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ARTICLE

Overnight ovarian tissue transportation for centralized cryobanking: a feasible option

BIOGRAPHY

Jana Liebenthron obtained her PhD at the University Clinic Bonn under the supervision of Professor Markus Montag. In 2014, she assumed the position of Director of the IVF-Lab and Cryobank for human gametes and ovarian tissue. She is an active executive board member of the network FertiProtekt, highly specialized for ovarian tissue-banking and supports national and international hospitals in this field of fertility preservation. She has managed the new department UniCareD at the University clinic Düsseldorf since January 2018.

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KEY MESSAGE

Ovarian tissue cryopreservation is gaining more acceptance in clinical practice as an established option for fertility protection. Nevertheless, as the techniques require high expertise for reproducible success rates, the concept of centralized cryobanking can be carried over by fertility preservation networks to allow optimal tissue freezing and storage for everyone.

ABSTRACT

Research question: Is overnight transportation of ovarian tissue before cryopreservation in a centralized cryobank from the FertiPROTEKT network feasible?

Design: Data from 1810 women with cryopreserved ovarian tissue after overnight transportation from December 2000 to December 2017 were analysed with a focus on transportation, tissue activity parameters and pregnancy, and delivery rates after transplantation.

Results: A total of 92.4% of tissue samples arrived at ideal temperatures of 2–8°C, 0.4% were transported at temperatures lower than ideal and 6.4% were transported at temperatures that were too high, generally due to mishandling of the inlayed cool packs of the transportation boxes. In 62 women, 78 tissue transplantations were carried out. A subgroup of 30 women who underwent a single orthotopic transplantation with fulfilled criteria of a complete follow-up after transplantation until the end of study, a premature ovarian insufficiency after gonadotoxic therapy as well as the absence of pelvic radiation, was further analysed. In this group, transplantations into a peritoneal pocket accounted for 90%. Transplants were still active at 1 year and above after transplantation in 93.3%. Pregnancy and delivery rates were 46.7% and 43.3%, respectively, with one ongoing pregnancy at the end of the study.

Conclusions: Overnight transportation for central cryobanking is a feasible concept that results in high reproducible success rates through standardized professional tissue freezing and storage.

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KEYWORDS

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Fertility preservation
Gonadotoxic therapy
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Ovarian tissue transplantation
Overnight transportation

INTRODUCTION

The awareness of ovarian tissue freezing as an option for fertility preservation has steadily increased (*von Wolff et al., 2015; Winkler-Crepaz et al., 2017*) since the first successful transplantation of cryopreserved ovarian tissue resulted in a live birth in 2004 (*Donnez et al., 2004*). To date, more than 140 live births have been documented (*Dittrich et al., 2015; Donnez and Dolmans, 2015a; van der Ven et al., 2016; Jadoul et al., 2017; Jensen et al., 2017; Pacheco and Oktay, 2017; Donnez et al., 2018; von Wolff et al., 2018a*), and it is now considered an established procedure (Association of the Scientific Medical Societies in Germany [AWMF] joint Guidelines from the German Society of Gynaecology and Obstetrics [DGGG], the German Society of Urology [DGU] and the German Society of Reproductive Medicine [DGRM], 2017) and as one of several options for women before gonadotoxic therapies (*von Wolff et al., 2018*b).

Freezing of ovarian tissue, however, still faces many challenges. The risk of transferring malignant cells needs further evaluation (*Dolmans et al., 2013; Dolmans and Masciangelo, 2018*), and several issues regarding transplantation need to be resolved, such as the best location of transplantation and the amount of tissue needed for transplantation to regain sufficient ovarian function. Furthermore, cryobanks need to meet several safety regulations and should carry out complex viability assays on tissue samples before cryopreservation and after thawing (*Beckmann et al., 2018*).

Therefore, it would be clinically and scientifically desirable to prepare, freeze and store tissue in highly specialized and centralized cryobanks that meet all these requirements. Such centralized cryobanks have already been set up in several countries, and their success rates, as well as their research output, confirm the value of this concept (*Jensen et al., 2015; van der Ven et al., 2016*).

Importantly, improvements in ovarian tissue transportation to central cryobanks will enable more women undergoing oncological treatment, a sensitive and emotional time, to undergo ovarian biopsy in their local hospital while still having their tissue stored in a centralized and expert cryostorage centre.

