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## The fate of frozen human embryos when transferred either on the day of thawing or after overnight culture

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### ABSTRACT

**Objective:** To study the performance of thawed zygotes and cleavage stage embryos transferred either on the day of thaw or after overnight culture. **Methods:** A retrospective study of 864 frozen embryo transfer cycles. Cryosurvival rates per thawed embryo and implantation rates were analysed for embryos frozen on Day 1, Day 2 or Day 3 relative to oocyte collection (Day 0) and transferred on the day of thaw or after overnight culture, together with clinical pregnancy rates and prevalence of multiple gestations. **Results:** Survival of Day 3 embryos was significantly lower than those frozen on Day 1 ( $P=0.017$ ) or Day 2 ( $P=0.015$ ). Following overnight culture, resumption of mitosis of zygotes was more frequent than Day 2 ( $P=0.000$ ) which are in turn higher than Day 3 ( $P=0.000$ ) embryos. The implantation rate for Day 2 embryos dividing overnight was significantly higher than those that did not divide for women  $<35$  yrs ( $P=0.001$ ) but not those women  $\geq 35$  yrs ( $P=0.055$ ). There were no differences in the implantation rates for those dividing or not after culture, for embryos frozen on Day 3 for women  $<35$  yrs ( $P=0.254$ ) or  $\geq 35$  yrs ( $P=0.403$ ). **Conclusions:** Later cleavage stage post-thaw embryos survive and resume mitosis less frequently compared to earlier stages. Embryos not resuming mitosis after culture overnight can implant, particularly Day 3 embryos, suggesting that they can further increase the cumulative pregnancy rate per oocyte collection and that discarding them is wasteful. Overnight culture is best used for logistical reasons rather than a strategy to improve pregnancy rates.

## 1. Introduction

The cumulative pregnancy rate per oocyte collection has been significantly improved for IVF cycles since the introduction of cryopreservation techniques to store supernumerary embryos after transfer<sup>[1]</sup>. Embryos can be cryopreserved at a range of developmental stages to suit the practice of the clinic, from zygotes through to blastocysts<sup>[2]</sup>, and cleavage stage embryos continue to be frozen in large numbers<sup>[3]</sup> in spite of the introduction of blastocyst culture<sup>[4]</sup>.

Much work has been carried out to optimise the outcome following the transfer of embryos frozen at early cleavage stages<sup>[5–13]</sup>, and it is well known that the damage sustained by early cleavage embryos during freezing and thawing clearly affects adversely their implantation potential<sup>[5,14,15]</sup>.

Furthermore, the ability of a thawed embryo to cleave prior to transfer can be used as a test of functional viability rather than just post-thaw morphology, with those resuming mitosis having a higher implantation rate<sup>[16,17]</sup>. Whilst the resumption of mitosis by an embryo may be used to predict the likelihood of implantation, the culture of thawed early cleavage embryos overnight is not straightforward and requires practical considerations including (i) embryos that do not divide overnight can still implant, albeit with an often reduced probability<sup>[18]</sup>, and so discarding them is potentially wasteful, (ii) having a selection of embryos to choose from after overnight culture may result in a surplus of surviving embryos<sup>[16]</sup> that should not be wasted, and (iii) thawing more embryos the following day should an embryo fail to divide is not ideal due to possible asynchrony of the embryo and endometrium<sup>[19]</sup>.

The current study has retrospectively assessed the fate of embryos when transferred on either the day of thaw or after overnight culture, according to the age of the woman and the stage of the embryo at the freeze. The decision when to transfer was principally made for logistical reasons

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(eg no transfers on Sunday or clinician availability) rather than in response to the embryo quality. The rate of survival, implantation per embryo transferred and clinical pregnancy rates were determined and compared to decide whether the culture overnight of thawed embryos provides any advantage in achieving pregnancies.

