Slow freezing and vitrification of mouse morula and early blastocysts

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Abstract

Purpose To assess the relative success of morula and early blastocyst slow freezing and vitrification in regards to survival and implantation rates utilising protocols which could be clinically implemented as a viable alternative to expanded blastocyst stage freezing.

Methods Mouse morula and early blastocysts were either slow frozen/thawed or vitrified/warmed. Their subsequent survival, blastocyst development and blastocyst cell number and allocation to either the inner cell mass, trophectoderm or epiblast was assessed. In addition blastocysts were also transferred to pseudopregnant recipients and implantation and fetal development was determined.

Results Vitrification of both morula and early blastocysts resulted in significantly higher rates of survival and blastocyst development compared to slow freezing. In addition slow frozen early blastocysts had significantly reduced blastocyst cell number compared to control however vitrified morula and early blastocysts and slow frozen morula had equivocal blastocyst cell numbers. Transfer of blastocysts from both methods of cryopreservation resulted in similar implantation rates however the placentas created from slow frozen early blastocysts were significantly lighter than control ($95.5 \text{ g} \pm 5.4$ vs. $122.0 \text{ g} \pm 4.2$ respectively).

Conclusions Vitrification resulted in significantly higher rates of morula and early blastocyst survival and blastocyst development compared to slow freezing. In addition this study has

validated the use of a closed DMSO free vitrification protocol which could then be investigated for use in the clinical setting as an alternative to expanded blastocyst freezing.

Keywords Vitrification · Slow freezing · Morula · Early blastocyst · Implantation · Closed system vitrification · DMSO free · Fetal development · Epiblast

Introduction

Improvements in embryo culture techniques that enable extended culture to the morula (day 4) and blastocyst stage (day 5 and 6) have resulted in increased pregnancy rates after IVF [1–3]. As a result there has been a shift towards single embryo transfer and therefore, to maximise cumulative pregnancy rates, cryopreservation of super nummery embryos needs to be optimised. Furthermore with the emerging evidence that pregnancy and offspring outcomes are improved in a natural cycle following the transfer of a cryopreserved embryo, there is a renewed interest in the ability to cryopreserve embryos with minimal loss in viability [4–6]. However, although increased pregnancy rates are seen after blastocyst transfer, survival rates after blastocyst cryopreservation are variable, possibly due to the presence of the large fluid filled blastocoel cavity [7–9]. It is hypothesized that during the cryopreservation process, the water in this cavity may form ice thus inducing cellular damage, where the likelihood of ice crystal formation is directly proportional to volume and inversely proportional to viscosity and the cooling rate [9, 10]. It has been shown that manual collapsing of the cavity results in increased survival rates and this is often performed in clinics resulting in increased rates of survival and thus implantation; however the methods of reducing the water filled cavity can be invasive and may induce other forms of damage to the embryo such as changes to imprinting [9, 11].

Capsule Vitrification of morula/early blastocysts results in increased survival rates compared to slow freezing.

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An alternative to manipulating the blastocoel cavity is to cryopreserve embryos at the morula/early blastocyst stage. This would avoid many of the issues associated with dehydration of water in the blastocoel cavity. In the literature day 4 transfer of morula/early blastocysts has been shown to maintain the benefits of blastocyst culture, i.e. culture past embryonic genome activation, resulting in increased embryo selection and thus producing pregnancy rates equivalent to day 5 expanded blastocyst, however has received little attention in regards to cryopreservation [12]. The aim, therefore, of this study was to identify the optimum cryopreservation technique for morula and early blastocyst stage embryos that yields the highest survival and ultimately implantation rate, which could be implemented clinically as a viable alternative to expanded blastocyst stage freezing. The efficacy of slow freezing and vitrification on mouse morula and early blastocyst embryos was assessed and survival rates, blastocyst development and pregnancy outcomes after transfer were determined.

Materials and methods

Embryo collection and culture media

The media used for embryo culture was the G-1v3 and G-2v3 sequential culture system (Vitrolife, Kungsbacka, Sweden) and G-MOPS (Vitrolife) was used as a collection and handling medium. All culture media was supplemented with 5 mg/ml HSA (human serum albumin) and culture media drops were overlaid with Ovoil (Vitrolife). All plastic-ware consumables were pre-screened for their ability to support embryo development using a 1-cell mouse bioassay [13].

