Introduction

Vitrification is the gold standard for female oocyte cryopreservation and has become more common over the last 15 years [1-3]. Oocyte cryopreservation using slow freezing is not very effective, and results in low pregnancy rates and a high likelihood of oocyte damage [4]. Therefore, the methodology transitioned to vitrification with fertilization and pregnancy rates similar to those in in vitro fertilization cycles using fresh oocytes [5-8].

One study reported that in embryos, a longer duration since vitrification may be associated with decreased clinical outcomes, including decreased pregnancy, clinical pregnancy, and live birth rates, without any detrimental effects on neonatal outcomes [9]. Several mechanisms may explain this phenomenon, including possible toxic effects related to cryoprotectants used in vitrification, which may increase DNA fragmentation in biological cells [10]. Furthermore, the solidification of cells from vitrification results in a glass-like state, which may reduce thermomechanical stress, leading to cell fracturing [11]. Finally, there may be small molecular movements within the glass-like state that can cause relaxation and ultimately cell degradation [12]. These mechanisms may also be involved in vitrification of oocytes. However, it is difficult to determine whether the negative effects of vitrification on embryos translate into oocytes.

Most studies on oocyte cryopreservation have demonstrated positive outcomes after short vitrification durations. The exceptions are two case reports in the literature that demonstrated live births after 12 and 14 years of vitrification respectively [13,14]. Based on these case reports, we cannot determine if success after this duration of time would be common or an exception. The effects of long-term cryostorage on the biological competence of thawed oocytes and their effect on clinical outcomes, such as fertilization, pregnancy, and live birth rates, need to be further explored.
Here, we present four cases in which long-term storage of vitrified oocytes for 11, 12, and 13 years for social freezing or cancer has led to four successful live births, and two cases, one with partial thawing at 13 and then 14 years and one with warming 15 years after vitrification of oocytes that failed to result in a live birth.

All cases underwent the same vitrification and thawing protocols.

**Case report**

1. **Case 1**
In May 2008, a 38-year-old single woman, gravida 0 para 0, visited the McGill University Reproductive Center for oocyte cryopreservation for social freezing. She underwent routine screening tests that demonstrated a multifibroid uterus with small intramural fibroids (<1 cm in diameter) without involvement of the uterine cavity. The ovaries were polycystic, with follicle counts greater than 12 on each side. All the other tests indicated a normal fertility profile. She requested multiple cycles of oocyte preservation to obtain enough banked oocytes to conceive two children in the future.

She underwent treatment in our clinic consisting of controlled ovarian hyperstimulation (COH) under a gonadotropin-releasing hormone (GnRH) antagonist protocol with recombinant follicle stimulating hormone (r-FSH) (75 IU daily; Follitropin beta, Merck, Canada) and human menopausal gonadotropin (hMG) (75 IU daily; Repronex, Ferring, Canada). Pituitary down-regulation was achieved with Ganirelix Acetate 250 mcg (Orgalutron, Merck, Canada) subcutaneous injections daily as part of a variable start protocol, with the antagonist being initiated on day 5 of stimulation when the lead follicles measured 13 mm in diameter. Thirty-six hours after administration of 5,000 IU of human chorionic gonadotropin (hCG) (hCG; Merck Serono, Montreal, Canada), oocytes were retrieved transvaginally using a 17-gauge single-lumen needle (Cook Inc, Brisbane, Australia) under ultrasound guidance (Voluson S8; General Electric Corp., Boston, MA, USA) and conscious sedation. Three stimulation cycles were performed with no complications, using the same doses of medication and protocol. A total of 48 metaphase-II oocytes were vitrified. The oocytes were suspended in equilibration medium containing 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide for 5 to 15 minutes at room temperature, then transferred to vitrification medium containing 15% (v/v) EG, 15% (v/v) dimethyl sulfoxide, and 0.5 M sucrose at room temperature for 45 to 60 seconds. The oocytes were then loaded onto a McGill Cryoleaf (Medicult, Copenhagen, Denmark) and immediately plunged into liquid nitrogen for storage. More information on our vitrification protocol is available in the following publication [15].