In Denmark, one centralized cryobank located in Copenhagen receives transported tissue from different parts of the country by air, a process that can require up to 5 h in total (*Jensen et al., 2015*). In Germany, most of the tissue is sent to centralized cryobank institutions, such as Bonn, Düsseldorf or Erlangen, which mostly requires overnight transportation (*van der Ven et al., 2016; Liebenthron and Montag, 2017*). The success rates of all centres (*Jensen et al., 2015; van der Ven et al., 2016*) have shown that transportation is a possible option. More data, however, are required to fully evaluate overnight transportation before it can be recommended for implementation in other countries.

We, therefore, systematically and retrospectively reviewed and analysed data from one university institution that started centralized cryobanking services and logistics in 2000. We present data on all patients that had ovarian tissue cryopreserved after overnight transportation to restore fertility after gonadotoxic cancer treatment. We report pregnancy and delivery rates achieved after orthotopic ovarian transplantation as well as tissue activity rates after 1 year. To reduce heterogeneity of our analysis, we focused on patients who underwent a single orthotopic transplantation, who had oligomenorrhoea or amenorrhoea for at least 4 months before transplantation and FSH concentrations of 20 IU/l or greater, underwent two assessments at an interval of at least 4 weeks (premature ovarian insufficiency [POI]), had not undergone radiotherapy of the pelvis and who underwent a complete follow-up to the end of statistical analysis.

MATERIALS AND METHODS

Before starting ovarian tissue cryopreservation, an ethical vote, approved by the Institutional Review Board of the University of Bonn (No. 007/09, dated 3rd July 2009), was obtained to offer a central cryobanking service and to use up to 10% of the incoming tissue for quality-control measures and patient-related research. Ovarian tissue was cryopreserved between 2000 and 2017.

Surgical retrieval of ovarian tissue and overnight transportation to the Cryobank

Between 25 and 50% of tissue from one ovary was removed by laparoscopy, without using electric coagulation. The side of the corpus luteum or a pre-ovulatory follicle was avoided. Transportation to the Cryobank Bonn was either by direct or by overnight transportation using specialized commercial providers.

The tissue was placed in a 30-ml sterile flask, filled with 2–8°C cold Custodiol® (Dr Franz Köhler Chemie GmbH, Bensheim, Germany) perfusion solution. The flask was placed in a special isolated cooling box (FIGURE 1), in the middle part of three pairs of cooling packs, precooled to 2–8°C; a temperature logger (delta T Gesellschaft für Medizintechnik GmbH, Fernwald, Germany) (FIGURE 1) inside the box was used for continuous monitoring of the temperature. The temperature remained stable at 2–8°C for a maximum of 22 h. In addition, a patient's data sheet was added.

The tissue was scheduled to reach the Cryobank within a maximum of 22 h. Upon arrival at the Cryobank, the tube containing the tissue was temporarily stored at 2–8°C until preparation. Data from the temperature logger were checked and logged into the patient's records.

Preparation of ovarian tissue for cryopreservation

According *Beckmann et al. (2018)*, tissue were prepared in a sterile class II lamina air flow in a contamination-free environment. The entire ovarian piece was placed in a culture dish containing fresh Custodiol® at a convolution cooling plate (UKH602, FRYKA Kältetechnik GmbH, Esslingen, Germany) pre-cooled to a temperature of 2–8°C. Precision 22′scalpels and anatomical forceps were used to gently remove the medulla, leaving a tender layer of the medulla on the cortex surface to facilitate revascularization of the graft after transplantation (*Donnez and Dolmans, 2015b*). Cortex stripes (about 8 × 4 × 1 mm) were prepared for later transplantation and cryopreserved as described below.

From the remaining cortical tissue, six small-standardized biopsies were obtained from different areas of the

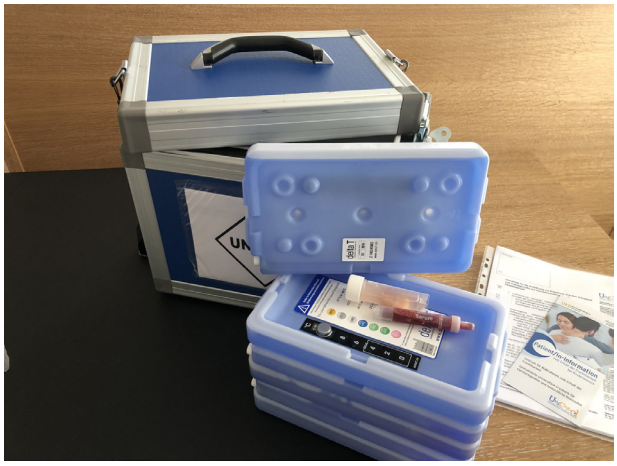

FIGURE 1 Transportation box for overnight transportation with cooling elements (at 2–8°C), temperature logger, flask with transportation medium and ovarian tissue, blood for analysing anti-Müllerian hormone concentration in serum and instruction sheets.

prepared cortex using a two-mm diameter biopsy punch (PFM Medical AG, Cologne, Germany) for quality control.