## 2. Materials and methods

### 2.1. Oocyte collection and embryo cryopreservation

This retrospective study included 864 consecutive frozen embryo transfer cycles at Fertility North between January 2008 and September 2011. The original cycle in which the embryos were cryopreserved had oocytes collected on Mondays, Wednesdays or Fridays, and inseminated by conventional IVF or intracytoplasmic sperm injection (ICSI); this was designated Day 0. Oocytes were then checked 16–18 hours after insemination to confirm fertilisation on Day 1. Zygotes or embryos were transferred around midday on Day 1, Day 2 or Day 3 depending on different clinical schedules. Embryo Transfers and cryopreservation therefore took place on Wednesdays, Fridays or the following Monday to avoid weekend procedures. Cryopreservation of supernumerary embryos at any stage was performed using a single protocol with one embryo per straw. Briefly, 1,2-propanediol (PROH) and sucrose were used as cryoprotectants to dehydrate embryos at 37 °C [20]. A programmable freezer (Kryo 360–1.7; Planer Products, Sunbury-on-thames, UK) was used for cooling starting at 20 °C, and initially cooling the straws down to –7 °C at a rate of –2 °C/min. Manual seeding was done at –7 °C before the temperature was held for 10 min. Thereafter, straws were cooled down to –35 °C at a rate of –0.3 °C/min. Finally, the temperature was lowered to –140 °C at –50 °C/min before the straws were removed from the freezer and plunged into liquid nitrogen.

### 2.2. Embryo transfer cycles and thawing

Embryos were transferred either in natural cycles, stimulated cycles using GnRH analogues (Lucrin, Abbott Australasia Pty Ltd, Botany NSW 2019, Australia; Synarel, Pfizer Australia Pty Ltd, West Ryde NSW 2114, Australia; Orgalutran, Merck Sharp & Dohme (Australia) Pty Limited, South Granville NSW 2142, Australia) and recombinant FSH (Puregon, Schering-Plough, North Ryde NSW 2113, Australia; Gonal-f, Merck Serono Australia Pty Ltd, Frenchs Forest NSW 2086, Australia), or hormone replacement cycles using oestradiol valerate (Bayer Australia Ltd, Pymble NSW 2073, Australia) and progesterone injections (Stenlake Pharmacy, Bondi Junction NSW 2022, Australia) and pessaries (Orion Laboratories, Balcatta WA 6021, Australia). Zygotes frozen on Day 1 were thawed 2 days after ovulation was triggered by an LH surge or hCG administration in natural or stimulated cycles, or two days after progesterone was commenced in a hormone replacement cycle. Embryos frozen on Days 2 or 3 were thawed one or two days later respectively. Embryos transferred on the same day were thawed between 8am and 10am for transfer between 11am and 2pm. Embryos for overnight culture were thawed between 12pm and 2pm for transfer between 11am and 2pm the following day. The number of cells was scored at the time of freeze and also after thaw, and embryos showing an increase in cell number were said to have resumed mitosis.

Embryos were thawed on a one-by-one basis until the number of surviving embryos desired for transfer had been reached. Straws were taken out of liquid nitrogen swiftly and exposed to room temperature for 45 s followed by 30 °C water bath for 30 s. The embryo was then released from straw into 0.5 M/mL sucrose media and kept for 10 min then 0.2 and 0 M/mL sucrose media for 10 mins each. Thereafter, the embryo was checked to confirm survival. Surviving embryos were transferred to Quinn's Advantage® Cleavage (QAC) or Blastocyst (QAB) media (SAGE, Trumbull, USA) in accordance of their age after being washed in corresponding media. Zygotes were considered to have survived the thaw if there was no obvious damage to the zona pellucida, and the cytoplasm was clear and after rehydration had re-expanded to its original volume. Surviving Day 2 and Day 3 embryos had ≥50% of their blastomeres intact. Embryos for overnight culture were placed individually in 20 µL droplets containing either QAC or QAB overlaid by oil (SAGE, Trumbull, USA) in the MINC incubators (William A. Cook Australia Pty. Ltd, Eight Mile Plains, QLD 4113, Australia) supplied with 5%CO<sub>2</sub>, 5%O<sub>2</sub> and 90%N<sub>2</sub>. Embryos were assessed between 7:30am and 8am the following day to confirm the number of cells. The laboratory is enrolled in an External Quality Assurance programme for embryos assessment (EQASRM, Northlands, Western Australia), and embryo assessment compared regularly between scientists as part of an internal quality control programme.

### 2.3. Pregnancies

Serum hCG was measured by immunoassay (Siemens Centaur CP automated analyser) 14 days after ovulation, with a concentration of >25 IU/L considered positive. However, a clinical pregnancy was confirmed by the presence of a fetal heart detected on ultrasound 5 weeks after ovulation. Clinical pregnancy rate was determined by dividing the number of fetal heart detections by total number of embryo transfers while implantation rate was calculated by dividing the total number of fetal heart beats by the total number of embryos transferred.

