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Healthy Monozygotic Twins Born from a Vitrified Blastocyst Derived from a Vitrified Oocyte, and a Highly Efficient Vitrification for Freezing Human Oocytes and Blastsocysts

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Abstract: We used simplified oocyte/embryo vitrification and warming protocols (Irvine Scientific) combined with vitristraws (SciTech Invention) to freeze and thaw human oocytes and blastsocysts. Throughout the year of 2014, twelve recipients were transferred embryos developed from vitrified donor oocytes, and fourteen recipients were transferred embryos developed from fresh donor oocytes at the North Carolina center for reproductive medicine (NCCRM). There were no statistically significant differences in donor age (25.9 ± 3.6 vs 24.9 ± 3.2) and recipient age (43.0 ± 5.4 vs 41.4 ± 6.8), fertilization rates (86.2% vs 87.0%), blastocyst development rates (50.0% vs 53.8%), number of embryo transferred (1.7 ± 0.8 vs 1.9 ± 0.4), clinical pregnancy rates per transfer (75.0% vs 71.4%) and live birth rates per transfer (66.7% vs 57.1%) between vitrified and fresh oocyte cycles, respectively. The results demonstrate that vitrification techniques can be used to cryopreserve human oocytes for future use. We are also reporting the live birth of healthy monozygotic twins resulted from a re-vitrified blastocyst derived from a vitrified oocyte. Oocytes from a 30-year-old donor were vitrified in vitristraws. Seven out of eight oocytes survived after thawing on November 16, 2013. Those seven oocytes were inseminated by intracytoplasmic sperm injection (ICSI) at about 2 hours post thawing. All seven oocytes were tested as fertilized by pronuclear check at 18 hours after ICSI. Those fertilized oocytes showed normal cleavage on day 2 and day 3. Four of them developed to blastsocysts by culturing in continuous single culture medium in a tri-gas incubator for 5 days. Two blastsocysts were transferred to a 43-year-old recipient, but that did not result in a pregnancy. The other two blastsocysts were re-vitrified in a vitristraw. The re-vitrified blastsocysts were thawed and then transferred to the same recipient on May 8, 2014. The patient achieved a normal pregnancy on her second transfer. On June 14, 2014, an ultrasound scan detected two heartbeats in one gestational sac. Two healthy monozygotic boys (weighing 2466g and 2353g) were born on January 13, 2015. To our knowledge, this is the first report of monozygotic twins born from an embryo by twice vitrification at oocyte and blastocyst stage.

Keywords: Human Oocyte, Blastocyst, Vitristraw, Twice Vitrification, Monozygotic Twins

1. Introduction

In 1986, the first successful attempt of human oocyte cryopreservation was reported by using slow-freezing [1]. In 1999, a live birth was achieved by vitrification of human oocytes [2]. As vitrification is superior to the slow-freezing [3-5], vitrification techniques have been widely used in cryopreservation of oocytes [5-12] and blastsocysts [13-17] in human in vitro fertilization (IVF). By using DNA fingerprinting, Forman et al have shown that oocyte vitrification does not increase the rate of aneuploidy or diminish the implantation potential of viable blastsocysts [18]. The evidence that aneuploidy rate does not increase in vitrified oocytes is a welcome endorsement of the safety of the vitrification technique and a watershed in assisted reproductive technology [19]. Many studies show that vitrified oocytes result in similar fertilization, development and pregnancy rates compared to fresh oocytes [20-23].

Oocyte vitrification techniques can be employed to enable women to use their own eggs in their future pregnancy for medical or social reasons [24-28], and also establish egg banks for donation [29-35]. Two re-vitrified day 2 embryos derived from in-vitro matured and vitrified oocytes were transferred, but there was no pregnancy [35]. Healthy babies born from re-vitrified embryos in donation cycles conducted with vitrified oocytes have been reported [16, 36]. Moreover, after studying a large series of transfers of vitrified embryos generated from previously vitrified oocytes, Cobo et al show that double vitrification has no impact on delivery rates [37]. We report the first live birth of healthy monozygotic twins (born on January 13, 2015) resulted from a re-vitrified day 5 embryo derived from a vitrified oocyte.