Embryo collection and culture

Zygotes were collected from superovulated prepubescent F1 female mice (C57Bl6 × CBA), following an intra-peritoneal injection with 5 IU of eCG (Folligon, Intervet, Bendigo, Victoria, Australia), and 5 IU hCG (human Chorionic Gonadotropin, Pregnyl, Organon, Oss, Holland) 48 h later. Immediately following the hCG injection females were placed with F1 male mice. Mating was determined the following morning by the presence of a vaginal plug. At 23 h post-hCG, zygotes were collected into G-MOPS medium and denuded following incubation with 1 mg/ml hyaluronidase (Sigma Aldrich, St Louis, MO). Putative zygotes were washed twice in G-MOPS and once in G-1v3, before being placed into culture. Embryos were cultured in groups of 10 in 20 µl drops of medium, under Ovoil. All embryos were cultured for 43 h in G-1v3 at 37 °C in 6%CO₂:5%O₂:89%N₂ and then washed in G-2v3 and cultured in G-2v3, for a further 49 h, to the blastocyst stage. All embryo culture dishes were prepared 4 h prior to embryo

culture so as to allow gas and temperature equilibration. All procedures were conducted in accordance with the National Research Council guide for the care and use of laboratory animals [14] and ethics was obtained from the Institute of Medical and Veterinary Science (IMVS) animal ethics committee (Northern Adelaide).

Assessment of morphology

Embryo morphology was assessed at 51 h (late day3) and 66 h (early day4) of culture. Embryos were classified as on-time if they displayed the following morphology: at 51 h of culture; morula (fully compacted embryo) and at 66 h culture; early blastocyst (early cavity formation, ≤ half of the embryo volume). After cryopreservation and thawing/warming, morula embryos were cultured for a further 41 h and early blastocysts for 26 h (total of 92 h culture) before being assessed for development to the expanded blastocyst or hatching blastocyst (trophectoderm is clearly herniating through the zona pellucida). Any embryo displaying morphology that was not considered on-time development was classified as delayed and embryos which did not survive cryopreservation were classified as degenerate.

Slow freezing and thawing

The slow freezing technique was based on a previously published method for day 4 embryos and is described below [15, 16] with two modifications (base medium used and the storage vessel). The base medium for freezing and thawing media was G-MOPS supplemented with 10 mg/ml HSA (G-MOPS+10 %). Initially embryos were placed into G-MOPS+10 % in a Nunc 4-well dish for 5 min at room temperature. Embryos were then moved sequentially for 5 min each through freezing solutions containing 0.8 M propandiol and 0.05 M sucrose, 1.0 M propandiol and 0.075 M sucrose and finally 1.5 M propandiol and 0.1 M sucrose. In the final well, up to 5 embryos were loaded into sterile 0.25 ml freezing straws (Minitub, Germany). The straws were heat sealed at both ends and then placed into a programmable freezer set to initiate at 20 °C (Cryobath CL3300; Cryologic, Mulgrave, VIC). Cooling started at a rate of −2 °C/minute. At −7 °C ice nucleation was induced by touching the meniscus at the top of the column holding the embryos with forceps that had been cooled in liquid nitrogen (LN₂). The straws were held at −7 °C for 10 min and then were cooled at a rate of −0.3 °C/minute to −30 °C before free falling at a cooling rate of 50 °C/minute^{−1}. Embryos were then plunged into LN₂ when the temperature had reached −120 °C.

After holding at −196 °C for a minimum of 30 min, embryos were thawed at room temperature for 30 s followed by 30 s in a 30 °C water bath. The embryos were then expelled from the straw and incubated for 3 min in 1 M propandiol and

0.2 M sucrose and moved sequentially through 0.5 M propandiol and 0.2 M sucrose, 0.15 M sucrose, 0.075 M sucrose each for 3 min. Embryos were then moved to G-MOPS+10 % where the dish was warmed to 37 °C; the embryos removed, washed 3× through G-2v3 and then placed back into culture.