The patient subsequently moved to California and requested that 18 of her vitrified oocytes be transported to the Pacific Fertility Center in San Francisco, California. She underwent robotic myomectomy in San Francisco, California, for an enlarged fibroid uterus. She became pregnant in 2011 with a natural transfer cycle, with donor sperm insemination using vitrified oocytes at 42-year of age. Her pregnancy was complicated by preeclampsia and required a cesarean section, given her history of myomectomy. The patient’s daughter was healthy. The thawing protocol used at the California in-vitro fertilization center is unknown.

She returned to our clinic in October 2019 at 50-year of age and returned to Montreal. The patient had no relevant medical issues. Thirty oocytes were stored at our center. She had a new male partner who was 52-year-old and otherwise healthy with normal semen analysis according to the 2010 World Health Organization (WHO) guidelines.

As the patient was perimenopausal with a history of amenorrhea for 6 months, a “random start” cycle was performed in August 2020. She was 51-year-old at the time of the procedure, 12-year from the time of oocyte cryopreservation. Endometrial preparation was achieved using oral estradiol valerate 6 mg daily (Estrace; Acerus Pharmaceutical Corp., Mississauga, Canada). On day 11, endometrial thickness of 8 mm was achieved. Oocyte warming was planned, and progesterone effervescent tablets (100 mg; Endometrin, Ferring, Canada) three times daily were commenced 120 hours before embryo transfer.

For warming, the McGill Cryoleaf was directly inserted into a warming medium containing 1.00 M trehalose for 1 minute at 37°C. Warmed oocytes were transferred into diluent medium-I containing 0.50 M trehalose for 3 minutes and then into diluent medium-II containing 0.25 M trehalose for 3 minutes. Oocytes were washed twice in washing medium for 3 minutes each. Surviving oocytes were inseminated by intracytoplasmic sperm injection (ICSI), and zygotes were further cultured in LifeGlobal medium (Guilford, CT, USA) up to day 6. Embryo transfer was performed on day 5, and any
good-quality blastocysts were vitrified on day 5 or 6, depending on when blastulation occurred.

Ten oocytes were successfully warmed (all survived) and ICSI was performed 2 hours later. Fertilization was assessed 16 to 19 hours after insemination for the appearance of two distinct pronuclei and two polar bodies. At this point, eight oocytes were successfully fertilized and two had degenerated. The zygotes were cultured in embryo maintenance medium (Cooper Surgical, Trumbull, CT, USA). Four blastocysts were obtained with Gardner’s grades as given: 4AA (day 5), 4BA (day 5), 4AB (day 6), and 4AB (day 6). No other lower-quality blastocysts were created. Embryo transfer using the 4AA (day 5) blastocyst with assisted hatching was performed under ultrasound guidance on September 27, 2020. Eleven days after embryo transfer, serum \( \beta \)-hCG level was 493 IU/L.

She underwent obstetrical ultrasonography at 6 weeks and 2 days gestational age, confirming a singleton intrauterine gestational sac with a positive fetal heart tone. She delivered a healthy child via cesarean section.

2. Case 2

In February 2005, a 37-year-old woman, gravida 0 para 0, presented to our clinic for social oocyte freezing. She underwent routine fertility screening tests with no abnormalities. She underwent three cycles of controlled ovarian stimulation (COH) as part of a GnRH antagonist cycle with r-FSH 225 IU daily (Follitropin Alpha; Merck Serono) and recombinant luteinizing hormone 75 IU daily (Lutropin Alpha; Merck Serono). Pituitary down-regulation was achieved with ganirelix acetate 250 mcg subcutaneous injections daily as part of a variable start protocol, with the antagonist being initiated on day 6 of stimulation when the lead follicles measured 13 or 14 mm in diameter, in each case. Thirty-six hours after administration of 250 \( \mu \)g recombinant hCG (Ovidrel; Merck Serono), pre-ovulatory oocytes were retrieved transvaginally under ultrasound guidance, as previously described. A total of 32 metaphase II oocytes were vitrified using the same protocol as previously described.

