Live birth of a healthy baby from slow-freezing cryopreserved pronuclear stage embryos warmed using a standard devitrification protocol: Case report

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Running Title: Live birth from devitrified slow-frozen embryos

ABSTRACT

This paper reports on the birth of a healthy child after the transfer of pronuclear stage embryos cryopreserved through slow-freezing method, stored for nine years, and warmed through a standard devitrification protocol. Surviving embryos were transferred to a 45-year-old patient, resulting in clinical pregnancy and the birth of a live child. This case report demonstrated that long-term-stored pronuclear stage embryos cryopreserved through the slow-freezing method may successfully present cleavage, implantation and developmental viability after being warmed using a standard devitrification protocol.

Keywords: Case report; Cryopreservation; Devitrification; Slow-freezing.

INTRODUCTION

Recently, a new ultra-rapid cryopreservation technique, known as vitrification, has emerged as a more effective alternative to more traditional methods used in the preservation of human oocytes and embryos (Liebermann et al., 2002; Stehlik et al., 2005; Vatja et al. 2006; Stachecki, 2008; Almodin et al, 2010). Thus, there has been an increasing trend for the use of vitrification in cryopreservation processes in replacement to slow-freezing methods (Al-Hasani et al., 2007; Saragusty et al., 2011; de Moraes et al., 2009). Due to this shift towards vitrification, companies that produce media used in the cryopreservation of human cells are losing interest in producing solutions for slow-freezing protocols. As a result, it is possible that, in the near future, warming kits for the slow-freezing method may disappear from the market altogether. Besides, not only the technique, but also the type and the concentrations of cryoprotectants used in vitrification are totally different from those used in the slow-freezing methods (Rall et al.; 1985; Park et al., 2000). These have raised some concern in many human reproduction centers all over the world on how to warm human cells that have been cryopreserved through the slow-freezing method and been kept stored for long periods of time.

This paper reports on the birth of a healthy child after the transfer of long-term-stored pronuclear stage embryos cryopreserved through the slow-freezing method, stored for nine years, and warmed through a standard devitrification protocol.

CASE REPORT

Patient consent

Before a new embryo transfer attempt was performed, both the patient and her husband were fully informed on the circumstances involving the procedure, and consented to having their embryos warmed through the standard devitrification procedure.

Slow-freezing cryopreservation

A 36-year-old patient began infertility treatment in 2003 due to male factor. The stimulation cycle was initiated with 300 UI/day of GnRH analog (Synarel – Zodiac, São Paulo, Brazil) during 21 days, followed by 150 UI of hMG (Merional – Mezler, São Paulo, Brazil). Serial transvaginal ultrasound was performed to monitor and control follicular growth and endometrial thickness. Recombinant hCG (Ovidrel – Merk, São Paulo, Brazil) was administered on the night of the eleventh day of the cycle, followed by transvaginal ultrasound--guided oocyte retrieval 35 to 36 hours later. After oocyte aspiration, cumulus cells were removed from all oocytes and twelve mature metaphase II (MII) oocytes were selected out of twenty three. Intracytoplasmic sperm injection (ICSI) was performed in all twelve MII oocytes, and after 16h in controlled culture (37oC and 6% CO2 atmosphere), they were checked for fertilization.

Two pro-nuclei were observed in 10 out of the 12 injected oocytes. Six pronuclear stage embryos were cryopreserved while four embryos were kept in culture until day 3, when they were loaded into a Sydney IVF embryo transfer catheter (Cook IVF – Brisbane, Australia), and transferred into the uterine lumen under trans-abdominal ultrasound guidance. β-hCG serum levels were measured 12 days after embryo transfer to determine biochemical pregnancy. Transvaginal ultrasound was performed at 6 weeks of gestation to confirm clinical pregnancy, which consisted of two gestational sacs and one heartbeat. The result was the birth of a healthy boy.

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The exceeding embryos were cryopreserved through a slow-freezing method using FREEZE-KIT™ (Vitrolife – San Diego, USA), which consists of three solutions: Cryo-PBS (phosphate-buffered saline), EFS 1 (1.5M 1,2-propanediol) and EFS 2 (1.5M 1,2-propanediol and 0.1M Sucrose). Embryos were washed for 5 minutes in Cryo-PBS, incubated for 20 minutes in EFS 1 and washed quickly in EFS 2. They were then loaded into straws (two per straw), which were sealed and placed in a programmable freezer. The temperature was programmed to decrease to -7oC at 2oC/min rate, held at -7oC to allow manual seeding, and subsequently dropped to -30oC at 0.3oC/min rate. Samples were then plunged into liquid nitrogen for storage until warming.

WARMING

In January 2012, the couple returned to the clinic to try to achieve a new pregnancy using the embryos that had been kept stored in liquid nitrogen for the past nine years. The patient, now 45 years old, had her endometrium prepared for ET through the administration of estradiol valerate (2mg/day), during 16 consecutive days, until the endometrial lining reached 10 mm. The vitrification-warming system developed by Ingamed (Maringá, Brazil) was used in this case (Vitringá-Kit®). The warming kit is constituted of three solutions: DV-I (warming), DV-II (diluent) and DV-III (rinsing). Their chemical composition is based on different concentrations of sucrose (Almodin et al., 2010).