Cryopreservation protocol

A slow-freezing protocol for cryopreserving ovarian tissue was carried out, modified from the procedure initially described by *Gosden et al. (1994)* (*Isachenko et al., 2007*; 2012; *Bastings et al., 2016*). In short, ovarian tissue strips (around 10 pieces per patient) were pre-incubated for 30 min in 2°C pre-cooled sterile filtered freezing solution (Leibovitz's L-15 Medium without phenol red, Gibco by Life Technologies, Paisley, UK) containing 10% dimethyl sulfoxide (DMSO) (Cryo-Sure DMSO, WAK-Chemie Medical GmbH, Steinbach, Germany) and 10% human serum albumin (HSA) (Irvine Scientific, Santa Ana, CA, USA). Tissue pieces were transferred each into one 1.8-ml cryogenic vial (Nunc; Thermo Fisher Scientific, Denmark), and a controlled freezer (IceCube 14S-A, SY-LAB, Neupurkersdorf, Austria) was used for slow freezing, which supports automatic seeding. Tissue pieces in cryogenic vials were cooled at a rate of –2°C per min and automatic seeding was started as soon as the medium in the sample vial reached the temperature of –6°C. After successful seeding, the temperature was held for another 5–8 min, and was then slow cooled at –0.3°C per min to –40°C followed by fast cooling at –10°C per min to –140°C. Cryogenic vials were plunged into liquid nitrogen and transferred into storage boxes. A vapour phase storage tank (MVE 1500 Series Storage) was used for storage at –90°C (Chart MVE BioMedical Industries, Inc., Garfield Heights, USA).

Quality control

Three 2-mm diameter biopsies were used for the quality analysis of density and viability of primordial and primary follicles embedded in the cortical tissue (*Beckmann et al., 2018; Liebenthron et al., 2018*) after transportation, preparation and before cryopreservation and after thawing of the cryopreserved control biopsies before transplantation. Tissue was digested with collagenase (Sigma-Aldrich Chemie GmbH, Munich, Germany), follicles were stained with calcein–acetoxymethylester (Promega GmbH, Mannheim, Germany) and follicle density was analysed by fluorescence microscopy (*Liebenthron et al., 2013*).

Follicle density as well as serum anti-Müllerian hormone concentrations, antral follicle count and patient's age at tissue removal were regarded as the ovarian reserve parameters. These were used to calculate the amount of ovarian tissue required for transplantation. The tissue-removing units carried out histological analysis of a small piece of tissue to exclude microscopically detectable malignant cells.

Thawing and transplantation of ovarian tissue

Before transplantation, ovarian function was estimated through evaluation of menstrual cycle, antral follicle count and endocrine parameters by the patient's reproductive physician. Transplantation took place in 13 different centres. For this purpose, the frozen samples, stored until then at the centralized Cryobank Bonn, were transferred in a suitable MVE CryoShipper (Chart MVE BioMedical Industries, Inc., Garfield Heights, USA) and transported to the corresponding transplant centres by only one of the personnel of the Cryobank, who was completely involved in the thawing procedure on-site at the transplant centres (called 'mobile thawing service', which was established for clinics and centres that did not have appropriately qualified personnel).

The thawing protocol used was modified from published procedures (*Isachenko et al., 2007; Rosendahl et al., 2011; Dittrich et al., 2012; Isachenko et al., 2012a; Bastings et al., 2014*). Cryogenic vials were kept at room temperature for 30 s and then immersed in a 37°C water bath for about 2 min until only a centrally located thin ice spindle was left. Tissue pieces were transferred into 0.75-M sucrose (Sucrose ACS reagent, Sigma-Aldrich Chemie GmbH, Munich, Germany) in Dulbecco's phosphate buffered saline solution (DPBS) (CTS, Gibco by Life Technologies, Paisley, UK), containing 10% HSA (Irvine Scientific, Santa Ana, CA, USA) for 15 min, followed by 15-min incubation in 0.375 M sucrose, DPBS and HAS, and another 15-min incubation in 0.125 M Sucrose, DPBS and HSA. Finally, tissue was washed twice (10 and 5 min) in DPBS and HSA. The entire thawing process was carried out at room temperature using an orbital shaker.

Before transplantation, tubal patency was assessed with sodium chloride solution to exclude tubal blockage (*Beckmann et al., 2018*). Tissue was then

immediately transplanted by laparoscopy to the remaining ovary, into a peritoneal pocket, or both, which was prepared close to the fimbria. Ultrasound and endocrine monitoring took place after transplantation at regular intervals.

