Comparing thaw survival, implantation and live birth rates from cryopreserved zygotes, embryos and blastocysts

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ABSTRACT

CONTEXT: Most *in vitro* fertilization (IVF) programs employ embryo cryopreservation to enhance pregnancies from a single ovarian stimulation. More embryos are created, some of which are not transferred to the uterus immediately, generating a need for improved cryopreservation protocols. One protocol may involve growing embryos to a further stage of development, allowing only embryos with proven developmental capabilities to be cryopreserved. Here we examined thaw survival, implantation and live birth rates of embryos cryopreserved at different stages. **AIMS:** We examined thaw survival, implantation and live birth rates of embryos cryopreserved at the zygote, day 3 (D3) embryos or blastocyst stage. **SETTINGS AND DESIGN:** This is a retrospective study from a single academic IVF program. **PATIENTS AND METHODS:** A retrospective study of all patients who had frozen embryos transferred to their uteri from year 2002 to 2008 at a single academic IVF program was conducted. **STATISTICAL ANALYSIS USED:** Analysis of variance followed by Fisher's Exact Test was performed to compare the survival after thaw, implantation and live birth rates between the three groups. **RESULTS:** One thousand nine hundred and ninety-one zygotes, 2880 D3 embryos and 503 blastocysts were frozen using a slow freeze technique, thawed and transferred. Significantly more D3 embryos and blastocysts survived the thawing process compared to zygotes and significantly higher implantation rate per number of thawed blastocysts was achieved than that for zygotes. Live birth rates were similar between the three groups. **CONCLUSIONS:** Growing embryos to blastocyst stage prior to cryopreservation is associated with fewer frozen embryos but does not appear compromise patients' chance of achieving pregnancy

KEY WORDS: Blastocyst, embryo cryopreservation, frozen embryo transfer, implantation, *in vitro* fertilization, slow-freeze, zygote

INTRODUCTION

The first birth from a thawed blastocyst occurred 1983.[1] It is now routine for *in vitro* fertilization (IVF) programs to use embryo cryopreservation to enhance pregnancy yields from a single ovarian stimulation cycle,[2-4] to eliminate or decrease the risk of ovarian hyperstimulation syndrome, and to minimize high order gestations by limiting of the number of transferred embryos.[4-9] Transferring cryopreserved embryos offers several other major advantages. It is less expensive and less invasive than repeating an ovarian stimulation required for a fresh embryo transfer (ET) and there is no risk of ovarian hyperstimulation.[2-4,10] With more couples choosing to cryopreserve excess embryos, it becomes increasingly important to optimize cryopreservation protocols.

Although pregnancy rates with cryopreserved embryo cycles may be lower than those achieved from fresh cycles,[3,11,12] the cumulative effect of adding pregnancies achieved with thawed cycles to those attained with fresh cycles must also be considered. Overall outcomes from a single oocyte retrieval can be significantly enhanced when cryopreservation is used.[3,4] Moreover, there is no apparent negative effect on perinatal outcome or health of children born as a result of these procedures.[13-17]

Human embryos can be cryopreserved at several developmental stages, including zygotes, cleaved embryos (day 3 embryos, will be referred to as D3 embryos) or blastocysts.[18,19] The term zygote refers to a fertilized egg showing 2 pro-nuclei 16 hours after fertilization. Cleavage stage (or D3) embryo refers to an embryo having 4-8 cells of roughly equal size. Blastocyst refers to an embryo grown to day 5 or 6, which has a clearly defined blastocele cavity, inner cell mass and trophectoderm. The inner cell mass consists of small, compacted cells that will give rise to the embryo proper and the trophectoderm cells located at the periphery of the embryo give rise to the placenta [Figure 1].

While several groups reported cryopreserving blastocysts quite successfully, some have found better results with freezing at earlier stages.[4,12,19-21] Since improved culture media have been developed to better support *in vitro* embryo growth, more blastocysts have become available for cryopreservation.