Vitrification and warming

All vitrification and warming took place at 37 °C. Vitrification was performed using solutions containing propandiol, sucrose, ethylene glycol and Ficoll. Initially embryos were placed into G-MOPS+10 % HSA for 5 min and then moved to an equilibration solution for 2 min (G-MOPS+10 % with 8 % propandiol and 8 % ethylene glycol). The embryos were then placed in a 20 µl drop of vitrification solution (G-MOPS+10 % with 16 % propandiol and 16 % ethylene glycol, 0.65 M sucrose, 10 mg/ml Ficoll) pipetted up and down at least 3 times and then loaded onto the inner hemistraw of a High Security Vitrification Straw (Cryo Bio Systems, Paris, France) in approximately 300 nl of fluid (with a maximum of 5 embryos/straw). The straw was then placed into its outer straw, sealed and plunged into LN₂. The process from immersion into Vitrification solution to plunging into LN₂ was between 30 and 45 s.

After plunging, the embryos were warmed after 2 h (to adjust for the time taken for slow freezing and thawing to be performed). Warming was performed at 37 °C using solutions containing decreasing concentrations of sucrose. While in LN₂ the straws were cut and the inner straw was immediately removed and the end immersed into G-MOPS+10 % and 0.65 M sucrose, removed within 30 s then placed into G-MOPS+10 % and 0.325 M sucrose for 1 min, G-MOPS+10 % and 0.125 M sucrose for 2 min and then into G-MOPS+10 % for 5 min. The embryos were then washed 3 times through G-2v3 and placed back into culture.

Differential staining

Allocation of cells in the blastocyst to the inner cell mass (ICM) or trophectoderm (TE) was assessed at 92 h of culture using a differential staining protocol [17, 18]. All chemicals were purchased from Sigma unless otherwise stated. Briefly, after 92 h of culture blastocysts were placed in 0.5 % pronase to dissolve the zona. After this blastocysts were incubated in 10 mM TNBS (2,4,6-Trinitrobenzenesulfonic acid) at 4 °C and then transferred to 0.1 mg/ml α-DNP BSA (anti-dinitrophenyl-BSA,) at 37 °C and placed in guinea pig serum (IMVS, Adelaide, SA) with 10 µg/ml propidium iodide at 37 °C. Blastocysts were then stained with 6 µg/ml bisbenzimidazole in 100 % ethanol overnight. The following day the embryos were washed in 100 % ethanol, mounted in a glycerol drop on a siliconised slide and viewed on

a fluorescent microscope at 400×, under a UV filter where the ICM nuclei appeared blue and TE nuclei stained pink.

Epiblast staining

Allocation of cells in the blastocyst to the inner cell mass (ICM) and the epiblast was assessed at 116 h of culture using immunohistochemistry as previously described [19]. All chemicals were purchased from Sigma unless otherwise stated. Briefly after 116 h of culture, blastocysts were fixed in 4 % paraformaldehyde at 4 °C overnight. Blastocysts were then neutralised in 0.1 M glycine/PBS, permeabilised using 0.25 % TritonX-100/PBS (Tx-PBS) and then blocked with 10 % Normal Donkey Serum/PBS (Sapphire Biosciences, Redfern, NSW, Australia). Blastocysts were then washed through Tx-PBS and incubated in 1° antibody mixture containing Nanog rabbit anti-mouse polyclonal antibody (Sapphire Biosciences Cat#120-21603) at 1:200 and Oct 3 / 4 (N19) goat anti-mouse polyclonal antibody (Santa Cruz Biotechnology inc, Santa Cruz, CA; sc-8628) at 1:100 in PBS. Blastocysts were washed in Tx-PBS and then incubated in 2° antibody mixture containing donkey anti-rabbit (fluorescein isothiocyanate, Australian Laboratory Services, Homebush NSW, Australia) and donkey anti-goat (Rhodamine Red, Jackson ImmunoResearch, West Grove, PA; 705-025-003) at 1:100. Finally blastocysts were washed twice in Tx-PBS, incubated in the nuclear stain 6 µg/ml bisbenzimidazole in PBS and then imaged using fluorescence microscopy at 400× where Nanog positive cells were counted using the FITC (fluorescein isothiocyanate) channel (518 nm emission), Oct4 was counted using the TRITC (tetramethylrhodamine-5-(and 6)-isothiocyanate) channel (546 nm emission) and nuclear stain was counted using UV (358 nm emission). A negative control was performed with each replicate omitting the primary antibody.