2. Materials and Methods

2.1. Oocyte Retrievals, Fertilization and Embryo Culture

Oocytes were retrieved from donors through transvaginal ultrasound-guided puncture of the follicles in 36 hours after lupron trigger. Cumulus-oocytes complexes were cultured in insemination medium (IM, human tube fluid (HTF, Irvine Scientific) containing 10% of serum substitute supplement (SSS, Irvine Scientific)) for about 4 hours in fresh cycles or 2 hours in vitrification cycles. All mature (fresh/vitrified) oocytes were fertilized by ICSI using standard ICSI protocols. Fertilization checks were completed by performing pronuclear checks under a stereo microscope at 18 hours post ICSI. Fertilized oocytes were moved to 45 µl droplets (covered with mineral oil) of continuous single culture medium (CSCM, Irvine Scientific) containing 10% of SSS in a 60-mm culture dish (Fisher Scientific) in order to culture further for blastocyst development till 5 or 6 days. All cultures were performed at 37°C in benchtop incubators (Cook Medical) in the tri-gas of 4% O_2, 6% CO_2, and 90% N_2.

2.2. Oocyte Cryopreservation

Two hours after retrieval, cumulus cells were removed from cumulus-oocytes complexes before vitrification, and only mature oocytes in metaphase II (MII) were used for vitrification. Oocytes were vitrified by following the simplified MII oocyte vitrification protocols (Irvine Scientific) and using the Vit Kit-Freeze media (Irvine Scientific) containing washing solution (WS, a HEPES buffered solution of M-199 containing gentamicin sulfate (35 µg/ml), and 20% (v/v) of dextran serum supplement (DSS)), equilibration solution (ES, a HEPES buffered solution of M-199 containing gentamicin sulfate (35 µg/ml), 7.5% (v/v) of each DMSO and ethylene glycol and 20% (v/v) of DSS)), and vitrification solution (VS, a HEPES buffered solution of M-199 containing gentamicin sulfate (35 µg/ml), 15% (v/v) of each DMSO and ethylene glycol and 20% (v/v) of DSS and 0.5 M sucrose). All vitrification procedures were done at room temperature (22-27°C). Oocytes were placed into a 20 µl drop of WS for 1 minute. A 20 µl drop of ES was merged to the WS drop, with spontaneous mixing for 2 minutes (using tip of transfer pipette to move ES towards WS until drops merge). The second 20 µl ES drop was merged into the WS+ES drop, with spontaneous mixing for other 2 minutes. Then, oocytes were transferred from the merged drop to the third 20 µl drop of ES and exposed undisturbed for 6-10 minutes. During the 6-10 minute exposure, a 50 µl drop of VS was aseptically dispensed separately. Oocytes were transferred from the third ES drop to the VS drop for 50 seconds before loading, and gently pipetted once within the VS drop to ensure complete rinse in VS. A vitristraw (SciTech Invention) was pre-labeled with the patient's information. Two or three oocytes were loaded to the tip of one vitristraw with about 0.5 µl of VS within 10 seconds and then the vitristraw was plunged into the liquid nitrogen (LN2) immediately. The tip of the vitristraw was inserted into a clear sleeve and twisted tightly within LN2.

2.3. Embryo Cryopreservation

Embryos were vitrified by following the simplified Embryo vitrification protocols (Irvine Scientific) and using the Vit Kit-Freeze media containing WS, ES and VS. All vitrification procedures were to be done at room temperature (22-27°C). Embryos were placed into a 50 µl drop of WS for 1 minute and then transferred into a 50 µl drop of ES for 6-10 minutes. During the 6-10 minute exposure, a 50 µl drop of VS was aseptically dispensed. Embryos were transferred from the ES drop to the VS drop for 50 seconds before loading, and gently pipetted once within VS drop to ensure complete rinse in VS. A vitristraw was pre-labeled with patient's information. One or two embryos were loaded onto the tip of one vitristraw with about 0.5 µl of VS within 10 seconds and then the vitristraw was plunged into LN2 immediately. The tip of the vitristraw was inserted into a clear sleeve and twisted tightly within LN2.