Subgroup analysis for calculating the success rates after transplantation of frozen–thawed tissue after overnight transportation

According to *van der Ven et al., (2016)*, for reducing data with heterogeneity, analysis focused on those patients with a follow-up of at least 1 year after orthotopical transplantation and those who underwent a single transplantation and were diagnosed with POI after gonadotoxic treatment and without radiation of the small pelvis. The presence of POI was defined as oligomenorrhoea or amenorrhoea for at least 4 months before transplantation, and FSH concentrations were 20 IU/l or over, with two assessments at an interval of at least 4 weeks.

Outcomes

The outcome 'biochemical pregnancy' was defined as a pregnancy ending before confirmation by ultrasound through no visualization of a gestational sac and a noticed rapid fall in serum beta-HCG concentration. Clinical pregnancy outcomes were described by the presence of a fetal heartbeat after 6–7 weeks of pregnancy, and the definition of a miscarriage was selected if a clinical pregnancy ends before the 20th week of gestation.

RESULTS

In total, 1972 tissue samples were cryopreserved, 1810 (91.8%) of those samples after overnight transportation. The tissue had been removed by 71 collaborating external centres in Germany, Switzerland and Austria, all members of the network *Ferti*PROTEKT (www.fertiprotekt.com). The time interval between removal of tissue and arrival at the cryobank for samples transported overnight was 16–22 h. Patient characteristics and temperature of the tissue on arrival at the cryobank are shown in TABLE 1. A total of 1672 out of 1810 (92.4%) tissue samples reached the cryobank at 2–8°C (defined as an ideal temperaturebecause lower temperatures can result in freezing and higher temperatures in degradation of the tissue during transportation). In seven (0.4%) cases, the temperature was less than 2°C owing to mishandling of tissue dispatch by the centres. The cooling packs were stored in the freezer instead of a fridge as shown by the logged start temperature of –22 and –18°C. These samples were non-viable and were discarded. In 91 (5.0%) cases, temperature was 9–15°C and, in 25 (1.4%) cases, the temperature was 16–21°C; in all these samples, the follicles were still viable. Increased temperature was caused by insufficient cooling of the cooling packs. Temperature was not recorded owing to malfunctioning or missing data logger in 15 (0.8%) samples, but follicles were found to be viable in all these samples.

Transplantation was carried out 78 times in total (62 patients) with tissue that had been transported overnight before cryopreservation (TABLE 2). Three patients received a non-orthotopical transplantation. One patient in a pocket of the sacrouterine ligament, one patient in an abdominal pocket (subperitonealy into adipose tissue) and one patient on the upper arm (subcutaneously into adipose tissue) – the latter transplantation was carried out twice. Eleven patients repeated transplantations, nine of which had two transplantations and two patients that received three transplantations. Seven patients (two of them each had two transplantations) were lost to follow-up after transplantation, and no data were available for those cases. Four patients underwent a radiation of the small pelvis and seven patients had no defined POI at the time of transplantation. A subgroup analysis was carried out after the exclusion of 32 women. This analysis included 30 women who underwent a single or first orthotopic transplantation with a defined POI after gonadotoxic treatment, but without pelvic radiation and with a complete follow-up until end of study.

In 27 out of 30 women (90%), tissue was transplanted into a peritoneal pocket and, in three women (10%), in both a peritoneal pocket and into the ovary. Ideal transport conditions (2–8°C) were recorded in 26 (86.7%) cases, whereas, in four cases (13.3%), temperatures were between 9 and 15°C (TABLE 2). In this group, all biopsies for quality testing before and after cryopreservation were viable, and vitality parameters were within normal range (*Liebenthron et al., 2018*); detailed data not shown here.

In 28 out of 30 cases (93.3%), tissue was still active 1 year after transplantation. Fourteen patients achieved clinical pregnancies and 13 women delivered at least one child; in one patient, pregnancy was still ongoing until the end of study. Outcome among the full set of patients (n = 62) was as follows: 16 women achieved first-time pregnancy after single or first transplantation biochemical pregnancies, 14 of which developed into clinical pregnancies. Thirteen delivered a child, whereas one pregnancy was still ongoing (TABLE 3). Fourteen out of these 16 (87.5%) patients that achieved a pregnancy were 35 years of age or younger at the time of cryopreservation, 15 out of 16 (93.8%) received transplantation only subperitoneally, and 14 out of 16 (87.5%) became pregnant spontaneously. Two women achieved a pregnancy after IVF, one of whom also conceived a second pregnancy spontaneously, which resulted in the live birth of twins. In addition, two other women conceived a second pregnancy spontaneously after the first transplantation, one of which resulted in the birth of a healthy baby and the other in a miscarriage. In all woman, a minimum of 33–50% of one ovary had been removed and, on average, 5.0 ± 1.7 tissue pieces were transplanted (TABLE 3).