### 2.4. Statistical analysis

Statistical analysis by *Chi*-square test was performed using the online interactive tool [21] with Yates' correction applied if any expected frequency is below 1 or if the expected frequency is less than 5 in more than 20% of cells. Differences were considered significant if  $P < 0.05$ .

## 3. Results

### 3.1. Embryo survival and division after culture

The fate of thawed embryos is summarised in Table 1. There was no difference in the rates of embryo survival between the two groups of women according to age at the time of the freeze for embryos frozen on Day 1 ( $\chi^2=0.607$ ,  $df=1$ ,  $P=0.436$ ), Day 2 ( $\chi^2=0.454$ ,  $df=1$ ,  $P=0.500$ ) or Day 3 ( $\chi^2=3.648$ ,  $df=1$ ,  $P=0.056$ ). After combining the two age groups, as shown in Figure 1, there was a difference in survival between the three categories of embryo ( $\chi^2=9.633$ ,  $df=2$ ,  $P=0.008$ ) with Day 3 embryos being significantly lower than those frozen on Day 1 ( $\chi^2=5.751$ ,  $df=1$ ,  $P=0.017$ ) or Day 2 ( $\chi^2=5.901$ ,  $df=1$ ,  $P=0.015$ ).

**Table 1**

The overall fate of thawed embryos according to the stage at which they were frozen, and the woman's age at the time of the freeze. Survival after thawing was defined as the cell remaining healthy (Day 1) or 50% or more blastomeres being intact (Days 2 and 3), and implantation resulted in a fetal heart seen on ultrasound.

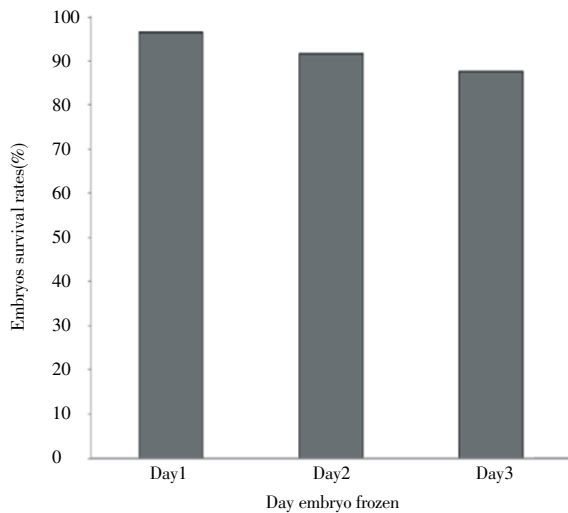
No.embryos	Day 1		Day 2		Day 3	
	<35 yrs	≥35 yrs	<35 yrs	≥35 yrs	<35 yrs	≥35 yrs
Thawed	38	47	527	478	232	228
Survived and transferred*	36 (94.7%)	46 (97.9%)	480 (91.1%)	441(92.3%)	210 (90.5%)	193 (84.7%)
Implantation rate (%)**	10 (27.8%) <sup>a</sup>	4 (8.7%) <sup>a</sup>	111 (23.1%) <sup>b</sup>	68 (15.4%) <sup>b</sup>	51 (24.3%) <sup>c</sup>	27 (14.0%) <sup>c</sup>

\*As a proportion of embryos thawed. All embryos surviving were transferred, either with or without overnight culture.

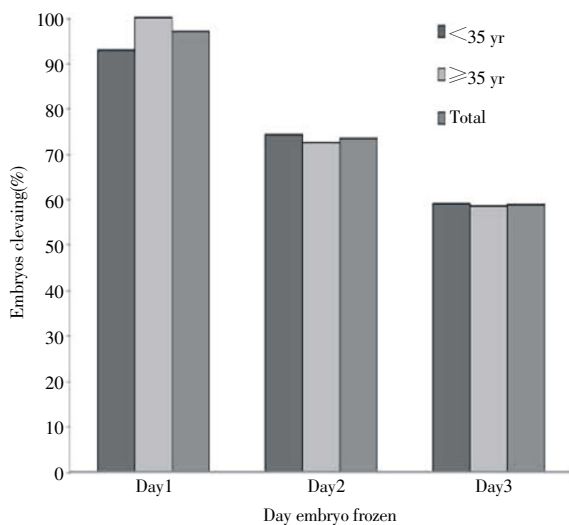
\*\*As a proportion of those transferred.

Proportions with the same superscript are significantly different from each other (all  $P < 0.05$ ).