2.4. Oocyte/Embryo Thawed

Embryos or oocytes were thawed by following the simplified embryo and oocyte warming protocols (Irvine Scientific) and using Vit Kit-Thaw media (Irvine Scientific) containing WS, dilution solution (DS, a HEPES buffered solution of M-199 containing gentamicin sulfate (35 µg/ml), 0.5M sucrose and 20% (v/v) of DSS), and thawing solution (TS, a HEPES buffered solution of M-199 containing gentamicin sulfate (35 µg/ml), 1.0M sucrose and 20% (v/v) of DSS). One ml of TS was aseptically dispensed in the center of an organ dish (Fisher Scientific) and warmed to 37°C (in an incubator without CO_2 or on a heating stage) at least 30 minutes prior starting warming procedure. The tip of the vitristraw (carrying embryos/oocytes) was separated from the clear sleeve within LN2 and then immediately plunged into TS (37°C) in the center of the organ dish and gently swirled to detach embryos/oocytes. The embryos/oocytes were then kept in TS for 1 minute at room temperature. Embryos/oocytes were transferred from TS to DS for 4 minutes at room temperature, undisturbed. During the 4 minutes exposure in DS, 2 drops (50 µL) of WS (WS1, WS2)

were aseptically dispensed. Embryos/oocytes were transferred from the DS drop to the WS1 drop for 4 minutes at room temperature, undisturbed, and then moved from the WS1 drop to the WS2 drop for other 4 minutes at room temperature, undisturbed. Embryos were transferred to pre-equilibrated IM with 20% (v/v) of SSS and then cultured at 37°C for about 2-4 hours prior to embryo transfer. Oocytes were transferred to pre-equilibrated IM with 20% (v/v) of SSS and then cultured at 37°C for about 2 hours before ICSI.

2.5. Embryo Transfer

Assisted hatching (AH, creating a hole in the zona pellucid) was performed on all vitrified embryos (or fresh embryos generated from vitrified oocytes) prior to embryo transfer (ET). One or two embryos were transferred to a recipient using a Wallace embryo transfer catheter (Origio Inc.) by ultrasound-guide.

3. Results

3.1. Fresh and Frozen Oocyte Cycles

From January to December of 2014, twelve vitrified oocyte cycles and fourteen fresh oocyte cycles were performed in our clinic through oocyte donation programs. After thawing, all oocytes were cultured in IM containing 20% of SSS for about 2 hours, and only survived oocytes (figure 1) would be inseminated by ICSI (figure 2). Figure 3 shows day 5 blastsocysts developed from fresh oocytes, and figure 4 shows day 5 blastsocysts developed from vitrified oocytes. Morphology of blastsocysts from vitrified oocytes (figure 4) looks as good as that (figure 3) from fresh oocytes. The data were analyzed with a chi-squared test of independence, and P-values lower than or equal to 0.05 were regarded as an indicator of significant difference. The results demonstrate in table 1 that there were no statistically significant differences in donor age (25.9 ± 3.6 vs 24.9 ± 3.2) and recipient age (43.0 ± 5.4 vs 41.4 ± 6.8), embryo number for transfer (1.7 ± 1.3 vs 1.9 ± 0.9) between vitrified and fresh oocyte cycles. The fertilization and blastocyst development rates in the fresh oocyte cycles were higher than that in the vitrified oocyte cycles, (87.0% vs 86.2% and 53.8% vs 50.0% respectively); nonetheless, there were no statistically significant differences between two groups. The clinical pregnancy and live birth rates per transfer in vitrified oocyte cycles were higher than that in fresh oocyte cycles (75.0% vs 71.4% and 66.7% vs 57.1% respectively), however, there were still no statistically significant differences between two groups.

