

ORIGINAL ARTICLE

First Live Birth in Germany after Re-Transplantation of Cryopreserved Ovarian Tissue: Original Device for Initiation of Ice Formation

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SUMMARY

Background: The problem of post-cancer infertility is of significant concern. The cryopreservation of ovarian tissue before cancer therapy with retransplantation after convalescence is the key to solving this problem.

Methods: Cryopreservation of ovarian tissue was performed in 2005 after surgical operation, post-operative low-temperature 22 hour transportation, and freezing using a special, original design block constructed for the initiation of ice formation (ice-seeding). We present the construction and function of this block.

Results: In 2011, it was noted that a baby was born after thawing and re-transplantation of ovarian tissue. The technical and biological aspects of initiated crystals formation in the process of cryopreservation are emphasised and discussed.

Conclusions: The first live birth in Germany after re-transplantation of cryopreserved ovarian tissue was noted. This cryopreservation was performed using the protocol described here. Block for auto-seeding of principally new construction recommended.

(Clin. Lab. 2012;58:933-938. DOI: 10.7754/Clin.Lab.2012.120224)

KEY WORDS

human, ovarian tissue, cooling, cryopreservation, ice-formation, live birth

INTRODUCTION

In USA only, overall cancer incidence rates in women have been declining by 0.6% annually since 1998. A total of approximately 774,000 women were diagnosed with malignant tumors [1]. Between 1998 and 2007, the overall incidence rate for cancer in children aged 14 years and younger increased by 0.6% per year [2]. Similar dynamics are observed in Europe. For example, the current estimate for 2010 is approximately 204,000

cancer cases for women in Germany. Every year in Germany, around 800 girls under age 15 are diagnosed with cancer [3].

In the same time, the death rate has decreased by 1.0% per year. Over the past 25 years, there have been significant improvements in the 5-year relative survival rate for all of the major childhood cancers due to new and improved anti-cancer treatments. The 5-year relative survival rate for children for all cancer sites combined improved from 58% for patients diagnosed between 1975 and 1977 to 82% for those diagnosed between 1999 and 2006 [4].

Due to the increasing effectiveness of cancer treatments and good long-term prognosis for young women, the

Manuscript accepted April 8, 2012

problem of post-cancer infertility plays an important role. Chemotherapy, depending on the treatment regime chosen, can be gonadotoxic and lead to the functional death of ovaries. The cryopreservation of ovarian tissue before cancer therapy with retransplantation after convalescence is the key to solving this problem [5-7].

Now a live birth after thawing and transplantation of ovarian tissue is reality [8-19].

In 2011 in Germany, the first baby was born after the surgical operation, post-operative low-temperature 22-hour transportation, cryopreservation, and re-transplantation of ovarian tissue [20,21].

Here we describe the detailed methodology of the transport of ovarian fragments at low temperatures and freezing of ovarian strips.

MATERIALS AND METHODS

Low-temperature transportation and preparation of strips

Except where otherwise stated, all chemicals were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA).

Biopsies of ovarian tissue were obtained from patient W with indications for anticancer therapy on August 22, 2005 at Dresden University Maternal Hospital. In accordance with the protocol of the network FertiPROTEKT, the ovarian tissue was sent to Bonn University Maternal Hospital.

This 22-hour transport of ovarian fragments from Dresden to Bonn was carried out in a transport container for blood in solution of Brahma I medium (Cryo Bio System, Paris, France). Currently, Cryo Bio System does not produce this solution. Instead of Brahma I solution, we recommend the following solution, which can be used for transportation of ovarian tissue and also for preparations of ovarian strips: Leibovitz L-15 medium with 5% Serum Substitute Supplement (SSS) (Irvine Scientific, Santa Ana, CA, USA).

On the next day, 23 August 2005, ovarian tissue was received in Bonn.

Using tweezers and scalpel No 22, ovarian fragments were partially separated from the medulla in Brahma II medium. At present, Brahma II is not produced by Cryo Bio System. Instead of Brahma II, we also recommend the use of self-prepared medium (see above).

All manipulations to prepare ovarian strips were performed in the medium cooled to 4°C in an ice bath. Toward the end of the preparation of the strips (about 30 minutes) the temperature of the medium was increased to 12°C.