Embryo transfers

Female Swiss mice aged between 8 and 12 weeks were mated to vasectomised males, and embryos were transferred on the morning of day 4 of pseudo-pregnancy. Females were anaesthetised with 2 % Avertin (0.015 ml/g body weight, Sigma). Six blastocysts were then transferred to each uterine horn with either vitrification or slow freezing in one horn and the matched control in the contralateral horn. On day 18 of pregnancy, the number of implantations and fetuses were recorded as was fetal and placental weights and crown–rump length. Different treatment groups were transferred into the same mother to control for mother as a covariate with studies demonstrating that in the mouse embryo uterine transmigration does not occur due to the separation caused by a media septum [20].

Statistical analysis

For binomial data, each replicate was expressed as a proportion for analysis. Embryo survival, blastocyst development, the number of blastocysts with no epiblast and implantation were assessed using Fishers exact test (Graphpad software, La Jolla, CA). Blastocyst total cell number and ICM (for both differential and epiblast staining) and TE (for differential staining) were not normally distributed and were therefore analysed using Kruskal-Wallis with Dunn's post hoc. The %ICM/total (differential staining) as well as epiblast cell number and epiblast percentage were normally distributed so were analysed using one way ANOVA with Tukey's post hoc tests and fetal and placental data were analysed using univariate generalised linear modelling followed by LSD post hoc (SPSS 15.0). For fetal and placental data mouse number, implantations per horn, fetuses per horn, implantations per mother and fetuses per mother were treated as covariates. Significance for all experiments was set at $P<0.05$.

Results

Effect of vitrification or slow freezing of morula/early blastocysts on blastocyst development

Embryos were cultured from the zygote until the morula stage (late day3–51 h of culture) and early blastocyst stage (early day4–66 h of culture) where morula was defined as a completely compacted embryo lacking any other morphological anomalies such as vacuoles or excluded cells and an early blastocyst was classified as an embryo with a cavity $\leq$ half of the volume of the embryo also lacking in any morphological anomalies. The morula and early blastocysts were then allocated randomly to control, vitrification and slow freezing treatment groups.

For both vitrification and slow freezing, a ≥ 93 % recovery rate was obtained. The rate of embryo degeneration (as defined by >70 % of the cells within the embryo being degenerate) after

vitrification was 2.2 % for morula and 5.4 % for early blastocyst which was significantly lower than slow freezing (17.0 % and 29.5 % respectively, Table 1; $P<0.01$). Total blastocyst development was significantly higher for control and vitrified embryos compared to slow freezing at both stages of cryopreservation (Table 1; $P<0.01$). In addition vitrified morula and early blastocysts had significantly increased rates of hatching blastocysts compared to slow frozen however all treatment groups had lower rates of blastocyst hatching compared to control and hatching rates were significantly higher in the cryopreserved (both vitrification and slow freezing) morula group compared to cryopreserved early blastocysts (Table 1; $P<0.01$).

Effect of vitrification or slow freezing of morula/early blastocysts on subsequent blastocyst cell number and allocation after 92 h of culture

There was a significant reduction in total cell number after cryopreservation of early blastocyst by slow freezing when compared to control; in comparison vitrification and slow freezing of morula and vitrification of early blastocysts did not alter total blastocyst cell number (Table 2; $P<0.01$). In addition vitrified morula also had significantly increased inner cell mass (ICM) cell number compared to slow frozen and vitrified early blastocysts and the reduction in total blastocyst cell number after early blastocyst slow freezing was due to decreased ICM cell number (Table 2; $P<0.01$).