In February 2016, the patient returned to our clinic at the age of 47 years requesting the use of the cryopreserved oocytes. She had a male partner at the time. He was otherwise healthy; however, his semen analysis detected teratozoospermia with 2% normal forms on strict morphological evaluation.

In August 2016, the patient underwent a natural cycle with no estrogen or progesterone medication for planned embryo transfer. She was 48-year-old at the time of the procedure and 11 years from the time of oocyte cryopreservation. Ultrasound monitoring was performed until cycle day 11, when the endometrial lining measured 11 mm with a trilaminar stripe and a dominant follicle was present. Triggering was performed using 250 \( \mu \)g recombinant hCG. Oocyte warming was performed as previously described. No other luteal support is provided.

Of the seven thawed oocytes, five were subjected to ICSI as described above, whereas two oocytes had degenerated. Fertilization was assessed 16 to 19 hours later where four oocytes had successfully fertilized and one had degenerated. After three days of incubation, two embryos at the 8-cell stage were transferred with assisted hatching under ultrasound guidance. The remaining embryos were then arrested. The resultant serum \( \beta \)-hCG level was 378 IU/L on day 16 of embryo development. She underwent obstetrical ultrasonography at 6 weeks and 5 days of gestation, confirming a single intrauterine gestational sac with positive fetal heart tones and a crown-rump length measured at an appropriate size. She went on to deliver a healthy baby, with no complications at term.

3. Case 3

In January 2008, a 37-year-old single woman, gravida 0 para 0, presented to our clinic for social oocyte freezing. She underwent routine fertility screening tests with no abnormalities. She underwent two cycles of COH and oocyte banking, using a GnRH antagonist cycle with 225 IU of hMG daily for both cycles. Pituitary down-regulation was achieved with Ganirelix Acetate 250 mcg subcutaneous injections daily as part of a variable start protocol, with the antagonist being initiated on day 6 of stimulation when the lead follicles measured 13 or 14 mm in diameter, respectively. Thirty-six hours after administration of 250 \( \mu \)g recombinant hCG, oocytes were retrieved transvaginally under ultrasound guidance, as previously described. A total of 28 metaphase-II oocytes were vitrified using the same protocol described previously.

In 2012, she spontaneously conceived on her own at 42-year of age, with the live birth of a healthy child. This was followed by three early first trimester miscarriages and an ectopic pregnancy, all of which were conceived spontaneously.

In May 2018, the patient returned to our clinic at 47-year
of age, requesting to use her cryopreserved oocytes. She developed hyperthyroidism, which was poorly controlled with propylthiouracil (Phebra, Richmond Victoria, Canada); therefore, she was referred for medical optimization by an endocrinology specialist. Once her thyroid function was within the normal limits on medication, embryo transfer was performed. She had a male partner at that time, who was otherwise healthy with normal semen parameters according to the 2010 WHO criteria.

In July 2019, she was started on estradiol valerate 6 mg daily for planned embryo transfer. She was 48-year-old at the time of the procedure and 11 years from the time of oocyte cryopreservation. The estradiol valerate was increased to 10 mg daily from days 11 to 15 owing to a thin endometrial lining. On day 15 of the cycle, the endometrial lining was deemed adequate for embryo transfer and was measured at 9.7 mm. Oocyte warming was performed as described previously, and progesterone effervescent tablets 100 mg were administered vaginally three times daily 120 hours before embryo transfer.

All 28 oocytes were thawed at the patient’s request. Only one oocyte was degraded, and the remaining 27 oocytes were subjected to ICSI, as previously described. Fertilization was assessed 16 to 19 hours after insemination, and 21 oocytes were successfully fertilized, whereas the remaining six oocytes had degenerated. The zygotes were cultured in embryo maintenance medium. Eleven blastocysts were obtained with Gardner’s grades of 4AA (1; day 5), 4AB (1; day 6), 4BAs (4; day 6), 4AB (1; day 7), 4BBs (2; day 5), 4BB (1; day 6), and 3BB (1; day 5). No low-quality blastocysts were obtained. Embryo transfer using 4AA (day 5) blastocysts was performed under ultrasound guidance. The remaining embryos were then frozen. Approximately 2 weeks after embryo transfer, the serum $\beta$-hCG level was 409 IU/L. She delivered a healthy girl with no complications at term.