Details of all patients who achieved a pregnancy (n = 16) after transplantation are presented in TABLE 4. In general, 19 pregnancies were documented, 17 clinical (with heart activity) and two biochemical (without an amnion sac). From these 17 clinical pregnancies, 17 babies were born (two pairs of twins), one pregnancy was ongoing and one pregnancy terminated with a miscarriage. Maximum age at time of cryopreservation was 36 years, 14 out of 16 (87.5%) of patients were 35 years or younger at the time of cryopreservation; however, a live birth could be documented for all three of them.

DISCUSSION

We focused our analysis on a well-defined group of patients, 30 transplantations in 30 women (only one/first transplantation per women), according to the study design by *van der Ven et al. (2016)*, to reduce heterogeneity. Our study reveals that pregnancy and delivery rates are around 46.7 and 43.3% after overnight transportation in this defined subgroup. These results are similar to rates of other centres and networks without

TABLE 1 CHARACTERISTICS OF PATIENTS WHO HAD OVARIAN TISSUE CRYOPRESERVED AFTER OVERNIGHT TRANSPORTATION

n	**1810**
Age at cryopreservation (mean age, years ± SD)	27.7 ± 7.1
Main diagnosis (malignant diseases), *n* (%)	1620 (89.5)
Breast cancer	821 (45.4)
Hodgkin's lymphoma	410 (22.7)
Non-Hodgkin's lymphoma	69 (3.8)
Leukaemia	45 (2.5)
Sarcoma	87 (4.8)
Cerebral cancer	33 (1.8)
Gastrointestinal cancer	40 (2.2)
Gynaecological cancer	95 (5.2)
Other types of malignancies	20 (1.1)
Main diagnosis (non-malignant diseases), *n* (%)	53 (2.9)
Thalassemia	25 (1.4)
Lupus erythematosus	18 (1.0)
Turner syndrome	5 (0.3)
Multiple sclerosis	2 (0.1)
Other types of non-malignant diseases	3 (0.2)
Unknown diseases, *n* (%)	137 (7.6)
Temperature of tissues at arrival, *n* (%)	
<2°C	7 (0.4)
2–8°C	1672 (92.4)
9–15°C	91 (5.0)
16–21°C	25 (1.4)
Not recorded	15 (0.8)

overnight transportation, and support the concept that centralized cryobanking with overnight tissue transportation is a feasible option.

Even though our data from this mainly descriptive work support this concept, we cannot exclude some bias. The number of transplantations is limited; furthermore, the number of samples in this retrospective study without overnight transplantation was insufficient to serve as a control group. Therefore, the success rates after overnight transportation can only be compared with historical data of other centres and networks. Data from other centres are quite heterogeneous, hampering a direct comparison.

Van der Ven et al. (2016) reported on 95 transplantations in 74 women. In 40 women with the same strict inclusion criteria as being used in our analysis (one transplantation per woman), nine deliveries corresponding to a delivery rate of 22.5% were found. *Jensen et al. (2015)* reported on 53 transplantations in 41 women. In 32 infertile women, 42 transplantations were carried out (1.3 transplantations per woman) and 10 women delivered at least one child corresponding to a delivery rate of 31.3%.

Meirow et al. (2016) reported on 21 transplantations in 20 women (1.1 transplantations per woman); six deliveries were reported corresponding to a delivery rate of 30.0%. *Diaz-Garcia et al. (2018)* reported on 44 transplantations in 44 women (one transplantation per woman); 10 deliveries were reported corresponding to a delivery rate of 22.7%.

A comparison of our data with these cohort studies revealed that success rates were not lower after overnight transportation, thereby demonstrating that overnight transportation does not seem to have a clinically relevant negative effect on the viability of the tissue. The delivery rate in our cohort, however, was lower if all transplanted women (n = 62) were analysed. The delivery rate was 21.0% (1.3 transplantations per woman) compared with 43.3% in our selected group of women (n = 30). This difference is due to the high number of women either lost to follow-up, with radiation of the pelvis or with repeated transplantations.

