Short-term storage of tripronucleated human embryos

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Abstract

Purpose To determine the survival and subsequent in vitro development of human cleavage stage embryos and hatched blastocysts following varying periods of short-term storage at 4 °C, using tripronucleated human embryos (TPN) as a model. **Methods** TPN cleavage embryos and hatched blastocysts short-term stored at 4 °C for 0 h (control), 24 h and 48 h. The main outcome measures were: survival rates (SR) and in vitro developmental ability (blastocyst rate and blastocyst-re-expansion rate) in each of the groups after storage. **Results** Cleavage-stage TPN survived at comparable rates to controls, regardless of storage time (average: 97.3 %). The in vitro development of cleavage-stage TPN stored for 24 h was comparable to that of controls (average 64.7 %), but was significantly impaired when storage lasted 48-h (20.8 %). After artificial shrinkage, SR was comparable in 24-h-stored and non-stored hatched blastocysts (85.7 %; $p>0.05$), but was significantly impaired in the 48-h-stored group (20.0 %). Following 24-h storage, the re-expansion rate of hatched blastocysts was similar to that of controls (average: 57.1 %; $p>0.05$), but was higher than that of the 48-h-stored group (15.0 %; $p<0.05$).

Capsule Tripronucleated human cleavage embryos and blastocysts can be stored for up to 24 h at 4 °C without detrimental effect on survival or in vitro development.

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Conclusions TPN human cleavage embryos and blastocysts can be successfully stored short-term for up to 24 h at 4 °C without using cryoprotectants without any significant negative impact on survival or subsequent in vitro development.

Keywords Short-term storage · Human · Cleavage embryos · Hatched blastocyst · Refrigeration

Introduction

Human embryo cryopreservation has been successfully applied at different points of embryo development, from the one-cell stage to the blastocyst stages. It has been shown to be beneficial for the reproductive management of infertile patients, allowing the door of future maternity to be kept open. This method represents a step forward in the technological capacity of in vitro fertilization (IVF) laboratories, and complements other reproductive techniques such as preimplantation genetic diagnosis (PGD) or screening (PGS) [5, 39, 40]. Currently, vitrification is a well-accepted technique for cryopreserving human oocytes [8, 10, 11, 28, 29], early cleavage-stage embryos and blastocysts [9, 27–29] and is increasingly applied in IVF centers worldwide. However, its results are still less than optimal for zona-drilled cleavage-stage embryos [6, 24, 25, 31, 43, 50, 51] and hatching or zona-free hatched blastocysts [9, 13, 18, 20–23, 26, 33, 42, 46, 49]. The reduced survival rates observed tend to be due to the absence of a continuous zona pellucida (ZP), which acts as a partial water barrier that prevents embryos from rupturing due to rapid rehydration during thawing/warming procedures and avoids samples sticking to the carrier.

In a PGD/PGS program, the results of genetic diagnosis or screening of biopsied blastomeres or trophoctoderm cells are usually available 24 h after biopsy, though it can take longer when samples need to be transported to another laboratory for analysis

or when confirmation of results is required. In these cases, biopsied embryos must be cryopreserved or cultured *in vitro*.

When PGD/PGS is to be performed on cleavage-stage embryos and extended *in vitro* culture is not available, cryopreservation of biopsied embryos is the only option. However, biopsied cleavage-stage embryos survive cryopreservation at significantly lower rates than non-biopsied embryos, usually due to blastomeres near the hole of the ZP being lysed and/or escaped during the addition and removal of cryoprotective solutions [6, 25, 31, 43, 50, 51].

PGD/PGS of blastocysts consists of the removal of a number of protruding trophoctoderm cells from artificially zona-drilled expanded blastocysts or naturally hatching blastocysts. In all cases, the biopsied blastocyst, which has a discontinuous ZP, must be cryopreserved or cultured until day 6 or 7 of development while waiting for the results of the genetic analysis [5, 39, 40]. There are few case reports of embryos being cultured for a longer period (until day 7 of development); however, this is not a usual practice [17–19, 37, 41]. Following vitrification, the survival rates of warmed hatching blastocysts and zona-free hatched blastocysts are lower (particularly so in the latter case) than those of blastocysts at earlier stages of development (expanded or earlier blastocyst stages; [9, 49]) in which the volume of the blastocoel cavity is much smaller and the zona pellucida is still intact. Despite efforts to reduce the volume of the blastocoel by artificial shrinkage [23, 26, 33, 42, 46], the post-warming survival of hatching and hatched blastocysts is hampered by the discontinuity of the zona pellucida, which is incapable of protecting against the osmotic stress produced during addition and removal of concentrated cryoprotective solutions [27–29, 48], rapid rehydration during warming, adhesion of the embryo membrane to the polypropylene strip surface and/or the cryoprotective solution-air interphase [12], all of which can result in embryo lysis.