Table 1. *Oocyte donation program in NCCRM in 2014.*

	Vitrified oocyte cycles (N=12)	Fresh oocyte cycles (N=14)	P-value
Donor age	25.9± 3.6	24.8± 3.2	0.406
Recipient age	43.0± 5.4	41.4± 6.8	0.540
Oocyte survival (%) (post thawing)	65/90 (72.2%)	N/A	N/A
Fertilization (%)	56/65 (86.2%)	160/184 (87.0%)	0.934
Blastocyst development (%)	28/56 (50.0%)	86/160 (53.8%)	0.230
Embryo number for transfer	1.7±0.8	1.9±0.4	0.421
Clinical pregnancy (%)	9/12 (75.0%)	10/14 (71.4%)	0.845
Live birth (%)	8/12 (66.7%)	8/14 (57.1%)	0.635

3.2. Monozygotic Twins (a Case Report)

Oocytes (from a 30-year-old donor) were vitrified in vitristraws in Vit Kit-Freeze media. Seven out of eight oocytes survived after thawing in Vit Kit-Thaw media on November 16, 2013. Those seven oocytes were inseminated by ICSI at about 2 hours post thawing. All seven oocytes were tested as fertilized by pronuclear check at 18 hours after ICSI. All seven fertilized oocytes were cultured in CSCM in a tri-gas incubator and cleaved normally to embryos (figure 5) at day 2, but only four of them developed to blastsocysts (figure 6, figure 7) at day 5. The AH was performed on two blastsocysts (figure 6) before they were transferred to a 43-year-old recipient, but this did not lead to a pregnancy. The other two blastsocysts (figure 7) were re-vitrified in a vitristraw in Vit Kit-Freeze media. The re-vitrified blastsocysts were thawed in Vit Kit-Thaw media, and AH was performed. The thawed blastsocysts (figure 8) were then transferred to the same recipient on May 8, 2014. The patient achieved a normal pregnancy on her second transfer. On June 14, 2014, an ultrasound scan detected two heartbeats in one gestational sac. Two healthy monozygotic boys (weighing 2466g and 2353g) were born on January 13, 2015. From a survey in 2018, the two boys were normal growth within 3 years.