4. Case 4

In 2006, a 19-year-old gravida 0 para 0 woman was referred for emergency fertility preservation after being diagnosed with leukemia that required chemotherapy. A “random start” GnRH antagonist cycle with stimulation using 225 IU daily of hMG was performed. The GnRH antagonist was initiated 6 days after the initiation of COH. After 10 days of ovarian stimulation, the production of recombinant hCG was triggered. Thirty-six hours after administration of 250 μg of recombinant hCG, oocytes were retrieved transvaginally under ultrasound guidance, as previously described. A total of 26 metaphase II oocytes were vitrified using the previously described protocol.

Three years later, at the age of 22 years, after undergoing chemotherapy (medications unknown), she no longer menstruated, and her serum FSH level was 147 IU/L, confirming premature ovarian insufficiency. Oral contraceptives were then administered.

She subsequently returned to our clinic in 2018, 13 years after oocyte vitrification, at 31 years of age, with a partner desiring pregnancy. Baseline testing revered a semen analysis with 3.2 mL, a concentration of 10.7 mil/mL, 8% forward motility, and 1% strict normal forms. Her serum FSH level was 120 IU/L. She had stopped oral contraceptives 3 years prior and claimed to have menstruation several times per year. All other test results were normal.

She was started on estradiol valerate 6 mg daily for planned embryo transfer, and on day 10 of medication, the maximum endometrial thickness was 5.5 mm. The estradiol level increased to 10 mg daily from day 11 because of a thin lining. On cycle day 17, the endometrial lining was 6.9 mm and a decision was made to plan embryo transfer despite the sub-adequate lining. Progesterone effervescent tablets 100 mg were started vaginally three times daily 120 hours before embryo transfer. Oocyte warming was performed as described previously.

Twenty-six oocytes were thawed at the patient’s request. Nine oocytes were degraded and the remaining 17 oocytes were subjected to ICSI, as previously described. Fertilization was assessed 16 to 19 hours after insemination, and eight oocytes were successfully fertilized. The remaining oocytes were fertilized abnormally with four 0 pronuclei (PN), two 1PN, two 3PN, and one 4PN, all of which failed to cleave. The zygotes were cultured in embryo maintenance medium. On day 3, an 8 cell, grade 1 embryo was transferred under ultrasound guidance. The remaining embryos were then frozen. Approximately 2 weeks after embryo transfer, the serum $\beta$-hCG level was 319 IU/L. She went on to deliver a healthy boy at 35 weeks of gestation via cesarean section due to placental abruption.
Table 1. Summary of the three patients’ outcomes