Freezing

Standard 2 mL cryovials (Nunc, Roskilde, Denmark) were filled with 1.8 mL of cryopreservation medium containing L-15 Medium (Leibovitz) with L-glutamine +1.5 mol/L dimethyl sulphoxide (DMSO) +10% serum substitute supplement (SSS, Irvine Science, St. Ana,

CA, USA) and were cooled in iced water (0°C). Ovarian strips were then transferred to the cryovials and held in iced water for 30 minutes. Cryovials were subsequently placed in an IceCube 14S freezer (SyLab, Neupurkersdorf, Austria), where the freezing chamber had previously been stabilized to 2°C for 20 - 30 minutes. The cryopreservation program was as follows: (i) the starting temperature was 2°C because the temperature of the cryovials increased from 0°C to 2°C after their manipulation and transfer from ice-water to the auto-seeding block; (ii) samples were cooled from 2°C to -6°C at a rate of -2°C/minute; (iii) samples were then held at -6°C for 10 minutes; (iv) samples were cooled from -6°C to -40°C at a rate of -0.3°C/minute; and (v) cooling to -140°C at a rate of -10°C/minute followed by plunging of cryovials into liquid nitrogen.

Additionally to the cooling process, at -6°C the initiated ice-formation (agitated seeding) during 60 seconds was performed.

The auto-seeding block was specially developed for this study in collaboration with the manufacturers (SyLab), generating a novel piece of equipment (Figure 1). The location of this block in the freezer chamber guarantees full contact of the upper part of the cryovial wall with the tube that conducts liquid nitrogen (Figure 1), so that ice formation is initiated at a certain temperature. The reliability of the auto-seeding block was tested before starting the cryopreservation of ovarian tissue of patient W.

With seeding at -6°C, the liquid nitrogen begins to enter and cool the conducting tubes, and these tubes cool the cryovials containing freezing medium with ovarian strips (Figure 1).

Recommended protocol for freezing

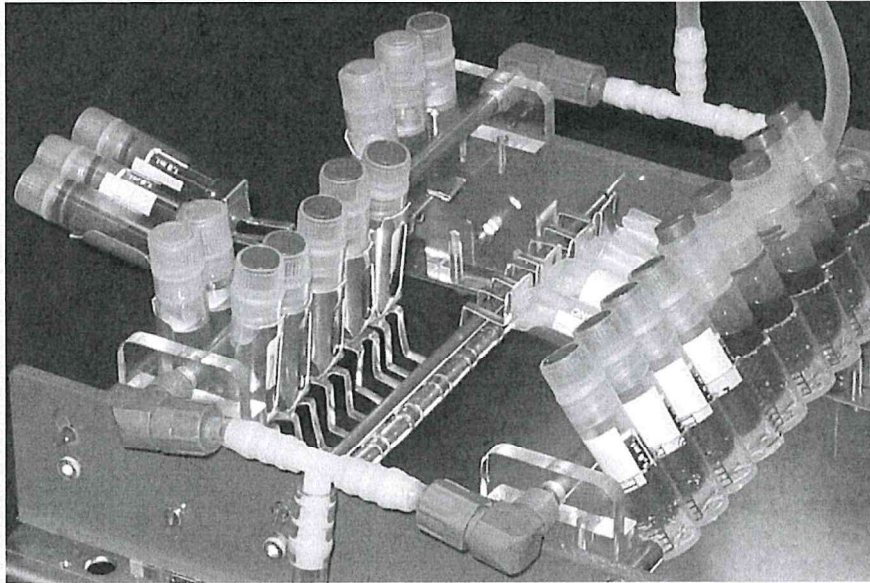
At present, the recommended freezing solution for ovarian tissue based on Isachenko et al. [22-28] is: L-15 Medium (Leibovitz) with L-glutamine +10% serum substitute supplement (SSS, Irvine Science, St. Ana, CA, USA) +6% dimethyl sulfoxide +6% ethylene glycol +0.15 M sucrose. Cologne group recommends for cryopreservation of ovarian strips to use 5 mL cryovials.

Recommended protocol for thawing

The procedure for thawing includes holding the cryotubes for 30 seconds at room temperature and then immersion in a 100°C (boiling) water bath for 60 to 75 seconds (because of differences in construction of cryovials of different manufacturers and temperature in laboratory) and expelling the contents of tubes into solution for the removal of cryoprotectants. Theoretically, there is a risk of overheating of ovarian strips during thawing in boiling water. To avoid this overheating, the following is recommended. Standard surgical cotton pad (tampon) (for example, Telaprep No.1, Paul Hartmann AG, Heidenheim, Germany) is placed on the bottom of 5 mL cryovials, and thereafter we place a strip on this pad. This mode prevents a direct contact of tissue with walls of cryovial and leads to a fast, and at the