The purpose of this study was to determine the relationship between stages of development at the time of cryopreservation and subsequent thaw survival and implantation rates.

PATIENTS AND METHODS

Patients

A retrospective review of all patients who underwent the transfer of cryopreserved embryos between 2002 and 2008 at the IVF program at a single academic institution was conducted. Informed consent for cryopreservation and subsequent thawing was obtained for all patients. The study was approved by Institutional Review Board for Human Research. Patients using oocytes obtained from donors were excluded.

Controlled ovarian hyperstimulation protocols

Controlled ovarian hyperstimulation was achieved by once or twice daily subcutaneous injections of gonadotrophin using either a luteal phase gonadotrophin-releasing hormone (GnRH-a) agonist protocol[22,23] or an antagonist protocol, once the absence of dominant follicular development was confirmed sonographically and serum estradiol (E_2) levels were reduced to less than 50 pg/mL. For patients using the luteal phase agonist protocol, pituitary down-regulation was achieved with 0.5 mg GnRHa (Lupron, TAP Pharmaceutical, North Chicago, IL) daily for 21 days to suppress endogenous gonadotrophin secretion. For the antagonist protocol, controlled ovarian hyperstimulation by injection of recombinant FSH was started on the third day of menstruation; the starting dose was decided according to the patient's age and ovarian reserve.[24,25] When the leading follicle reached 11-12 mm in diameter, patients began to receive a daily injection of 250 μg of ganirelix.[25] FSH administration was initiated at daily dosages ranging from 150 to 450 IU. Final follicular maturation was triggered by the administration of 250 μg of choriogonadotrophin α-injection (Ovidrel, Serono Laboratories, Rockland, MA USA) when at least three follicles were more than 16 mm in diameter.

Transvaginal oocyte retrieval was performed 36 hours after choriogonadotrophin α-injection. Oocytes were inseminated 4-6 hours after retrieval by coculture with motile sperm or by intracytoplasmic sperm injection (ICSI), depending on semen quality. Fertilization was assessed 15-18 hours after insemination. Embryos were cultured under mineral oil in 40 μL droplets of culture medium at 37°C in a humidified, 5% O_2, 5% CO_2 and 90% N atmosphere until day 3. Transfer was performed on day 3, or delayed until day 5 if more than five 8-cell embryos were available for transfer on day 3. Sequential media from SAGE Biopharma (Bedminster, NJ) or Irvine Scientific (Santa Ana, CA) were used for embryo culture. After extended culture, blastocysts developing on either day 5 or day 6 were cryopreserved for later use.

Endometrial priming

To more accurately control the uterine environment for ET, patients underwent endometrial priming with estrogen and progesterone.[2-4,19] Ovarian steroid supplementation consisted of 8 mg of oral estradiol starting on day 1 of the menstrual cycle and 100 mg of IM progesterone starting on day 9 as long as the uterine lining was greater than 6.5 mm. If the uterine lining was noted to be less than 6.5 mm, an additional 2-4 mg of estradiol was administered vaginally. ET was performed 5 or 7 days after starting progesterone, depending on the stage of the embryo that was going to be transferred. Pregnancies were initially detected by serum β-human chorionic gonadotrophin (hCG) concentrations