To overcome the abovementioned technical limitations of methods and gaining time while waiting for the results of PGD/PGS, we propose the short-term storage of cleavage-stage human embryos and hatched blastocysts as previously reported for other mammalian species. In fact, short-term storage of unfrozen ovine [3], bovine [30, 35], porcine [36], rabbit [2, 4], horse [7, 32], and mouse [15, 16, 34, 38, 44, 45, 47] embryos has been carried out successfully using different media, with or without cryoprotectant proteins, at different temperatures (from 0 °C to 10 °C), and for different time periods (from 12 h to 5 days).

Materials and methods

Institutional approval and informed consent

This work was approved by the ethics committee of the Instituto Universitario IVI (Valencia, Spain) and the Valencian regional

government (Conselleria de Sanitat, Generalitat Valenciana). Signed informed consent was obtained from patients prior to TPN embryo recruitment.

Origin of tripronucleated embryos and blastocysts

Three hundred and eighty-six zygotes confirmed as containing three pronuclei and one polar body (TPN) during fertilization assessment (18–22 h post-sperm microinjection –ICSI–) were individually cultured for 3 days in 50 μ L IVF-20TM drops (Scandinavian IVF, EMB, Barcelona, Spain) under paraffin oil at 37 °C in 5 % CO₂. On day 2–4 of development, 75 TPN cleavage-stage embryos containing ≥ 6 cells, but with less than 20 % fragmentation, were randomly assigned to one of the three 4 °C-storage times (0 h, 24 h or 48 h) to be assayed.

In the case of blastocysts, TPN embryos were transferred on day 3 of development from IVF-20TM drops to CCMTM (Scandinavian IVF, EMB, Barcelona, Spain) drops and cultured in the aforementioned conditions for three additional days. On day 6 of development, 62 TPN-derived hatched blastocysts were selected and their blastocoel artificially shrunk by a laser pulse [22, 23, 33] before being assigned at random to one of the three storage periods (0 h, 24 h or 48 h).

Embryo storage and return to culture conditions

Before storage, >6-cell embryos and shrunken zona-free blastocysts were washed three times in 100 μ L drops of HEPES Quinn's Advantage medium (QAM, Cook) and then placed individually in wells (Reproplate, Kitazato, Biopharma, Japan) containing 300 μ L QAMTM and covered with mineral oil (Lite oil, Life Global, Belgium) at room temperature (25 °C). They were then cooled to 4 °C by directly transferring the plate to a laboratory refrigerator at 4.0 $\pm$ 1.5 °C where it was left for 24 or 48 h.

Immediately prior to returning the embryos to normal culture conditions (37 °C), the cooled plates were left for 10 min at room temperature and then washed three times in fresh CCMTM. Subsequently, the embryos were transferred to 50 μ L CCMTM drops covered with mineral oil for further culture at 37 °C in 5 % CO₂ in order to assess their ability to progress to the blastocyst stage. This further culture period lasted 3 days. Alternatively, after adaptation to room temperature, the 4 °C-stored zona-free blastocysts were transferred to Embryoscope slides (Embryos Slide® Unisense Fertilitect A/S, Aarhus, Denmark, EU) containing 12 individual microwells filled with 25 μ L CCMTM with an overlay of 1–4 mL mineral oil. Blastocysts were then cultured for 1 day at 37 °C in 5 % CO₂ air in an EmbryoScope incubator (Unisense FertiliTech A/S, Aarhus, Denmark, EU) equipped with a monochromatic Leica camera (1280 $\times$ 1024 pixels; 200x magnification) which recorded individual digital images every 15 to 20 min in 7 focal

Table 1 Survival and subsequent in vitro developmental ability to progress to the blastocyst stage of cleavage-stage tripronucleated embryos after 4 °C storage for different time periods (0 h, 24 h and 48 h)

	0 h	24 h	48 h
Initial number of embryos stored	32	19	24
Survival rate (n, %)	32 (100)	19 (100)	22 (91.7)
Blastocyst rate (n, %)	20 (62.5) ^a	13 (68.4) ^a	5 (20.8) ^b

^{a,b} Different superscripts within a row indicate $p < 0.05$

planes. Screening of these recordings allowed us to assess blastocyst re-expansion as an indicator of blastocyst viability.

The control group (0 h) was composed of thirty-two cleavage-stage embryos and 14 shrunken zona-free blastocysts, which were washed with storage medium and transferred to CCMTM for subsequent culture for 3 or 1 additional days in order to assess their progression to the blastocyst stage or blastocyst re-expansion, respectively.

To determine the influence of the time storage on survival and developmental ability of each developmental stage, data were analyzed by Chi-square tests. Differences were considered to be statistically significant when the p -value was lower than 0.05. All statistical analyses were performed using SPSS 17.0.