Effect of vitrification or slow freezing of morula/early blastocyst on epiblast development after 116 h of culture

To further investigate the impact of cryopreservation on the ICM, epiblast staining was performed after 116 h of culture to confirm the presence of pluripotent epiblast cells. Slow freezing of either morula or early blastocyst stage embryos decreased epiblast cell number compared to control however vitrification of morula or early blast did not alter epiblast cell number (Fig. 1(b); $P<0.05$). In addition when the number of epiblast cells was

Table 1 The effect of morula and early blastocyst cryopreservation on day 5 blastocyst development. Data expressed as the proportion of embryos cryopreserved after 20 h of culture. $n\geq 103$ embryos per treatment (≥ 5 replicate experiments) group a–e: Different superscripts

Treatment	% degenerate	% delayed	% $\geq$ expanded blastocyst	% hatching blastocyst
Control	1.9 ^a	1.1	97.0 ^a	65.2 ^a
Vitrification CM	2.2 ^a	2.8	95.0 ^a	56.8 ^b
Slow freezing CM	17.0 ^b	3.5	79.5 ^b	39.3 ^c
Vitrification EB	5.4 ^a	1.3	93.3 ^a	27.4 ^d
Slow freezing EB	29.5 ^c	1.9	68.6 ^c	17.7 ^e

within a column are significantly different ($P<0.01$) CM: compact morula, EB: early blastocyst, delayed embryos are all embryos $<$ expanded blastocyst however not degenerate

Table 2 The effect of morula and early blastocyst cryopreservation on day 5 blastocyst cell number and allocation. Data expressed as mean $\pm$ SEM. $n \geq 19$ embryos per treatment (3 replicate experiments) a–d:Different superscripts within a column are significantly different ($P < 0.01$) ICM: inner cell mass, TE: trophectoderm CM: compact morula, EB: early blastocyst

Treatment	Total	ICM	TE	% ICM/total
Control	73.9 $\pm$ 2.8 ^a	16.1 $\pm$ 0.7 ^{a,b}	54.9 $\pm$ 2.2	23.1 $\pm$ 0.7 ^{a,c,d}
Vitrification CM	61.3 $\pm$ 2.2 ^{a,b}	16.2 $\pm$ 0.4 ^a	45.0 $\pm$ 2.0	27.0 $\pm$ 0.8 ^b
Slow freezing CM	60.6 $\pm$ 2.9 ^{a,b}	15.4 $\pm$ 0.7 ^{a,b}	45.2 $\pm$ 2.4	25.7 $\pm$ 0.8 ^{a,b,c}
Vitrification EB	61.7 $\pm$ 3.6 ^{a,b}	13.3 $\pm$ 1.1 ^{b,c}	48.4 $\pm$ 2.8	21.6 $\pm$ 1.1 ^{c,d}
Slow freezing EB	57.4 $\pm$ 3.2 ^b	11.8 $\pm$ 0.8 ^c	45.6 $\pm$ 2.7	20.8 $\pm$ 0.9 ^d

assessed as a proportion of inner cell mass cells, slow frozen morula had a significantly reduced epiblast percentage compared to control blastocysts (Fig. 1(c); $P < 0.01$). Although blastocysts were evident in every group that were negative for Nanog staining, indicative of no epiblast, there were significantly more blastocysts lacking Nanog staining in the slow frozen morula group compared to vitrified early blastocysts and control (Fig. 1(d), $P < 0.05$).

Effect of vitrification or slow freezing of morula/early blastocyst on viability

To further assess the effects of cryopreservation on viability, expanded blastocysts were then transferred to pseudo-pregnant recipients and implantation and fetal and placental development was assessed on day 18. Vitrification/warming and slow freezing/thawing of both morula and early blastocysts did not significantly affect implantation of the resulting

Fig. 1 Day 6 blastocyst and epiblast cell number after cryopreservation of either compact morula or early blastocysts (a) Total cell number as shown by 4'-6-diamidino-2-phenylindole (DAPI) staining (b) Epiblast cell number as shown by Oct4 and Nanog staining (c) Percentage of ICM cells (Oct 4 staining only) that are epiblast cells (d) Percentage of

blastocysts that did not contain any Nanog positive cells. Data is expressed as mean $\pm$ SEM $n \geq 14$ embryos per treatment group (3 replicates) a–c: Different superscripts significantly different ($P < 0.05$) # indicates trending significance compared to control ($P = 0.07$) CM: compact morula, EB: early blastocyst

Fig. 2 Implantation and fetal development of day 5 blastocysts after transfer to pseudopregnant recipients for control, vitrified/warmed and slow frozen/thawed compact morula early blastocysts. **a** Percentage implantation/embryo transferred **b** Percentage fetal development per

successful implantation, n (embryos transferred/treatment) ≥ 24 blastocysts. **a–b**: Different superscripts indicate significantly different results ($P < 0.05$) CM: compact morula EB: early blastocyst

expanded blastocysts (Fig. 2(a)). However slow freezing of embryos at the morula stage did result in a significantly reduced fetal number per implantation (increased resorptions) when compared to vitrified morula or control embryos (Fig. 2(b); $P < 0.05$) likely reflective of an increased number of blastocysts without an epiblast.

