the time spent during warming, the higher the temperature at which recrystallization of intracellular ice became evident [21]. And that led to the view that if the warming rate was high enough, intracellular recrystallization could be suppressed, and the oocytes or embryos would survive. We confirmed that hypothesis in two subsequent papers [16,22]. The final step has been to extend this thinking to the possibility that with very high warming rates, one could substantially lower the concentrations of solutes in the vitrification medium. That has been the subject of the present work. After identifying three previous reports over the past 25 years that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use of very high cooling rates, presumably because they believed that the ability of cells to vitrify in dilute solutions demands the use

Table 5. Survival based on morphological normality and osmotic responsiveness of mouse oocytes suspended in various dilution of EAFS10/10, and cooled and warmed at indicated rates in straws or on Cryotops.

| Dilution of EAFS10/10 | Device | Warming rate (°C/min) | Cooling rate (°C/min) | 37 | 95 | 187 | 522 | 880 | 1827 | 69250 |
|-----------------------|--------|-----------------------|-----------------------|----|----|-----|-----|-----|------|-------|
| 1.00×                 | Straw 2170 | 15.8±12.3 A, a | - | - | 69.4±12.7 B, a | 62.9±9.2 B, a | - | 44.4±9.5 AB, a | - |
|                       | (4/28, N = 6) | (14/21, N = 6) | (22/35, N = 7) | (17/39, N = 8) |    |    |    |    |    |
| 1.00×                 | Straw 2950 | 65.8±10.7 A, a | - | - | 82.5±6.3 A, a | 88.9±7.7 A, a | - | 89.6±3.7 AB, a | - |
| (24/38, N = 8)        | (33/40, N = 8) | (27/31, N = 6) | (33/38, N = 6) |    |    |    |    |    |
| 0.875×                | Straw 2170 | 23.3±8.5 A, a | - | - | 10.0±10.0 B, b | 26.7±13.5 A, b | - | 30.0±16.2 A, B, b | - |
| (7/30, N = 5)         | (3/30, N = 5) | (8/30, N = 5) | (9/30, N = 5) |    |    |    |    |    |
| 0.875×                | Straw 2950 | 56.7±8.5 A, b | - | - | 33.3±14.9 A, b | 66.7±11.8 A, b | - | 46.7±14.3 A, B, ab | - |
| (17/30, N = 5)        | (10/30, N = 5) | (20/30, N = 5) | (14/30, N = 5) |    |    |    |    |    |
| 0.75×                 | Straw 2170 | 23.3±8.5 A, a | - | - | 0.0±0.0 B, b | 0.0±0.0 B, b | - | 3.3±3.3 A, B, b | - |
| (7/30, N = 5)         | (0/23, N = 4) | (0/24, N = 4) | (1/30, N = 5) |    |    |    |    |    |
| 0.75×                 | Straw 2950 | 52.8±10.0 A, b | - | - | 36.1±8.0 A, b | 47.2±8.0 A, b | - | 56.3±8.3 A, B, ab | - |
| (19/36, N = 6)        | (13/36, N = 6) | (17/36, N = 6) | (27/48, N = 8) |    |    |    |    |    |
| 0.50×                 | Straw 2170 | 0.0±0.0 A, a | - | - | 6.7±6.7 B, ab | 16.7±5.3 A, b | - | 3.3±3.3 A, B, b | - |
| (0/30, N = 5)         | (2/30, N = 5) | (5/30, N = 5) | (1/30, N = 5) |    |    |    |    |    |
| 0.50×                 | Straw 2950 | 25.0±9.4 A, b | - | - | 22.2±8.2 A, b | 44.4±7.0 A, b | - | 16.7±8.6 A, c | - |
| (9/36, N = 6)         | (8/35, N = 6) | (16/36, N = 6) | (6/36, N = 6) |    |    |    |    |    |
| 0.33×                 | Straw 2950 | 88.6±11.4 A, b | - | - | 89.6±6.3 A, b | - | 85.4±5.8 A, ab | - |
| (36/40, N = 7)        | (35/38, N = 8) | (39/46, N = 8) |    |    |    |    |    |    |
| 0.33×                 | Cryotop 117500 | - | - | 82.5±6.9 A, a | - | - | 75.0±14.4 A, a | - |
| (19/23, N = 4)        | (18/24, N = 4) | (44/48, N = 8) |    |    |    |    |    |    |