<table>
<thead>
<tr>
<th>Patient 1, social fertility preservation</th>
<th>Age at collection (yr)</th>
<th>Age at transfer (yr) and OT</th>
<th>No. of vitrified MII oocytes</th>
<th>No. of thawed oocytes (% of total vitrified oocytes)</th>
<th>No. of fertilized oocytes (2PN) (ratio fertilized to survived oocytes)</th>
<th>No. of degenerated oocytes (total)</th>
<th>Total % of oocytes surviving thawing 19 hours later (%)</th>
<th>No. of cleavage stage embryos on day 3 (ratio of day 3 cleavage stage embryos to 2PN)</th>
<th>No. of high quality blastocysts (total) (ratio of high quality blastocysts to remaining cleavage stage embryos not transferred)</th>
<th>No. of transfers</th>
<th>Pregnancy outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1, social fertility preservation</td>
<td>38</td>
<td>51 (OT)</td>
<td>48</td>
<td>10 (21 %)</td>
<td>8 (8/8)</td>
<td>2</td>
<td>80</td>
<td>8 (8/8)</td>
<td>4 (4/8)</td>
<td>1 OBT</td>
<td>Singleton live birth at term, healthy</td>
</tr>
<tr>
<td>Patient 2, social fertility preservation</td>
<td>37</td>
<td>48 (OT)</td>
<td>32</td>
<td>7 (22 %)</td>
<td>4 (4/4)</td>
<td>3</td>
<td>57</td>
<td>2 (2/4)</td>
<td>0 (0/0)</td>
<td>1 TCT</td>
<td>Singleton live birth at term, healthy</td>
</tr>
<tr>
<td>Patient 3, social fertility preservation</td>
<td>37</td>
<td>48 (OT)</td>
<td>28</td>
<td>28 (100 %)</td>
<td>21 (21/21)</td>
<td>7</td>
<td>75</td>
<td>21 (21/21)</td>
<td>11 (11/21)</td>
<td>1 OBT</td>
<td>Singleton live birth at term, healthy</td>
</tr>
<tr>
<td>Patient 4, fertility preservation pre-chemo for leukemia</td>
<td>19</td>
<td>32 (OT)</td>
<td>26</td>
<td>26 (100%)</td>
<td>17 (17/17)</td>
<td>9</td>
<td>65</td>
<td>6 (6/17)</td>
<td>0 (0/0)</td>
<td>1 OCT</td>
<td>Healthy singleton live birth at 35 weeks with placental abruption</td>
</tr>
<tr>
<td>Patient 5, social fertility preservation</td>
<td>35</td>
<td>50 (OT) &amp; 50</td>
<td>27</td>
<td>27 (100%)</td>
<td>20 (20/20)</td>
<td>3 at time of thawing 4 at fertilization check</td>
<td>74</td>
<td>9 (9/20)</td>
<td>3 (3/9)</td>
<td>2 TBT &amp; OBT</td>
<td>2 biochemical pregnancy losses</td>
</tr>
<tr>
<td>Patient 6, social fertility preservation</td>
<td>37</td>
<td>50 (OT) &amp; 50 &amp; 51 (OT)</td>
<td>20</td>
<td>10 (50 %) &amp; 10 (50%)</td>
<td>4 (4/9) &amp; 3 (3/7) (5 and 4 oocytes were 0PN after ICSI)</td>
<td>1 &amp; 3 (all were degenerated at time of thawing)</td>
<td>80</td>
<td>4 (4/4) &amp; 1 (1/3)</td>
<td>1 (1/2) &amp; 0 (0/0)</td>
<td>3 TCT, OBT, OCT</td>
<td>No pregnancy</td>
</tr>
</tbody>
</table>

High-quality blastocysts were defined as gardner grade BB or better. No low-quality blastocysts were obtained. Please note that if OT was not marked, the transfer was of a frozen blastocyst obtained after initial oocyte fertilization, which was not transferred in the first cycle. All thawed oocytes that survived seemed of good quality and potential. Note patient 1 had previously thawed 18 oocytes in California as well, resulting in a total of 58% of oocytes having been thawed in total. Please note that besides what has been detailed in the article, there were no other abnormalities observed through any of the patients’ basic testing. Among the patients who underwent thawing of oocytes that were subsequently fertilized and re-frozen as embryos (patients 5 and 6), no degradation was subsequently noted during embryo thawing.

OT, oocyte thaw; MII, metaphase II; PN, pronuclei; OBT, one blastocyst stage embryo transferred; TCT, two cleavage stage embryos transferred; OCT, one cleavage stage embryo transferred; TBT, two blastocyst stage embryos transferred; ICSI, intracytoplasmic sperm injection.
5. Common management of all patients
Per clinical policy, all women at least 45-year of age who were attempting to conceive a pregnancy underwent pre-clearance and counselling by a maternal-fetal medicine specialist and had a normal mammography and either an electrocardiogram (ECG) or stress ECG, which demonstrated a sinus rhythm without abnormalities. ECG type was ordered at the discretion of the treating physician.