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ORIGINAL DEVICE FOR INITIATION OF ICE FORMATION

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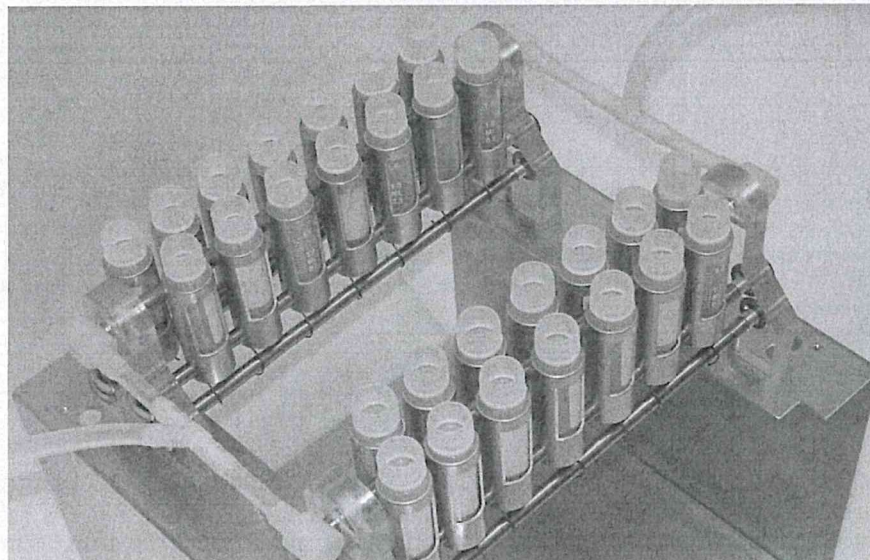


Figure 1. Auto-seeding block. (A) Prototype, (B) Manufactured by SyLab Company (Austria).

same time "delicate" heating of ovarian strips. We visually control the exposure time in the boiling water by the presence of ice in freezing medium; as soon as the ice is 2 to 1 mm from the apex, we remove the cryotube from the boiling water, at which point the final temperature of the freezing solution is between 4 and 10°C. Within 5-10 seconds after thawing, we expel the strips from the cryotubes into 10 mL thawing solution (0.5 M sucrose) in a 100 mL specimen container. The

stepwise dilution of cryoprotectants can be achieved using the same principle as for the saturation by ethylene glycol (see Figure 1 in Isachenko et al. [29]). We place the container on a shaker and continuously agitate at 200 osc/minute for 15 minutes at room temperature. For the stepwise rehydration of the tissue pieces in 30 minutes at room temperature, we use the same 'dropping' methodology [29]: slow addition of culture medium to the solution of sucrose with the ovar-

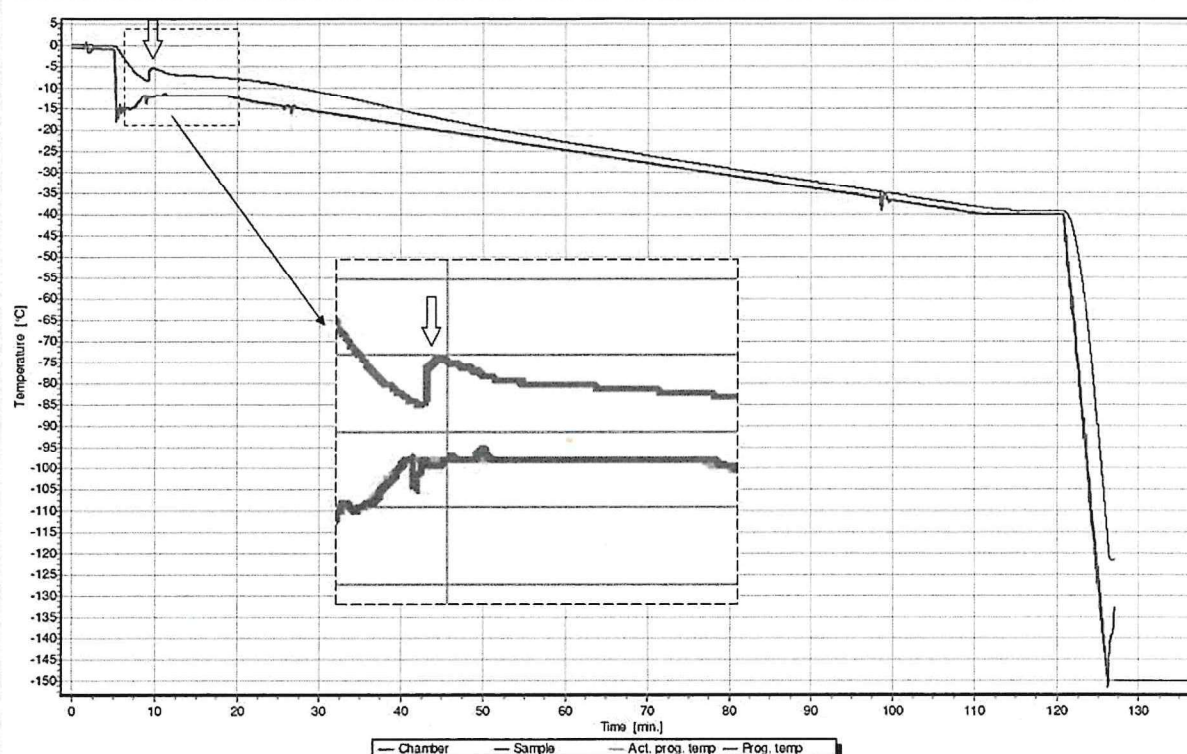


Figure 2. Temperature profile of freezing of ovarian tissue from Patient W on 23 August 2012.