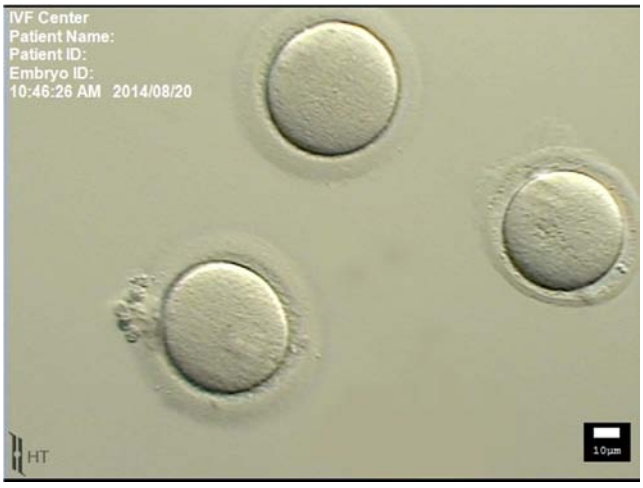

Figure 1. *Survived oocytes after vitrification/thawing.*

Figure 2. *A sperm (arrow) was injected into a vitrified/thawed oocyte by ICSI.*

Figure 3. *Two day 5 blastsocysts from fresh oocytes.*

Figure 4. *Two day 5 blastsocysts from vitrified oocytes.*

Figure 5. *Seven day 2 embryos from vitrified oocytes.*

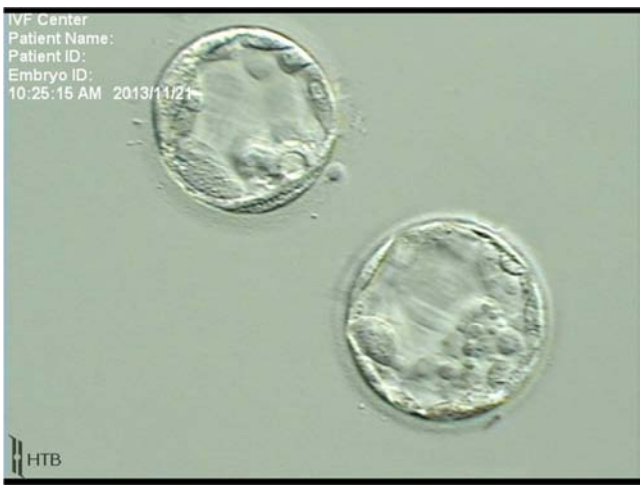

Figure 6. *Two day 5 blastsocysts from vitrified oocytes.*

Figure 7. *Two day 5 blastsocysts (going to be re-vitrified) from vitrified oocytes.*

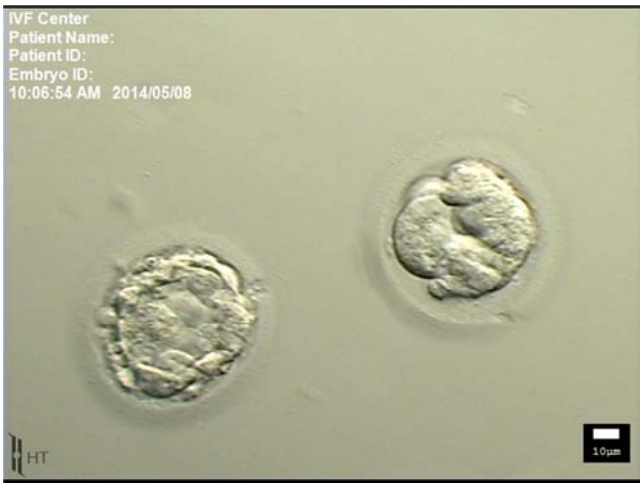

Figure 8. *Two thawed blastsocysts after twice vitrification at oocyte and blastocyst stage.*

4. Discussion

Many devices such as cryoloop [38], cryotip [39], cryotop [19], cryolock [40], cryoleaf [41], HSV [42], Rapid-i [44] and iVirti [41] etc., have been employed as oocyte/embryo carries for vitrification. In this study, we used vitristraws (SciTech Invention) as oocyte/embryo carries for vitrification and achieved a good result of 66.7% in live birth rate (table 1). Some studies show that vitrified oocytes result in similar fertilization, development and pregnancy rates compared to fresh oocytes [20-23]. Cobo's study demonstrates there was no difference in fertilization (76.3% and 82.2%), day 2 cleavage (94.2% and 97.8%), day 3 cleavage (80.8% and 80.5%), and blastocyst formation (48.7% and 47.5%) for vitrified and fresh oocytes, respectively [20]. From comparing frozen-thawed and fresh donor oocytes in recipients, it has been reported that 92.5% of vitrified oocytes survived after warming, and there were no statistically significant differences in fertilization rates (74.2% vs 73.3%), clinical pregnancy rates (55.4% vs 55.6%) between vitrified and fresh donor oocyte cycles [29]. Although our vitrified oocyte survival rate (72.2%) (table 1) was lower than the that (92.5%) from the study by Cobo et al [29], the fertilization rate (86.2%) and clinical pregnancy rate (75.0%) (table 1) were both higher than that of 74.2% and 55.4% [29], respectively. Our vitrification procedures may need to be modified to increase the vitrified oocyte survival rate post warming and also keep high fertilization and pregnancy rates per transfer in future study. Jones et al also shows that fertilization, blastocyst development, pregnancy and live birth rates are comparable in fresh and vitrified sibling oocytes from the same stimulation cycle [22]. They achieved a good result of 55% live birth rate in both vitrified oocyte and fresh oocytes groups [22]. In our study, eight healthy babies (66.7% of live birth rate) derived from vitrified oocytes in 12 frozen-thawed donor cycles were compared eight healthy babies (57.1% of live birth rate) from 14 fresh donor cycles in table 1. In conclusion, the simplified oocyte/embryo vitrification and warming protocol (Irvine Scientific) combined with a vitristraw (SciTech Invention) is a highly efficient vitrification procedure that can be used to freeze human oocytes for future use.