Oocyte degeneration was assessed both at the time of thawing and at the time of the fertilization check. Some thawed oocytes showed degeneration after ICSI procedure had been performed. This is likely due to damage from vitrification, perhaps combined with injury from ICSI, although this cannot be confirmed.

Table 1 that lists the outcomes of all patients who returned for fertilization and embryo transfer after vitrification of oocytes for at least 10 years, at our institution. This table includes all patients who did or did not conceive. As noted, of the 6/9 transfers, 5/6 patients were pregnant, of which 4/6 patients had live births. One patient experienced two miscarriages, whereas one patient failed to conceive. None of the patients had any issues or abnormalities in their tests performed as part of the basic examination.

The following protocol was used for blastocyst freezing and thawing.

To vitrify a blastocyst, an intact trophectoderm, blastocoel, and viable inner cell mass are used. The blastocysts were placed in an equilibration medium (Medicult, Copenhagen, Denmark) which contained 7.5% EG and 7.5% dimethylsulfoxide (DMSO) at room temperature for 3 minutes. Subsequently, embryos were transferred to a vitrification medium containing 15% EG, 15% DMSO, and 0.5 M trehalose solution for 1 mintue at room temperature. Subsequently, embryos were placed on a strip of Vitri straw (SciTech Inv., Tempe, AZ, USA) and submerged in liquid nitrogen.

For warming, the Vitri straw was rapidly submerged in 1 mL of 37°C warming solution containing 1.0 M trehalose for 1 minute. Subsequently, embryos were transferred to DS 1 solution (0.5 M trehalose) for 3 minutes and DS 2 solution (0.25 M trehalose) for 3 minutes. After two washes in basic medium at room temperature for 3 minutes each, blastocysts were transferred into a culture medium.

The time from thawing to transfer was 3-4 hours. After thawing, blastocysts that had greater than 50% of intact blastomeres were considered as having survived.

Discussion
The first successful live birth using thawed human oocytes after slow-freezing cryopreservation was described in 1986 [16]. Since then, vitrification techniques have largely replaced slow-freezing. Vitrification minimizes ice crystal formation, leading to higher rates of cell survival, fertilization, embryo quality, and pregnancy compared to slow freezing [17]. Despite advancements in freezing techniques, it remains unknown for how long oocytes can maintain their pregnancy potential after vitrification.

There is a need for assessment of oocyte viability over longer periods and involving more patients after vitrification. Our study confirmed that successful pregnancy outcomes can arise as a result of long-term storage. Other case reports have similarly revealed successful outcomes with the use of cryopreserved oocytes for up to 14 years after vitrification [13,14,18]. Swedish data on 1,254 women who underwent different methods of fertility preservation, either elective or for malignancy, suggest that more women have conceived or at least thawed oocytes after 10 or more years [19]. This can be determined by the ranges presented for return to the clinic after fertility preservation, which are listed as 0-13 years for social indications and 0-12 years for medical indications. However, from this article, it cannot be determined how many women returned after 10 or more years, nor if they conceived or had a live birth, because the data are presented in aggregate form. Therefore, the addition of four more cases of pregnancy after oocyte cryopreservation for more than 10 years clearly bolsters medical evidence. Cryopreservation is a valuable tool for social and medical reasons. Therefore, our experience supports the use of oocyte vitrification to preserve fertility past biological limits and for a relatively long time. The cryopreservation of oocytes can prolong reproductive potential during menopause. It can maintain the childbearing ability despite societal and social constraints. In particular, meeting a compatible long-term partner for reproduction is harder than it has been in the past, and there is sometimes a requirement to delay childbearing due to work or study limitations. Although some readers may conclude that the maximum duration for oocyte competency is 14 to 15-year after vitrification because the women in our series who thawed at 14 and 15 years failed to conceive, we believe this occurred by chance and that future studies will demonstrate oocyte survival beyond this time limit.
Conflict of interest

No potential conflict of interest relevant to this article was reported.

Ethical approval

None.

Patient consent

Informed consent is not required for retrospective case reports at our institution.

Funding information

None.

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