The proportion of embryos cleaving after overnight culture, as judged by an increase in the cell number, is shown in Figure 2. There were no differences between the women <35yrs and ≥35yrs for the embryos frozen on Day 1 ( $\chi^2=0.896$ ,  $df=1$ ,  $P=0.344$ ), Day 2 ( $\chi^2=0.328$ ,  $df=1$ ,  $P=0.567$ ), or Day 3 ( $\chi^2=0.009$ ,  $df=1$ ,  $P=0.924$ ), and the results for both age groups were then combined for each day of freezing. There was an overall difference in the proportion of embryos dividing overnight ( $\chi^2=215.687$ ,  $df=2$ ,  $P=0.000$ ), with those on Day 1 being greater than Day 2 ( $\chi^2=18.163$ ,  $df=1$ ,  $P=0.000$ ) which are in turn greater than Day 3 ( $\chi^2=177.558$ ,  $df=1$ ,  $P=0.000$ ).



**Figure 1.** Survival rates of thawed embryos according to the stage at which they were frozen.



**Figure 2.** The proportion of embryos that increased their cell number after culture overnight. Results were expressed according to the woman's age and the day of the cycle at the time of the freeze.

### 3.2. Embryo implantation

Table 1 shows there were significant differences in the implantation rates of embryos following transfer between the women <35 yrs and ≥35 yrs for embryos frozen on Day 1 ( $\chi^2=5.194$ ,  $df=1$ ,  $P=0.023$ ), Day 2 ( $\chi^2=8.715$ ,  $df=1$ ,  $P=0.003$ ) and Day 3 ( $\chi^2=6.830$ ,  $df=1$ ,  $P=0.009$ ). Considering only those embryos cultured overnight, shown in Table 2, only two Day 1 embryos that had not divided overnight were transferred and they did not implant. Day 2 embryos for the women <35 yrs old implanted less frequently if they did not divide overnight ( $\chi^2=10.857$ ,  $df=1$ ,  $P=0.001$ ), but for ≥35yr olds the trend did not reach significance ( $\chi^2=3.674$ ,  $df=1$ ,  $P=0.055$ ). There were no significant differences in implantation rates for the Day 3 embryos dividing or not for either age groups.

### 3.3. Clinical pregnancy rates for Single Embryo Transfers

As shown in Table 3, there were no significant differences in clinical pregnancy rates at any stage from Day 1 to Day 3 between those women having embryos transferred on the day of thaw or after overnight culture. However, Day 2 embryos cultured overnight resulted in a significantly higher clinical pregnancy rate in the women <35 yrs of age when the embryo divided compared with those without division ( $\chi^2=11.419$ ,  $df=1$ ,  $p=0.001$ ), whilst the trend in the women ≥35 yrs old did not reach significance ( $\chi^2=1.702$ ,  $df=1$ ,  $P=0.192$ ). The clinical pregnancy rates after the transfer of Day 3 embryos was similar whether there was division overnight or not for both age groups.

### 3.4. Clinical pregnancy rates for double embryo transfers

Table 4 shows that there was no difference in pregnancy rates for Day 2 or Day 3 embryos for any age group between those embryos dividing overnight and those not. The only significant difference was for those women ≥35 yrs of age having Day 2 embryos transferred either on the day of thaw or after overnight culture ( $\chi^2=5.943$ ,  $df=1$ ,  $P=0.015$ ).

### 3.4. Multiple pregnancies

The incidence of multiple pregnancy is shown in Table 5. All of the single embryo transfers that resulted in a pregnancy, irrespective of the woman's age at freeze, resulted in singleton pregnancies. Of all the pregnancies resulting from a double embryo transfer there were 33/152 (21.7%) twins, with the women <35 yrs being not significantly different (22/92, 23.9%) from the women ≥35 yrs (11/60, 18.3%;  $\chi^2=1.602$ ,  $df=1$ ,  $P=0.206$ ). Of the 15 pregnancies where the embryos showed no sign of division after culture overnight, 2 (13.3%) were multiple.

**Table 2**

The implantation rates of thawed embryos that had been cultured overnight, according to the stage at which they were frozen, and the woman's age at the time of the freeze. Survival after thawing was defined as the cell remaining healthy in appearance (Day 1) or 50% or more blastomeres being intact (Days 2 and 3), and implantation was confirmed by a fetal heart seen on ultrasound.