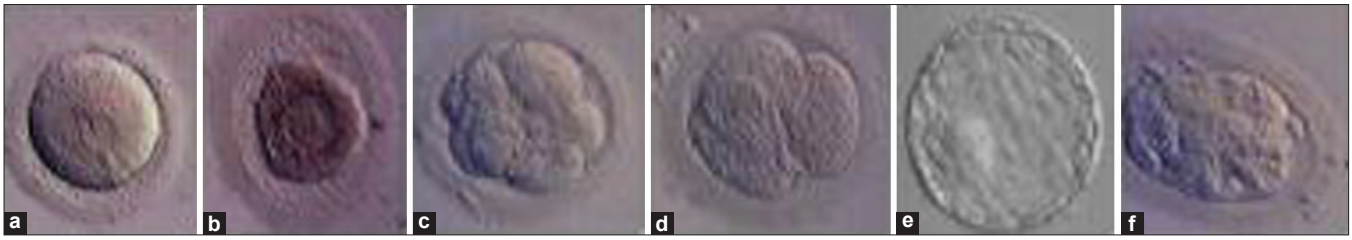

Figure 1: Post-thaw embryos: examples of (a) viable thawed zygote, (b) non-viable thawed zygote, (c) viable thawed D3 embryo, (d) non-viable thawed D3 embryo, (e) viable thawed blastocyst, (f) non-viable thawed blastocyst

and confirmed by transvaginal ultrasound. All pregnant women continued estradiol and progesterone therapy until 9 weeks of gestation.

Embryo cryopreservation techniques and transfer protocols

At our program, embryo cryopreservation was historically performed at the zygote stage when more than 15 zygotes were created. After ET on day 3, excess embryos were cryopreserved on the same day (before year 2005) or cultured to day 5 for cryopreservation at the blastocyst stage. Only good quality embryos and blastocysts were cryopreserved.

All zygotes, D3 embryos and blastocysts were cryopreserved using a slow freeze technique in freezing media, appropriate for embryo stage according to the manufacturer's instructions (media from Irvine Scientific). After equilibration in the cryopreservation media, zygotes, D3 embryos or blastocysts were loaded in groups of 1-3 inside a straw and frozen in a programmable freezer.[26] Briefly, the embryos were first cooled to –7° C at 2° C/minute, followed by "seeding" to induce ice crystal formation. The embryos were further cooled to –30° C at -0.3° C/min, and plunged into liquid nitrogen for storage.

At the time of thaw, zygotes, D3 embryos and blastocysts were removed from liquid nitrogen and held in the air at room temperature for 30 seconds, followed by immersion into a water-bath at 30° C for 40-50 seconds. Cryoprotectants were removed by sequential dilution in Embryo Thawing or Blastocyst Thawing solutions (Irvine Scientific) containing decreasing concentrations of cryoprotectants. Zygotes were thawed 2 days prior to a planned day 3 transfer and allowed to grow. D3 embryos were thawed the afternoon prior to the scheduled day 3 transfer and were examined the morning of the transfer to assess viability. Blastocysts were thawed the day of transfer and allowed to expand 2 hours prior to transfer [Figure 1]. ETs were then performed using transabdominal ultrasound guidance.[27,28]

Definition of outcomes

Thaw survival rate was calculated as the number or viable or developing embryos on the day of transfer divided by the total number thawed. For this study, implantation rate was defined as the number of gestational sacs per number of embryos transferred. Live birth was defined as a fetus exiting the maternal body and showing signs of life.

Statistical analysis

The primary endpoint for this study was thaw survival rates and the secondary endpoint was implantation rates. Total live birth rates and live birth rates per thawed embryo were also calculated. A power calculation with an α=.05 and a power of 80 determined that a minimum of 348 embryos in each group would be needed to detect a 10% difference in the thaw survival rate.

Descriptive data are presented as the mean±SD. Where appropriate, analysis of variance followed by Fisher's Exact Test was performed to compare the survival after thaw, implantation and live birth rates between the 3 groups. A *P*-value <0.05 was considered significant.