Results

One hundred and seventy-five of the 386 TPN zygotes developed to the >6-cell stage (45.3 %, cell-stage rate). Seventy-five were assigned to one of the three storage periods at the celled stage whereas the remaining TPN embryos ($n=100$) were subsequently in vitro cultured to the blastocyst stage; finally rendering 62 blastocysts that were used for the storage studies at such a stage.

The survival rate of the 75 stored celled embryos was comparable regardless of time during which they were stored (0–48 h: average 97.3 %; $p=0.113$). However, the capacity of embryos to progress in vitro to the blastocyst stage was significantly impaired when storage lasted 48 h (20.8 %) rather than 0 h or 24 h (average 64.7 %; $p < 0.05$; Table 1).

Table 2 Survival and subsequent in vitro developmental ability to blastocyst re-expansion of hatched, day 6 tripronucleated-derived blastocysts after 4 °C storage for different time periods (0 h, 24 h and 48 h)

	0 h	24 h	48 h
Initial number of hatched blastocysts stored	15	27	20
Survival rate (n, %)	14 (93.3) ^a	22 (81.5) a	4 (20.0) ^b
Blastocyst re-expansion rate (n, %)	10 (66.7) ^a	14 (51.8) a	3 (15.0) ^b

^{a,b} Different superscripts within a row indicate $p < 0.05$

On the other hand, 62 blastocysts of the 220 TPN zygotes (28.2 % blastocyst rate) and were assigned to one of the three aforementioned storage groups. Almost all blastocysts stored for 24 h survived, as did control blastocysts (average: 85.7 %), but survival rates were impaired when the storage period was extended to 48 h (20.0 %; $p < 0.05$). Similarly, the re-expansion rate of 24 h-stored blastocysts was statistically similar to that of controls (average: 57.1 %; $p > 0.05$), but higher than that observed for blastocysts stored for 48 h (15.0 %; $p < 0.05$; Table 2).

Discussion

This is the first study to show that TPN human cleavage embryos and blastocysts can be successfully stored short-term for up to 24 h at 4 °C without using cryoprotectants. We did not observe any significant negative impact on survival (100 % and 81.5 %, respectively) or subsequent in vitro development (68.4 % and 51.8 %, respectively); outcomes which were comparable to those in non-stored embryos (average of 62.5 % and 66.7 %, respectively). However, the in vitro capacity of cleavage stage embryos and blastocysts to develop further was impaired when the storage period was extended to 48 h (20.8 % and 15.0 %, respectively).

These results are in line with earlier reports of 24-h storage of different mammalian embryos at temperatures between 0 °C and 4 °C in the absence of cryoprotective or antifreezing proteins, in which survival rates and in vitro [3, 30, 35, 38, 44, 45] development were comparable with those of non-stored embryos.

To our knowledge, the only previous study of the effects of short-term embryo storage on development and molecular processes is that by Sakurai et al. [38] who reported that 12-h developmental arrest of fertilized murine zygotes at 4 °C resulted in the temporary cessation of developmental stages (pronuclear movement and cleavage of fertilized oocytes) and of molecular processes (DNA replication, cytoplasmic polyadenylation of maternal mRNAs, mRNA degradation of cell cycle-related genes and initiation of zygotic gene activation (ZGA), and elevated mRNA expression of ZGA-related genes), though no impact on the subsequent in vitro and in vivo development of the embryos was detected.

Although embryos can be stored at hypothermic temperatures for longer periods of time than at 37 °C, their viability can still be compromised [3]. In fact, the slowing down of metabolic rates in order to store embryos can be detrimental if exposure to hypothermic conditions is prolonged, as this can cause a rise in the total number of dead cells [32, 34, 35]. Indeed, lower survival rates and an undermined subsequent embryo viability has been reported when the cold storage period is extended to 48 h (this work; [1–4, 16, 30, 35, 38]) or longer [3, 16, 34, 44, 45]. However, such disruption may also depend on the species in question, embryo stage sensitivity to cold shock [16, 44, 45], the medium employed for storage [16, 32, 36, 44], the absence/presence of antifreezing supplements [2, 3, 34], or storage

temperature [3, 4]. In this sense, modifications to the refrigeration media or storage conditions can allow the 4 °C-storage period to be extended without compromising subsequent developmental capacity.

Despite conclusions obtained with TPN human embryos, further studies need to be followed-up with those normally fertilized, in order to evaluate and validate the short-term storage at 4 °C. The need to perform PGD/PGS on embryos and blastocysts necessitates their storage while results are pending. Therefore, if demonstrated that there is no discernible effect on embryo survival and developmental ability, it can be a very interesting tool to give more time for establishing the diagnosis (especially when a second analysis is required) without subjecting the embryo to cryopreservation. Moreover, delaying the embryo transfer one more day has no detrimental effect on the embryo-endometrium synchrony [14].

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