Some studies demonstrate embryos from vitrified oocytes could be re-vitrified and resulted in healthy babies [16, 36, 37, 44]. In Dorfmann's study, 57% of clinical pregnancy rate from transfer re-vitrified embryos derived from vitrified donor oocytes was compared 51% of that from transferring the embryos derived from vitrified donor oocytes [44]. Also, Lamb et al demonstrate there is no statistically significant difference in implantation (48% vs 64%), pregnancy (66% vs 70%) or live birth (56% vs 65%) rates between transferring fresh blastsocysts derived from vitrified oocytes and transferring re-vitrified blastsocysts derived from vitrified oocytes, respectively [36]. Moreover, after studying a large series of transfers of vitrified embryos generated from previously vitrified oocytes, Cobo et al show that double vitrification has no impact on delivery rate [37].

Compared with the 0.43% of monozygotic twins rate in the general population, the monozygotic twins rate in the IVF increased to 1.02%(94/9214) [45], 2.69%(93/3,463) [46], or 1.17%(122/10,470) [47], or 2.1%(131/6223) [51]. But the reasons for this are still unclear [53]. By analyzing 93 monozygotic twins from 3,463 pregnant women in IVF cycles, some procedures in assisted reproductive technique, such as the embryo number of transfer, assisted hatching, ICSI and vitrification did not increase probability of monozygotic twins, but extended embryo culture (from day 3 to day 4 and day 5/6) increased the rate of monozygotic twins (1.27%, 3.40%, 4.63% on day 3, day 4, day 5/6 transfer, respectively) [46]. Other studies also show the similar result of the increasing monozygotic twinning rate by comparing day 3 and day 5 embryo transfer [47-52]. But, after analyzing 1,951 fresh IVF/ICSI cycles, Papanikolaou, et al found out that the monozygotic twins rate was 2.6%(8/308) in day 3 transfer comparing 1.8%(5/271) in day 5 transfer [54]. Vitthala, et al demonstrate ICSI has 2.25 times higher risk of monozygotic twins than natural conception [50] and Mukaida et al show monozygotic twins rates were 2.7%(13/486) in fresh blastocyst transfer and 4.1%(15/363) in vitrified blastocyst transfer, although there was no statistical difference between two groups [55]. The risk factors of monozygotic twins involved in IVF still need more investigation.

We report the live birth of healthy monozygotic twins (born on January 13, 2015) resulted from a re-vitrified day 5 embryo derived from a vitrified oocyte, which was thawed, fertilized and cultured to a blastocyst. Dose re-vitrified oocytes/blastocyst increase risk of monozygotic twins? That remains a question for further study.

5. Conclusion

Vitrification techniques can be used to freeze human oocytes for future use. The fertilization, blastocyst development, clinical pregnancy and live birth rates in frozen/thawed oocyte cycles exhibited little functional difference with those in fresh oocyte cycles. Additionally,

extra embryos derived from vitrified oocytes can be re-vitrified. We report the first live birth of healthy monozygotic twins (born on January 13, 2015) resulted from a re-vitrified blastocyst derived from a vitrified oocytes. This result shows that an embryo, after being twice frozen/thawed, can still be split naturally into monozygotic embryos like a fresh embryo after transfer.

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