Vitrification and slow freezing of both morula and early blastocysts did not significantly alter resultant fetal weight or fetal:placental weight ratio however slow freezing of early blastocysts resulted in significantly lighter placenta than both control and vitrified early blastocysts and the crown rump length of these fetuses were smaller (Table 3, $P < 0.05$). The placentas from vitrified and slow frozen morula were unchanged compared to control.

Discussion

Extended culture is increasingly becoming standard practice to aid in selection of the most viable embryo within a cohort and therefore embryo cryopreservation technologies for later stage embryos requires further attention and refinement. The

results in this study have demonstrated that for morula and early blastocysts, survival after vitrification is significantly higher than that seen after slow freezing which is in agreement with meta-analysis concluding that vitrification at both the cleavage and expanded blastocyst stage is associated with significantly higher survival compared to slow freezing [21]. In further support of this, studies have also demonstrated increased metabolism in human day 3 embryos following vitrification compared to slow freezing, possibly indicative of decreased cellular trauma on the embryo [22] and slow frozen re-expanded blastocysts have significantly increased changes in global gene expression compared to vitrified and control blastocysts [23].

In this study it was demonstrated that slow freezing impacts on blastocyst differentiation with slow frozen early blastocysts having a significant reduction in inner cell mass (ICM) cells on day 5 and epiblast cells on day 6 compared to control. In comparison, vitrified morula and early blastocysts had comparable total cell number, cell differentiation and epiblast cell number to control. Epiblast cell number reflects the cell lineage that will contribute to the fetus while the remaining inner cell mass cells (primitive endoderm) and the trophoblast

Table 3 The effect of morula and early blastocyst cryopreservation on day 18 fetal and placental development. Data expressed as mean $\pm$ SEM. $n \geq 7$ fetal/placental pairs/treatment a–b: Different superscripts

Treatment	Fetal weight (mg)	Placental weight (mg)	Crown rump length (mm)	Fetal: Placental Ratio
Control	861.5 $\pm$ 32.4	122.0 $\pm$ 4.2 ^a	19.6 $\pm$ 0.3 ^a	7.2 $\pm$ 0.3
Vitrification CM	925.9 $\pm$ 36.5	127.1 $\pm$ 6.1 ^a	20.1 $\pm$ 0.5 ^a	7.4 $\pm$ 0.3
Slow freezing CM	844.1 $\pm$ 40.2	136.4 $\pm$ 17.5 ^a	19.1 $\pm$ 0.5 ^a	6.6 $\pm$ 0.9
Vitrification EB	782.4 $\pm$ 50.6	123.4 $\pm$ 10.9 ^a	19.1 $\pm$ 0.6 ^a	6.6 $\pm$ 0.7
Slow freezing EB	775.0 $\pm$ 29.8	95.5 $\pm$ 5.4 ^b	17.9 $\pm$ 0.5 ^b	8.3 $\pm$ 0.6

within a column are significantly different ($P < 0.01$) CM: compact morula, EB: early blastocyst

will form extra embryonic tissue [24]. Previous studies have demonstrated that a reduction in inner cell mass number correlates to decreased fetal development [18, 25–27] and although there are no current studies which have correlated epiblast number to pregnancy outcome, as the epiblast is the sole contributing cell lineage to the fetus it would be reasonable to assume that a significant reduction in epiblast cell number may result in a decrease in fetal development. Interestingly this was seen blastocysts derived from slow frozen morula which had reduced fetal development per implantation indicative of a higher number of resorption's, possibly due to the number of blastocysts that had no distinguishable epiblast. In addition we have previously validated and published this finding utilising in-vivo blastocysts all of which were positive for Nanog staining leading to the conclusion that blastocysts obtained from this experiment that had zero Nanog-positive cells is not a technical artefact [28].