Embryos after overnight culture	Day 1		Day 2		Day 3	
	<35 yrs	≥35 yrs	<35 yrs	≥35 yrs	<35 yrs	≥ 35yrs
Not divided	0/2	–	6/64a (9.4%)	4/56 (7.1%)	9/46 (19.6%)	4/43 (9.3%)
Divided	10/22 (45.5%)	4/32 (12.5%)	77/262a,b (29.4%)	40/229b (17.5%)	17/76 (22.4%)	10/68 (14.7%)

Proportions with the same superscript are significantly different from each other (all  $P < 0.05$ ).

**Table 3**

Clinical pregnancy rates per single embryo transfer according to the woman's age and day of cycle at the time of the freeze. Embryos were either transferred on the same day as the thaw, or after overnight culture. Those cultured overnight either showed signs of cell division the following day or did not.

Timing of transfer and embryo progress	Day 1		Day 2		Day 3	
	<35 yrs	≥35 yrs	<35 yrs	≥35 yrs	<35 yrs	≥35 yrs
Same day	0/2	0/2	3/16(18.8%)	0/6	4/13(30.8%)	3/8(37.5%)
Overnight culture:						
No division	0/2	–	0/30 (0.0%)a	1/14(7.1%)	4/20(20.0%)	0/13(0.0%)
Division	3/4	0/6	30/102(29.4%)a	14/49(28.6%)	6/22(27.3%)	2/14(14.3%)
Total	3/6	0/6	30/132(22.7%)	15/63(23.8%)	10/42(23.8%)	2/27(7.4%)

Proportions with the same superscript are significantly different from each other (all  $P < 0.05$ ).

**Table 4**

Clinical pregnancy rates per double embryo transfer according to the woman's age and day of cycle at the time of the freeze. Embryos were either transferred on the same day as the thaw, or after overnight culture. Those cultured overnight either showed signs of cell division the following day or did not.

Timing of transfer and embryo progress	Day 1		Day 2		Day 3	
	<35 yrs	≥35 yrs	<35 yrs	≥35 yrs	<35 yrs	≥35 yrs
Same day	0/3	0/3	7/28(25.0%)	10/25(40.0%)a	7/15(46.7%)	2/19(10.5%)
Overnight culture:						
No division	–	–	4/17(23.5%)	3/21(14.3%)	4/13(30.8%)	4/15(26.7%)
One divides	0/2	0/3	13/41(31.7%)	9/50(18.0%)	13/22(59.1%)	6/18(33.3%)
Both divide	4/9(44.4%)	3/13(23.1%)	32/80(40.0%)	15/90(16.7%)	8/27(29.6%)	8/27(29.6%)
Total	4/11(36.4%)	3/16(18.8%)	49/138(35.5%)	27/161(16.8%) <sup>a</sup>	25/62(40.3%)	18/60(30.0%)

Proportions with the same superscript are significantly different from each other (all  $P < 0.05$ ).

**Table 5**

The incidence of multiple pregnancy according to the woman's age and day of cycle at the time of the freeze. Embryos were either transferred on the same day as the thaw, or after overnight culture. Those cultured overnight either showed signs of cell division the following day or did not.

Timing of transfer and embryo progress	Single embryo transfer		Double embryo transfer	
	<35 yrs	≥35 yrs	<35 yrs	≥35 yrs
Same day transfer	0/7	0/3	3/14(21.4%)	1/12(8.3%)
Overnight culture	0/43	0/17	19/78(24.4%)	10/48(20.8%)
No division	0/4	0/1	2/8(25.0%)	0/7(0.0%)
One divides	0/39	0/16	3/26(11.5%)	6/15(40.0%)
Both divide	–	–	14/44(31.8%)	4/26(15.4%)
Total	0/50	0/20	22/92(23.9%)	11/60(18.3%)

## 4. Discussion

### 4.1. The profile of embryos frozen and surviving after thaw.

The number of embryos thawed reflects the practice profile of the Unit undertaking the current study. Zygotes frozen on Day 1 were primarily from cycles in which all embryos were electively frozen due to the risk of ovarian hyperstimulation syndrome[22], and this is now a relatively small group due to conservative ovarian stimulation strategies. The majority of

embryos thawed had been frozen on Day 2, as the main day of transfer, with a reduced number having been frozen on Day 3 from those women having oocytes collected on Fridays with the clinic avoiding Day 2 transfers on the weekend. It has been reported that zygotes survive better than Day 2 or Day 3 embryos in previous studies[2,23], which is consistent with the result of present study, while conflicting data has also been present elsewhere[24]. However, it should be acknowledged that other factors may influence the survival of early cleavage embryos other than the day of freezing

which were not identified in the present study, such as the growth rate of the embryos at the time of freeze[25]. Guidelines exist giving key performance indicators for the cryopreservation of early cleavage embryos[26], and competent laboratories should be able to achieve 60% survival and aim for 85% as a benchmark figure, and this compares favourably with the survival of 84.7%–92.3% seen in the current study.