Transportation of whole organs at 2–8°C has already taken place for many years. Hypothermia suppresses metabolism and enzyme activity by 50% for each 10°C drop, resulting in an approximate 10% remaining metabolic rate at 5°C (*Cantu and Zaas, 2011*). The maximum transportation time of whole organs varies owing to different tolerance for cold ischaemia and is around 6 h for hearts, 8 h for lungs, 12–15 h for livers and 24 h for kidneys (*Guibert et al., 2011*).

The effect of ovarian tissue storage at 4°C for around 24 h has been analysed in non-human as well as in human tissues. In mice, these conditions did

TABLE 2 CHARACTERISTICS OF PATIENTS WHO HAD OVARIAN TISSUE TRANSPLANTED AFTER OVERNIGHT TRANSPORTATION AND CRYOPRESERVATION

	All patients[a]	Subgroup analysis[b]
Number of patients, n	62	30
Number of transplantations	78	30
Age at cryopreservation (mean age ± SD)	31.8 ± 5.1	31.1 ± 5.0
Age at transplantation (mean age, years ± SD)	35.7 ± 4.5	34.8 ± 4.3
Main diagnosis (malignant diseases), n (%)	60 (96.8)	29 (96.7)
Breast cancer	28 (45.2)	13 (43.3)
Hodgkin's lymphoma	12 (19.4)	8 (26.7)
Non-Hodgkin's lymphoma	7 (11.3)	5 (16.7)
Leukaemia	1 (1.6)	0
Sarcoma	1 (1.6)	1 (3.3)
Cerebral cancer	0	0
Gastrointestinal cancer	5 (8.1)	0
Gynaecological cancer	4 (6.5)	1 (3.3)
Other types of malignancies	2 (3.2)	1 (3.3)
Main diagnosis (non-malignant diseases), n (%)	2 (3.2)	1 (3.3)
Thalassemia	0	0
Lupus erythematosus	2 (3.2	1 (3.3)
Turner syndrome	0	0
Multiple sclerosis	0	0
Other types of non-malignant diseases	0	0
Unknown diseases, n	0	0
Transplantation sites, n = 78		
Peritoneal pocket	66 (84.6)	27 (90)
Ovary	3 (3.8)	0
Ovary and peritoneal pocket	5 (6.4)	3 (10)
Heterotopic	4 (5.1)	0
Transport conditions (temperature at arrival of the tissue), n (%)		
<3°C	0	0
4–8°C	54 (87.1)	26 (86.7)
9–15°C	7 (11.3)	4 (13.3)
6–21°C	1 (1.6%	0
Not recorded	0	0

[a] Including all patients (n = 62) with orthotopical (n = 59) and non-orthotopical transplantation (n = 3) (one patient with tissue, transplanted in a pocket of the sacrouterine ligament, one patient in an abdominal pocket [subperitonealy into adipose tissue] and one patient on the upper arm [subcutaneously into adipose tissue]); the latter transplantation was performed twice; patients who underwent repeat transplantations (n = 11) (nine of whom had two transplantations and two patients who received three transplantations); patients lost to follow-up after transplantation (n = 7) — no data available for those cases (two of them each had two transplantations); patients who underwent a radiation of the small pelvis (n = 4); and patients who had no defined primary ovarian insufficiency (POI) at the time of transplantation (n = 7).

[b] For reducing data with heterogeneity, analysis focused on patients with a follow-up of at least 1 year after orthotopical transplantation and those who underwent a single transplant and were diagnosed with POI after gonadotoxic treatment and without radiation of the small pelvis. Presence of POI was defined as oligomenorrhoea or amenorrhoea for at least 4 months before transplantation and FSH concentrations were 25 IU/l or more, with two assessments at an interval of at least 4 weeks.

not have a negative effect on histological morphology, on the number of collected and fertilized oocytes and on the blastocyst development rate but the implantation rate and the rate of live pups was reduced (*Kamoshita et al., 2016*). In large animals such as goats (*Silva et al., 2000*) and cows (*Lucci et al., 2004*), the morphology of the primordial follicles also remained unaffected. The same was found in primates (*Hornick et al., 2012*). In humans, *Laronda et al. (2014)* also reported that ovarian tissue was almost unaffected by storage at 4°C for around 24 h, as analysed by histology and Tdt (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) assays to evaluate apoptosis. TUNEL-positive cells were mainly found at the edges of the tissue. *Isachenko et al. (2009)* used an in-vitro culture model to study human ovarian tissue quality and its follicles after a prolonged suprazero temperature exposure for 26 h. No negative influence on follicle quality and their in-vitro development competence,