In this study when cryopreserved embryos were cultured to the expanded blastocyst stage and transferred, implantation rates were equivalent for both slow freezing and vitrification regardless of stage suggesting that once an embryo has survived cryopreservation and resumed development (as determined after overnight culture), implantation rates are not impaired however slow frozen early blastocysts did produce significantly smaller placentas than all other treatment groups. The reason for this is not easily defined as placental growth can be influenced by a variety of factors although, interestingly as fetal weight was similar it may indicate altered placental efficiency, however this remains to be investigated. Alternatively the reduced total cell numbers measured in the slow freezing group correlate with this observation and thus cannot be ruled out influencing this outcome.

Cryopreservation of morula and early blastocysts has undergone limited investigation, primarily using animal models. Studies have determined that slow freezing of both mouse morula and early blastocyst results in increased survival compared to the more advanced embryo stages (blastocysts and expanding blastocysts) possibly due to the presence of water in the blastocolic cavity [29]. This is supported by human morula and early blastocysts having improved survival rates compared to fully expanded blastocysts after vitrification, however, when the cavity of expanded blastocysts underwent artificial shrinkage, survival rates were equivocal [9]. A further study demonstrated vitrification of early blastocysts resulted in improved survival compared to artificially collapsed expanded blastocysts while implantation and pregnancy rates were the same [30]. Overall the conclusion from these studies is that the morula/early blastocyst may be the best stage for cryopreservation as the embryo is better equipped to counteract osmotic changes, while the lack of the large fluid filled cavity circumvents the need to extended periods of exposure to cryoprotectants or artificial cavity collapse.

Interestingly this study showed differences between morula and early blastocyst cryopreservation with embryos that were vitrified at the morula stage having significantly higher hatching rates compared to vitrified early blastocysts. In addition slow frozen morula had significantly higher survival rates and increased inner cell mass cell numbers on day5 compared to slow frozen early blastocysts and fetal and placental parameters more similar to that of control. Together this data supports the hypothesis that the presence of a fluid filled cavity (however small) may impair successful cryopreservation.

Although this study demonstrated improved survival and blastocyst development with vitrification, it must be acknowledged that a potential limitation of this study is the use of a single protocol for slow freezing (which to date has the most successful outcome in a clinical human IVF program) [15]. Further optimisation of this current slow freezing protocol may improve outcomes such as those seen in a study by Edgar et al. where survival rates of slow frozen embryos for human cleavage stage embryos were improved by increasing the concentration of sucrose during the dehydration process [31].

One important aspect of this study was to assess cryopreservation systems that could be used in a clinical setting. The high security closed vitrification system used in this study was selected due to the additional clinical benefit of reduced cross contamination risk whilst in storage compared to open systems. The CBS closed vitrification carrier has been successfully implemented clinically and due to evidence of cross-contamination of embryos within liquid nitrogen cryobanks, closed system vitrification will likely become standard due to more stringent risk management for long term storage of embryos [32, 33]. Another additional important aspect of this study was the absence of dimethyl sulfoxide (DMSO) in the vitrification solutions via substitution with propanediol. The vast majority of embryo and oocyte vitrification reports to date have used DMSO and ethylene glycol, with comparable survival rates, however studies have demonstrated that DMSO can significantly alter cellular homeostasis and DNA imprinting in other cell types [34, 35] and vitrification using 1,2 Propanediol as compared to DMSO resulted in a gene expression profile closest to that of non-cryopreserved controls [23]. Vitrification on human cleavage stage embryos has been successfully employed using a DMSO free system and as with closed system vitrification, DMSO free vitrification is likely to become standard clinical practice due to safety concerns [22]. However that being said, vitrification of morula and early blastocysts could also be performed effectively using DMSO as a substitute for 1,2 Propanediol as a penetrating cryoprotectant due to the high survival and pregnancy rates achieved for blastocyst stages embryos.

In conclusion this study has investigated the relative success of morula and early blastocyst slow freezing and vitrification using protocols that could be implemented in a clinical

setting. The results from this study have demonstrated that vitrification of both morula and early blastocysts is superior to slow freezing in regards to survival and blastocyst development however whether this stage of vitrification results in increased survival and pregnancy rates compared to expanded blastocyst vitrification remains to be thoroughly investigated.

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