The data are % survival ± SEM. The first sets of parentheses are the ratios of the number of surviving oocytes to the number frozen or vitrified. N is the number of replicate. Values with different superscripts were significantly different (P<0.05) by one-way ANOVA. Capital letters shows the differences of survivals with the same warming rate, same diluted vitrification solution and various cooling rate, and small letters shows those with same warming rate and same cooling rate and various diluted vitrification solutions. *shows those with same cooling rate, same dilution of vitrification solution, and different warming rates of 2,170 or 2,950°C/min and †shows that it was not possible to do statistical analysis because the other survival was 0%. doi:10.1371/journal.pone.0036058.t005
On the applied and clinical side, the use of more dilute vitrification solutions may result in higher and more reproducible percentages of offspring developing from cryopreserved human oocytes or higher survivals of other cell types. For example, one adverse effect of high concentrations of protective solutes in oocytes appears to be hardening of the outer zona pellucida, a consequence of which is interference with the ability of sperm to enter the egg proper without the use of technically demanding ICSI (Intra-Cytoplasmic Sperm Injection). Another problem is that “open” devices like Cryotops, which were developed to achieve the very high cooling rates that were believed to be mandatory, place oocytes in direct contact with liquid nitrogen (LN2). There is concern that they could be contaminated by viable microorganisms in that refrigerant. Our finding that only moderate cooling rates are needed should eliminate that concern. By inserting the Cryotop tip with its adhering oocytes into its covering cap, it can be kept from contacting LN2 during cooling. The cap can then be removed while holding the device in cold vapor just above the LN2 to achieve extremely rapid subsequent warming when the now naked Cryotop is abruptly immersed into the sterile warming solution. Vanderzwalmen et al. [28] reported a similar approach to asepsis in 2009. Human blastocysts in vitrification solutions were placed in capillary VitriSafe devices which were hermetically sealed within straws and plunged into LN2. The double layered device slowed the cooling rate more than 10-fold from >20,000°C/min to <2000°C/min. To maintain a high rate of warming (>20,000°C/min), the straw constituting the outer surface was removed and the naked VitriSafe capillary plunged into the warming solution. They believed that the 10-fold lowering the cooling rate had to be compensated for by the use of a higher CPA concentration and/or a longer exposure to it. As we’ve noted, our findings differ; namely, one does not have to use a higher CPA concentration. In fact, our report deals with the successful use of concentrations of EAFS down to 1/3 of normal.

In stating that the findings in this report have clinical implications, we do not intend to imply that all the particulars we have found for mouse will apply to human oocytes. This may especially be the case for EAFS 10/10 since the acetamide it contains has been found to be somewhat toxic to the human egg. However, we believe that the matters of cooling rate vs. warming rate and warming rate vs. the dilution of vitrification solutions will apply. As we state, nearly all previously published papers have stressed the need to maximize the cooling rate, and many have attempted to increase the maximum. Our report shows that emphasis to be misplaced. It is the warming rate that needs to be maximized, and it is our hope that our paper will encourage some readers to attack the problem of obtaining even higher warming rates. If successful, we believe that will further facilitate the cryopreservation by vitrification procedures of difficult cell types like human oocytes and fish oocytes and embryos.

Beginning with our 2005 publication [19], we have based survival on the morphological/osmotic criteria summarized in Methods and Materials and detailed in [19]. This has left open the question of whether this reflects the functional survival of the oocytes. The current paper answers the question affirmatively to a considerable extent. The functional assay was the ability of the vitrified/warmed oocytes to undergo IVF and develop to the two cell stage. As we see in the far-right column of Table 6, between 63% and 97% of oocytes judged viable on morphological/osmotic grounds did develop to the 2-cell stage, provided that the cooling rate was 880°C/min or higher and the warming rate was 117,000°C/min.