The straws containing the embryos were removed from LN2 and left at room temperature for 30 seconds and then incubated in water bath at 37oC for 30 seconds. After the straws were wiped with sterile gauze, both ends were opened, the contents expelled in a petri dish and analyzed under a stereomicroscope. Embryos were collected and incubated in DV-I for 1 min at 37oC, and subsequently moved to DV-II, in which they were incubated for 3 min. Finally, the embryos were washed in DV-III twice for 5 min each, and then moved into pre-equilibrated G-1™ Plus medium (Vitrolife – San Diego, USA) at 37oC, with a survival rate of 75%.

The zygotes were kept in culture for 24 hours and three embryos were transferred to the uterus on day 2, each one grade A with four cells (symmetric blastomeres with no fragmentation). β-hCG serum levels were measured 12 days after embryo transfer to determine biochemical pregnancy. Luteal phase was supported by 600 mg of intravaginal progesterone per day until 12 weeks of gestation. Transvaginal ultrasound was performed at 6 weeks of gestation to confirm clinical pregnancy, which consisted of a gestational sac and one heartbeat. Nine months later the result was the live birth of a healthy baby boy.

DISCUSSION

This case report is interesting as it reports on an IVF live birth of a healthy boy resulting from the transfer of pronuclear stage embryos cryopreserved through a slow-freezing method, stored for nine years, and warmed using a standard devitrification protocol.

Several studies have been performed in order to minimize cryostorage duration and the risk of intracellular ice crystal formation, and thus optimizing embryo survival (Trad et al., 1999). The development of well-established ultra-rapid protocols (vitrification) was a practical solution to the matter (Liebermann et al., 2002). Vitrification is a cryopreservation strategy in which the water remaining inside and around the cells is converted into an amorphous crystal-free solid resembling glass (Vatja et al., 2006). This is achieved by the combination of high concentrations of cryoprotectants and ultra-rapid cooling (Almodin et al., 2010). The fact that there is no ice-crystal formation, which is one of the main problems found with more traditional cryopreservation techniques, has made vitrification a very attractive technique.

Although there is no ice crystal formation during the vitrification process, this may take place during warming. Seki and Mazur (2009) demonstrated that samples of mouse oocytes submitted to the vitrification procedure, when warmed at high rates had a survival rate higher than 80%. However, when samples were warmed at low rates survival was close to 0%, regardless the freezing rate used. They interpreted the lethality of slow warming as a consequence of allowing the growth of small intracellular ice crystals.

According to Mishima and Stanley (1998) three phenomena occur during warming: i) the conversion of amorphous ice into ultra-viscous water; ii) devitrification, which is the conversion of ultra-viscous water into clear ice; and iii) by increasing the temperature, ice goes through a third phenomenon, recrystallization. Hence, warming is also a critical process due to the occurrence of crystallization and recrystallization. Two factors are important in this process, warming rates and the concentration of solutes. Rapid warming is essential to minimize both the formation of intracellular ice crystals and their growth to lethal size by recrystallization (Seki et al., 2008). Therefore, with both the slow-freezing as well as vitrification protocols the formation of ice crystals during warming is possible, a problem that may be avoided by high warming rates. This is the reason why the straws removed from liquid nitrogen were submitted to a water bath at 37ºC before being added to the DV- I solution, as normally performed in the divitrification process.

The events that follow are the exit of cryoprotectants from the cell, as well as its rehydration. High sucrose levels are needed during warming to diminish the harmful effects of permeable cryoprotectants, by reducing osmotic stress and controlling cell water influx (Fabbri et al., 2000), and thus reducing the time of exposure of cells to the toxic cryoprotective agents. In general, the solutions used in slow-freezing methods are composed of 10% (1-2M) of permeable cryoprotectants, whereas the solutions used in vitrification protocols contain 30% (5-8M) (Shaw et al., 2003). As the concentration of cryoprotectants used in vitrification is much higher than that used in the slow-freezing method, it is fair to infer that the warming protocol used in vitrification has been standardized in such a way as to avoid cell osmotic chock even more than in the slow-freezing protocol. Thus the warming protocol used in vitrification could be safely used for the warming of cells cryopreserved with the slow-freezing protocol, a concept that has been fully demonstrated by this report.

This case report, therefore, demonstrated that long-term-stored pronuclear stage embryos cryopreserved through the slow-freezing method may successfully present cleavage, implantation and developmental viability after being warmed using a standard devitrification protocol. Prospective randomized comparative studies should be carried out to certify the viability and safety of devitrifcation of embryos cryopreserved with the slow-freezing methods.

AUTHORS' ROLES

RFE, RMS and PW were responsible for embryo warming and culture. FJL was responsible for embryo transfer and luteal phase support. RFE, and PMA were responsible for drafting the article, and CGA for revising the article critically for important intellectual content. CGA and PW were responsible for the final approval of the version to be published.

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CONFLICT OF INTEREST

None declared

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