TABLE 3 OUTCOMES OF PATIENTS WHO HAD OVARIAN TISSUE TRANSPLANTED AFTER OVERNIGHT TRANSPORTATION AND CRYOPRESERVATION

	All patients[a]	Subgroup analysis[b]
Number of patients, n	62	30
Number of transplantations, n	78	30
Number of transplanted tissue pieces (3–5 × 5–8 × 1 mm^3) (mean ± SD)	5.0 ± 1.	4.8 ± 1.3
Active tissue, n (%)	45/62 (72.6)	28/30 (93.3)
Patients who achieved a biochemical pregnancy, n (%)	16/62 (25.8)	14/30 (46.7)
Patients who achieved a clinical pregnancy n (%)[c]	14/62 (22.6)	14/30 (46.7)
Patients with live births, n (%)	13/62 (21.0)	13/30 (43.3)
Miscarriages, n (%)	0	0

[a] Including all patients (n = 62) who underwent non-orthotopical transplantation (n = 3) (one patient with tissue, transplanted in a pocket of the sacrouterine ligament, one patient in an abdominal pocket [subperitonealy into adipose tissue] and one patient on the upper arm [subcutaneously into adipose tissue]); the latter transplantation was carried out twice; ptients who underwent repeat transplantations (n = 11) (nine of whom had two transplantations and two patients who received three transplantations); patients who were lost to follow-up after transplantation — no data available for those cases (n = 7) (two patients had two transplantations each); patients who had radiation of the small pelvis (n = 4); and patients who had no defined primary ovarian insufficiency (POI) at the time of transplantation (n = 7).

[b] For reducing data with heterogeneity, analysis focused on those patients with a follow-up of at least 1 year after orthotopical transplantation and those who underwent a single transplant and were diagnosed with POI after gonadotoxic treatment and without radiation of the small pelvis. Presence of POI was defined as oligomenorrhoea or amenorrhoea for at least 4 months before the transplantation and FSH concentrations 25 IU/l or greater, with two assessments of at least an interval of 4 weeks.

[c] In one patient, pregnancy was ongoing until end of study.

and metabolic activity of cells from the growing follicles, was detectable. The tissue was not negatively compromised. In a second study from the *Isachenko et al. (2013)*, the viability of the follicles was even increased after 24 h at 5°C as analysed by immunohistochemical proliferation markers. In a further study, it was shown that these kinds of treated tissue induced an increased neo-vascularization on chorioallantoic membranes (*Isachenko et al., 2012b*).

Although different studies should only be compared with great care as too many confounders might affect the results, the hypothesis that tissue storage for 24 h at 2–8°C does not negatively affect tissue viability seems to be solid.

In the present study, and according to the work of the Isachenko *et al*., we used Custodiol® HTK (Histidine – Tryptophan – Ketoglutarate) medium, which is used for whole organ transportation (*Voigt and DeLario, 2013*). Corresponding to *Loganathan et al., (2010)*, *Lema Zuluaga et al., (2013), Gallinat et al., (2013)* and *Kahn and Schemmer, (2017)*, this medium prolongs the ischaemia tolerance. Nevertheless, the apparent robustness of ovarian tissue is surprising, as mature oocytes are sensitive to reduced temperature (*Sutton et al., 2003*). Primordial follicles and mature follicles and oocytes cannot be compared as their complexity and level of maturity is completely different. It could be speculated that physiologically, primordial follicles must be robust to withstand the numerous stressors, which they are exposed to from birth until the end of the reproductive phase. One exception is DNA damage, as oocytes have a unique and specialized quality-control system that eliminates primordial follicles efficiently after the detection of DNA double strand breaks (*Tuppi et al., 2018*).

What are the advantages and disadvantages of centralized cryobanking requiring transportation? The advantage of centralized cryobanking is that cryopreservation of tissue is carried out by highly specialized centres with a high degree of expertise. Furthermore, the high numbers of cases in centralized cryobanks involving transportation, cryopreservation and transplantation will allow further improvements of this still evolving technique. A disadvantage of centralized cryobanking is that long transportation time might result in degeneration of antral follicles. Therefore, in-vitro maturation of oocytes extracted from the tissue before cryopreservation (*Fatemi et al., 2011; Park et al., 2016*), which increases overall pregnancy chances, may no longer be possible.

The main challenge of overnight transportation is to set up a logistically reliable transportation system. In the present study, in 123 out of 1795 recorded transportations, (6.9%), the temperature was either too high or too low. Temperature that was too low (0.4%) was a result of mishandling the system, which had a devastating effect as the tissue was initially frozen during the transport. Temperature that was too high was not detrimental, as the viability assay revealed that the follicles were still viable. *Lucci et al. (2004)*, however, demonstrated a negative effect of higher temperatures in bovine tissue, as did *Silva et al. (2000)* in goats and *Duncan et al. (2016)* in bovine, feline and canine tissue. Furthermore, extension of the transportation time of over 24 h might also have negative effects as shown by *Silva et al. (2000)* in goat and *Lima et al. (2010)* in canine tissue. Therefore, effective education of the tissue-removing centres and stringent transportation logistics are required to avoid minor and major tissue damage.