(Red line) temperature of chamber, (blue line) temperature in cryovial with ovarian tissue, (white head arrows) increase in temperature after initiation of crystallization.

ian strips. The final sucrose concentration is 0.083M, resulting in almost isotonic conditions. Finally, we wash strips three times each in culture medium for 10 minutes and transfer, as required, for in-vitro culture or retransplantation.

RESULTS

In 2011, the first baby was born in Germany following the surgical operation, post-operative low-temperature 22-hour transportation, freezing, thawing, and retransplantation of ovarian tissue.

DISCUSSION

An IceCube 14S freezer was used for the conventional freezing of samples in a cryobank of University Maternal Hospital Bonn, because this freezer incorporates a

feature that initiates ice formation (seeding) automatically.

The cryopreservation program, with which we have frozen ovarian tissue of patient W, is described above (Figure 2). This program presupposes the initiation of ice-formation.

What happens during cryopreservation with spontaneous ice formation? When the temperature of the cryopreservation medium reaches -17 to -20°C , a sharp increase in temperature to -5 to -8°C is noted in the sample. After this increase in temperature, the cooling rate was stabilized to the normal $-0.3^{\circ}\text{C}/\text{minute}$ for 30 minutes (Figure 3 in Isachenko et al. [23]). During these 30 minutes, the rate of temperature decrease was changed from $-1^{\circ}\text{C}/\text{minute}$ (for a period of 10 minutes) to the given cooling rate of $-0.3^{\circ}\text{C}/\text{minute}$ (Figure 3 in Isachenko et al., [23]).

Seeding (i.e. initiated ice formation), usually carried out manually, is a central part of this protocol, followed by very slow cooling at a rate of $-0.3^{\circ}\text{C}/\text{minute}$ (Figure 2).

Seeding is necessary to control nucleation and propagation of ice crystals, minimizing the potential damage caused by the physical changes associated with ice formation, with the release of latent heat of fusion. The temperature of seeding varies from -6 to -9°C.

Based on an original protocol of cryopreservation by Demirci et al. [30], at a cooling rate of -2°C/minute, ice formation is initiated by the release of negative calories within the freezing chamber [30]. Two cryoprotectants, DMSO and propylene glycol, were tested at different concentrations of 1, 1.5, and 2 mol/L. Using 2 mol/L DMSO, spontaneous ice formation was observed at the temperature rise at -11°C: mortality rate of follicles was 8.4% [30].

The same authors later noted that in sheep ovaries the cryopreservation protocol is more effective with initiated ice formation (manual seeding) than with semi-automatic seeding, when ice formation begins without manual initiation [31]. Our results support this point of view [23].

An entire human ovary with its vascular pedicle was frozen [32,33] using the following cryopreservation procedure: the ovary was perfused and immersed in 10% DMSO, and placed in a large volume cryovial. The cryovial containing the ovary was then placed in a freezer and frozen with a cooling rate of -1°C/minute, without initiation of ice formation. The authors observed high survival rates of follicles, small vessels, and stromal cells, as well as a normal histological structure in all ovarian components after thawing. The results presented here show a negative effect of spontaneous ice formation in cryopreservation of ovarian tissue. The difference between the present results and those of Martinez-Madrid et al. [32] and Jadoul et al. [33] can be explained by the fact that in this study small fragments of ovary were used, whereas in the other studies whole ovaries were cryopreserved that had been equilibrated with cryoprotectant via perfusion. Based on the authors' observations, the use of initiated ice formation might have improved viability of frozen cells after thawing. The results of our research show a positive effect of low temperatures on cryopreserved ovarian tissue (not published results). To illustrate our point, the official protocol of cryopreservation for the cryobank of human ovarian tissue of Cologne University, Germany, presupposes the 22-24 hour cooling of ovarian tissue after surgical removal: currently, in our clinic, the operation is completed and immediately following the operation we store the ovarian tissue at 4°C and then freeze this ovarian tissue on the following day.

In conclusion, the first live birth in Germany after re-transplantation of cryopreserved ovarian tissue was noted. This cryopreservation was performed according to the protocol described above. Block for auto-seeding of principally new construction recommended.

Declaration of Interest:

The authors declare that they have no competing interests.

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