To obtain these percentages of fertilization and development, we had to partially dissect the zona pellucida. In 1997, Nakagata
Table 6. Morphological and functional survival of mouse oocytes suspended in various dilution of EAFS10/10, cooled at indicated rates on Cryotops, and warmed at 117,500 °C/min.

| Relative conc. | Cooling rate | % of Morphological Survival * | % of Oocytes develop to 2-cell | % of Morphol. Survivors |
|---------------|--------------|-------------------------------|-------------------------------|--------------------------|
| EAFS10/10     | (°C/min)     |                               |                               |                          |
|               | 1 ×          | +2 hr in M16                  | 0 hr in M16                   |                           |
| 1             | 69,000       | 91.7 ± 6.3                    | 91.7 ± 4.5                    | 80.8 ± 3.8 *             | 88.4                      |
| 880           | 75.0 ± 14.4  | 89.4 ± 5.5                    | 54.2 ± 9.2                    | 62.9                     |
| 95            | 82.5 ± 6.9   | 70.8 ± 9.9                    | 28.8 ± 8.8                    | 39.3                     |
| 0.875 ×       | 69,000       | 93.8 ± 4.4                    | Not determined                | Not determined            |                          |
| 880           | 81.0 ± 11.7  | Not determined                | Not determined                |                          |
| 95            | 88.1 ± 9.4   | Not determined                | Not determined                |                          |
| 0.75 ×        | 69,000       | 97.9 ± 2.1                    | 96.9 ± 3.1                    | 76.0 ± 4.3 *             | 77.8                      |
| 880           | 97.1 ± 2.9   | 96.7 ± 3.3                    | 63.3 ± 9.5 *                  | 66.7                     |
| 95            | 90.5 ± 9.5   | 75.9 ± 8.8                    | 39.3 ± 9.1 *                  | 52.5                     |
| 0.5 ×         | 69,000       | 85.4 ± 2.6                    | 93.8 ± 6.3                    | 67.3 ± 9.4 *             | 71.4                      |
| 880           | 89.6 ± 6.3   | 87.1 ± 3.4                    | 72.9 ± 5.8 *                  | 97.1                     |
| 95            | 88.6 ± 11.4  | 85.2 ± 7.7                    | 48.8 ± 6.3 *                  | 59.4                     |

**“Morphological survival” means an oocyte exhibiting normal osmotic responsiveness during the removal of EAFS and exhibiting normal morphology after 2 hr incubation in M16. That is, in the column labeled “+2 hr in M16”, after the vitrified oocytes were warmed and the EAFS was removed, the eggs were incubated in M16 medium for 2 hr before being assessed for morphological normality. These are the survivals shown graphically in Figures 1 and 2. In the column labeled “0 hr in M16”, as soon as the EAFS was removed, the eggs were scored for morphological survival. This was immediately followed by partial dissection of the zonae, and the mixing of eggs and sperm for IVF. In nearly all cases, each treatment was repeated 6 to 9 times with about 6 oocytes per repeat. In column of % of oocytes develop to 2-cell, values with different superscripts were significantly different (P < 0.05) by one-way ANOVA. Letters show the differences of survivals with the same warming rate, same diluted vitrification solution and various cooling rates, and there is no significant differences with same warming rate and same cooling rate and various diluted vitrification solutions.**

The number of oocytes developing to 2-cell embryos after IVF/the number recovered after vitrification.

*Column 5/Column 4.

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et al. showed that partial zona dissection (PZD) of denuded B37BL/6j oocytes increased the success of IVF from 12% to 73–88% [29]. Two years later, An et al. [30] reported that the percentage of ICR 2-cell embryos derived by IVF after PZD that develop to blastocysts is the same or higher than that of 2-cell embryos with intact zonae. A more recent example is the 2011 report by Macas et al. [31] that the percentage of human oocytes that develop to blastocysts after IVF is the same for oocytes with intact zonae, those with partially dissected zonae, and those with zonae in which various-sized holes has been created by laser. Thus, the use of PZD is not a problem. What is a remaining problem is that vitrified ICR oocytes will not develop beyond the 2-cell stage. They behave as though they have a two-cell block, but we do not as yet know the explanation or how to resolve it.

Author Contributions

Conceived and designed the experiments: SS. Performed the experiments: SS. Analyzed the data: SS. PM. Wrote the paper: SS. PM.
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