In conclusion, the idea of fertility-preservation networks, with their own designated highly qualified centralized cryobanks, is a well-functioning concept. The overnight transport of ovarian tissue in special transportation boxes using temperature data loggers does not seem to have any negative effects on the survival of follicles or the whole viability of the frozen–thawed ovarian tissue transplants, their function and the outcomes after transplantation. The advantages are obvious and should be considered in the future: same high standards and conditions for all patients through high-tech equipment and conditions for preparation; and cryopreservation, quality testing and storage of the incoming organ tissue

TABLE 4 CHARACTERISTICS OF PATIENTS AND OUTCOMES OF PREGNANCIES ACHIEVED AFTER OVARIAN TISSUE TRANSPLANTATION

Number	Diagnosis	Age at cryopreservation (years)	Age at transplantation (years)	Amount (%) of cryopreserved tissue (100% = one ovary)	Temperature at arrival of the tissue, °C	AMH before cryopreservation, ng/ml	Chemotherapy	POI	Transplantation site	Number of transplanted tissue pieces (~4 × 8 × 1 mm^3)	Tissue active 1 year after transplantation	Pregnancies, n	Clinical pregnancy outcome	Tissue still active
1	Breast cancer	33	38	33	7	0.54	Yes	Yes	Peritoneal pocket	7	Yes	1	Ongoing	Still pregnant
2	Lupus erythematosus	26	32	50	5	1.31	Yes	Yes	Peritoneal pocket	4	Yes	1	Live birth twins	Yes
3	Hodgkin's Lymphoma	27	32	50	7	NA	Yes	Yes	Peritoneal pocket	5	Yes	1	Live birth	Yes
4	Hodgkin's lymphoma	35	37	50	10	0.96	Yes	Yes	Peritoneal pocket	5	Yes	1	Live birth	Yes
5	Hodgkin's lymphoma	21	27	50	7	2.25	Yes	Yes	Peritoneal pocket	4	Yes	1	Live birth	Yes
6	Breast cancer	34	38	50	6	0.54	Yes	Yes	Peritoneal pocket	5	Yes	2	One live birth; one miscarriage	Yes
7	Hodgkin's lymphoma	31	36	50	6	NA	Yes	Yes	Peritoneal pocket	6	Yes	1	Biochemical	Yes
8	Hodgkin's lymphoma	33	37	50	8	0.83	Yes	Yes	Peritoneal pocket	5	Yes	2	Live birth; live birth	Yes
9	Breast cancer	33	37	50	4	2.21	Yes	no	Peritoneal pocket	4	Yes	1	Biochemical	Yes
10	Cystadenofibroma	20	27	33	7	NA	No	Yes	Peritoneal pocket	4	Yes	2	One live birth (IVF); one live birth of twins	Yes
11	Breast cancer	36	37	50	5	3.61	Yes	Yes	Peritoneal pocket	3	Yes	1	Live birth	Yes
12	Hodgkin's lymphoma	30	32	50	6	2.43	Yes	Yes	Ovary, peritoneal pocket	5	Yes	1	Live birth	Yes
13	Ovarian borderline tumour	21	28	50	5	NA	No	Yes	Peritoneal pocket	3	Yes	1	Live birth (IVF)	Yes
14	Ewing^s sarcoma	26	29	50	9	8.02	Yes	Yes	Peritoneal pocket	4	Yes	1	Live birth	Yes
15	Breast cancer	36	39	50	6	1.06	Yes	Yes	Peritoneal pocket	5	Yes	1	Live birth	Yes
16	Breast cancer	33	36	50	4	5.53	Yes	No	Peritoneal pocket	3	Yes	1	Live birth	Yes

All pregnancies were spontaneous and singletons unless otherwise stated (twins, after IVF).

Includes all patients with follow-up data (n = 55); patients who have underwent non-orthotopical and orthotopical transplantations; those who have undergone repeat transplantations; patients who have undergone radiation of the small pelvis; and patients who had no defined primary ovarian insufficiency (POI) at the time of transplantation.

AMH, anti-Müllerian hormone; NA, not analysed.

for later thawing and transplantation to achieve successful restoration of the endocrine function and induction of pregnancy.

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