#### 4.2. The cleavage of embryos cultured overnight.

The resumption of mitosis after overnight culture has been commonly regarded in several reports as a predictive factor to the subsequent implantation potential of thawed embryos[10,16,17]. However, not all embryos surviving the thaw go on to divide after culture. Zygotes were shown to cleave with the highest frequency in the present study (Table 1), and rates of division >70% have been described for zygotes frozen from routine IVF/ICSI cases because of legislative restrictions on the number of embryos allowed to be created[27], unavailability of freezing at the weekend[28] or as part of a freeze-all strategy due to the risk of ovarian hyperstimulation[29]. The introduction of vitrification methods may well improve this further[30]. Most of the reports on early cleavage embryos have focused on those frozen on Day 2. Embryos frozen on Day 2 with all blastomeres intact after thawing divide more frequently than if one or two blastomeres are damaged[5,16], whilst another study freezing all suitable Day 2 embryos reported a rate of division for all surviving embryos of 47%[17] compared to 73.4% in the present study. The highest rate of division is usually seen with fast growing embryos and 100% blastomere survival whilst the lowest rate is associated with slow growing embryos irrespective of the amount of blastomere loss[25]. The resumption of mitosis does not seem to be associated with the chromosomal constitution of the embryo[31].

#### 4.3. Implantation potential

Thawed embryos without blastomere damage after thawing appear to have the same implantation potential as fresh embryos[14]. However, reduced pregnancy rates are usually seen when 25% or more blastomeres are lysed after thawing[32], with reduced blastocyst formation *in vitro* occurring when the thawed embryo is partially intact compared to being fully intact[13]. The current study has not differentiated between early cleavage embryos on the extent of damage incurred, with surviving embryos having ≥50% cells intact. Day 3 embryos have been reported to have reduced on-going pregnancy rate due to lower survival rate and higher miscarriage rate[24]. Clinical pregnancy rates are said to be influenced by the ability of an embryo to resume mitosis in culture overnight, with low rates of blastomere loss at thaw and evidence of further division following culture giving the highest implantation potential[32] even after damage has been corrected for[18]. The poorest implantation rates are often seen with no division[10,16,17]. Division is generally regarded as cell number increasing by at least one[10,17], but the division of only one blastomere is not regarded as indicative of improved implantation[5]. The rate of implantation in the present study was good in the embryos frozen on Day 3 that did not divide overnight, and

the reasons for this are unclear. It is unlikely to be due to observer variability in the counting of cell numbers which generally has good reproducibility between scientists[33,34]. Assuming division must have occurred *in vivo* to allow implantation, one possible reason is the pattern of cleavage seen with Day 3 embryos where the 8-cell stage is held for 22–24hrs and cleavage cycles either side are rapid and last for less than 1 h[35]. It is therefore feasible that cleavage happened shortly after transfer.

#### 4.4. Thawing strategies

The use of overnight culture to identify the viable embryos has led some units to thaw up to 12 embryos for culture and to transfer the best ones the following day[16]. Whilst no mention was made of the fate of any supernumerary embryos that had divided, re-freezing embryos is always an option[36]. The present study thawed out embryos singly until the required number for transfer had been obtained. To implement an overnight culture protocol to identify the embryos most likely to implant would require further embryos to be thawed the next day to replace those not dividing, which may have the added complication of embryos becoming asynchronous with the endometrium. But the data has clearly shown that embryos failing to divide overnight can result in pregnancy, particularly Day 3 embryos, and that discarding embryos not dividing is not going to result in the maximum yield of off-spring and would therefore be wasteful.

#### 4.5. Conclusions

The present study has shown that later cleavage stage post-thaw embryos survive and resume cleaving less frequently compared to earlier stages. Embryos not resuming mitosis after culture overnight can implant, particularly Day 3 embryos, suggesting that they can further increase the cumulative pregnancy rate per oocyte collection and that discarding them is wasteful. Overnight culture is best used for logistical reasons rather than a strategy to improve pregnancy rates.

#### Conflict of interest statement

We declare that we have no conflict of interest.

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