RESULTS

Baseline characteristics and infertility diagnoses are summarized in Tables 1 and 2. A total of 1991 zygotes, 2880 D3 embryos, and 503 blastocysts were cryopreserved and thawed during this time period. The number of transfer cycles was 382 for zygotes, 768 for D3 embryos and 179 for blastocysts. The average patient age was 37.2 (range 26-47) years for cycles using frozen zygotes, 37.3 (range 25-47) years for cycles using D3 embryos, and 37.1 years for cycles using blastocysts (range 27-48). These differences were not statistically different. The survival rate was 69% for thawed zygotes, 85% for D3 embryos, and 88% for blastocysts [Table 1]. The implantation rate per number thawed was 10% for zygotes, 12% for D3 embryos, and 14% for blastocysts. The implantation rate per number of embryos that survived the thaw was 14% for zygotes, 14% for D3 embryos, and 15% for blastocysts. The implantation rate per transfer cycle was 50.2% for zygotes, 44.6% for D3

Table 1: Data on cryopreservation of zygotes, D3 embryos and blastocysts

Variable	Zygotes	D3 embryos	Blastocysts
Total thawed	1991	2880	503
# Transfer cycles	382	768	179
Age at transfer (±SD)	37.2 (5.1)	37.3 (4.6)	37.1 (5.1)
Age range	26-47	25-47	27-48
Avg # transferred (±SD)	2.7 (1.0)	2.6 (.93)	2.1 (1.1)
Age range	1-6	1-4	1-3
Thaw survival (%)	1374 (69)	2448 (85)	443 (88)
Implantation rate/Thaw (%)	192/1991 (10)	242/2880 (12)	68/503 (14)
Implantation rate/Thaw survival (%)	192/1374 (14)	343/2448 (14)	68/443 (15)
Implantation rate/Transfer cycle (%)	192/382 (50.2)	343/768 (44.6)	68/179 (38.0)

Table 2: Causes of infertility

Cause of infertility*	Zygotes	D3 embyros	Blastocysts
Male factor (%)	23	27	19
Tubal factor (%)	13.5	9.2	6
Decreased ovarian Reserve (%)	6.5	9.2	10
Ovulatory dysfunction-including PCOS (%)	7.6	26.4	17
Endometriosis (%)	6.9	6.7	6.6
Other/unexplained (%)	26	28.7	32

*Note: Patients may have more than 1 diagnosis for infertility

embryos, and 38.0% for blastocysts [Table 1]. Zygotes had a significantly higher implantation rate per transfer cycle compared to blastocysts. The number of thawed embryos transferred ranged from 1 to 6, with a mean of 2.7, and did not significantly differ between zygotes, D3 embryos and blastocysts. Significantly more D3 embryos and blastocysts survived the thawing process compared to zygotes ($P<0.05$, ANOVA, then Fisher's exact test), and a significantly higher implantation rate per number thawed was achieved for thawed blastocysts than that for thawed zygotes ($P<0.05$).

Live birth data is summarized in Table 3. From thawed zygotes, there were a total of 79 singletons born, 30 sets of twins and 2 sets of triplets, totalling 145 live births infants and a 7.3% live birth/thawed embryo rate. For D3 embryos, there were a total of 176 singletons, 53 sets of twins and 2 sets of triplets totalling 288 live births and a 10.0% live birth/thawed embryo rate. For blastocysts, there were 37 singletons, 11 sets of twins and 0 triplets, yielding a total of 59 live births and a 11.7% live born/thawed embryo rate. Although there was a higher implantation rate for zygotes, there were no statically significant differences in live births. However, there were no incidences of higher order multiples amongst the group in which thawed blastocysts were transferred [Table 3].

DISCUSSION

Cryopreserving embryos for future thaw and transfer is an important strategy for augmenting the cumulative pregnancy rate per retrieval cycle. This study compared the survival after thaw and subsequent pregnancy and live birth rates using cryopreserved zygotes, D3 embryos, and blastocysts. We found that cryopreserving embryos at the zygote stage was associated with lower survival rates and lower implantation rates compared with freezing at the blastocyst stage. Additionally, growing embryos to the blastocyst stage prior to cryopreservation was associated with a decrease in the total number of embryos cryopreserved, but did not appear to compromise patients' chance of achieving a pregnancy.

There is controversy over the benefits of cryopreservation of embryos at early or late developmental stages.[2-4,18,19,26,29-31] There are several reasons why freezing embryos at the blastocyst stage may be beneficial. First, blastocysts have many cells, thus the loss of a few cells during the freezing and thawing process may not compromise their integrity.[4] Moreover, the developmental capability of an individual embryo can be better established by growing it to the blastocyst stage. Any embryos which display developmental arrest would be identified and discarded prior to cryopreservation, leaving only the most competent embryos to be subsequently cryopreserved.[32,33]

Relatively few peer-reviewed reports have evaluated freezing blastocysts generated after extended culture in sequential media. Several studies have noted favorable pregnancy rates after freezing and thawing human blastocysts. Veek *et al*. reported that 76.3% of blastocysts survived thawing, and reported the subsequent implantation rate per blastocyst transferred to be 38.6%.[4] Langley *et al*. compared thawed day 3 embryos and blastocysts and reported a higher survival rate and subsequent implantation rate for blastocysts.[34]

However, cryopreservation at the pronucleate or zygote stage is still favored by some.[26,35-37] Cryopreserving at this early stage does give patients the best opportunity to have excess embryos in storage for subsequent ET attempts.[3,35,38] Additionally, early studies reported lower survival rates with blastocyst thaw.[12,19] Senn *et al*. compared the cumulative live birth rates obtained after cryopreservation of either zygotes or day three embryos and found a significantly higher implantation and pregnancy rate per transfer with zygotes. This was associated with a higher cumulative pregnancy and live birth rate.[26] Damario *et al*. reported that embryos cryopreserved at the zygote stage have similar potential for implantation and pregnancy compared with fresh embryos. However, in the study by Damario *et al*. all excess embryos were cryopreserved at the zygote stage.

The advantage of our study is that it compares all 3 stages of embryo development, namely zygote, D3 embryos, and blastocysts in a single embryology laboratory. Additionally, all non-donor oocyte patients were included, regardless of diagnosis and patient age [Tables 1 and 2]. The overall implantation and live birth rate achieved in our study is similar to those reported previously.[10,19,31]

A weakness of this study is that it is retrospective in nature. Additionally, embryos were not graded prior to transfer. Another study is currently underway in our unit to investigate embryo morphology pre-and post-thaw and assess its association with subsequent thaw survival and

Table 3: Live birth rate

	Total thawed	# Transfer cycles	Total # live born	Singleton	Twins	Triplets	# Live born/ # Thawed (%)	Live birth transfer cycle (%)
Zygote	1991	382	145	79	30	2	7.3	37.9
D3 embryo	2880	768	288	176	53	2	10.0	37.5
Blastocyst	503	179	59	37	11	0	11.7	33.0

implantation. We also did not account for the amount of time the embryos were in storage. However, a study by Riggs *et al*. suggests that cryostorage duration did not adversely affect post-thaw survival or pregnancy outcome.[5] This study only examined embryos that were cryopreserved using a slow freeze protocol. Human embryos can also be cryopreserved by vitrification. However, there is still limited data to definitively state that cryopreservation by vitrification would enhance the post-thaw survival rates for day 3 embryos and blastocysts.[39,40]

Our study shows that the number of cells at the time of cryopreservation is important both for survival at thawing and subsequent implantation and live birth. Earlier studies suggested that zygotes survived the freeze-thaw process better than more developed embryos. However, it now appears that with the addition of sequential media for growth and development, this may no longer be the case.[4] This retrospective study does not directly address the question of which freeze and thaw strategy ultimately results in the best pregnancy yield. Nevertheless, this embryo recovery data, implantation and birth rates support the belief that delaying cryopreservation until day 5 does not harm pregnancy rates. In summary, growing embryos after IVF to blastocyst stage prior to freezing is associated with fewer cryopreserved embryos and may not compromise patients' chance of achieving a pregnancy.

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