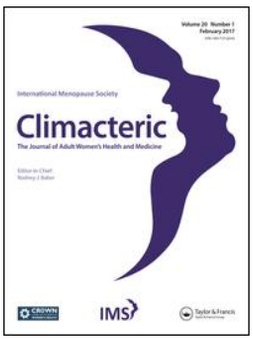

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ORIGINAL ARTICLE

Ovarian tissue cryopreservation and transplantation prevents iatrogenic premature ovarian insufficiency: first 10 cases in China

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ABSTRACT

Objective: The aim of this study was to report on the first 10 cases of ovarian tissue cryopreservation (OTC) and retransplantation (OTCT) in China.

Methods: A retrospective descriptive study was performed of 10 Chinese women with different diseases undergoing OTC/OTCT in the first specialized center in China. Patients' ovarian function and fertility were followed up.

Results: The 10 cases included five cases of cervical cancer and one case each of endometrial cancer, breast cancer, rectal cancer, myelodysplastic syndrome, and aplastic anemia, respectively. The average age at OTC was 33.70 years; the time from OTC to OTCT was 15 months. The average number of transplanted ovarian tissue pieces was 4.9, with 9.5 pieces remaining cryopreserved. The OTCT position for nine cases was in a right-sided peritoneal pocket of ovarian fossa, and for one patient was in bilateral pockets. The average time from OTCT to restoration of ovarian function was 3.4 months. One year after OTCT, all ovaries were still functioning normally. In the first case, the function now remains preserved for more than 3 years. So far, the woman who wishes to conceive has no pregnancy.

Conclusion: Regarding ovarian function, OTC and OTCT were successful and reliable in China's first cryobank. We expect to perform more retransplantations in the near future, which will add to the global data.

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Ovarian tissue cryopreservation; ovarian tissue retransplantation; fertility preservation; premature ovarian insufficiency; slow freezing; ovarian tissue transportation

Introduction

Advances in detection and treatment of cancer in reproductive-age women have improved significantly in recent years, increasing long-term survival and long-term quality of life[1]. Unfortunately, radiotherapy and/or chemotherapy can lead to premature ovarian insufficiency (POI)[2]. POI not only can cause infertility, but also increases the incidence of chronic diseases such as cardiovascular disease, osteoporosis, and dementia and increases the risk of early death[3]. The risk for such negative outcomes can be reduced by exogenous hormone replacement therapy (HRT). Ovarian tissue cryopreservation (OTC) and subsequent retransplantation (OTCT) may offer another means of restoring a normal hormonal environment to those women[4].

OTC and OTCT technology has some advantages compared to other fertility preservation methods, such as oocyte or embryo cryopreservation[5]. A piece of ovarian tissue contains hundreds or thousands of follicles. Therefore, OTC/OTCT can restore not only fertility, but also endocrine function. OTC/OTCT is the only fertility option for pre-pubertal girls, and is also the best choice for patients whose radiotherapy and/or chemotherapy cannot be delayed. OTC can be performed at any time during the menstrual cycle without delaying anticancer treatment[4].

The report of the first live birth after OTCT in 2004[6] and the second in 2005[7] greatly accelerated the implementation of this fertility preservation method. Rates of pregnancy and live births have continued to increase steadily, with over 140 live births reported worldwide and success rates of more than 95% after OTC/OTCT[8,9]. Pregnancy and delivery rates were 46.7% and 43.3%, respectively. Transplants were still active over 1 year after retransplantation in 63–93.3% of cases[8]. The mean duration of ovarian function after OTCT is 4–5 years, but the function can persist for up to 10 years[10], depending on the follicular density at the time of OTC[9]. OTC/OTCT should no longer be considered experimental[8,11].

The first International Fertility Protection Center in China was established in 2015[12,13]. In 2018, we were able to publish the first case of successfully cryopreserved ovarian tissue retransplantation in China in this journal[12], and organized the first consensus on this method with Chinese experts together with international advisors[5]. We have cryopreserved

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ovarian tissue for more than 300 patients from 15 hospitals within Beijing. The aim here is to report on the first 10 successful cases in China with different diseases (mostly cancer) using OTC before chemo/radiotherapy with subsequent tissue retransplantation to prevent iatrogenic POI.

Methods

Harvesting and transport of ovarian tissue and cryopreservation

Ovarian tissue was surgically removed from 10 patients from different Chinese provinces in two Beijing hospitals. The tissue was transferred into cold Custodiol® HTK solution (4–8 °C) and sent to the centralized ovarian tissue cryobank at the Beijing Obstetrics and Gynecology Hospital, Capital Medical University within 2 h at 4–8 °C. There is a comprehensive Standard Operation Procedure for each further step of cryopreservation performed during the same or next day of surgery, as reported elsewhere[5,12,14]. In short, the medulla of the ovarian tissue was removed under sterile conditions with a blade (Jinhuan Medical, No. 21; Shanghai, China) to obtain cortex with a thickness of 1 mm, which was cut into 4 mm × 8 mm pieces, and a punch with a diameter of 3 mm (Kai Medical, Germany) was used to obtain a round tissue piece for activity detection.

Slow freezing (SF) was used as the conventional method for OTC, according to a protocol published previously[15,16]. The processed cortex pieces were put into the cryopreservation tubes containing cryoprotectant to attain the precooling balance, and a controlled freezer (PLANER Kryo 360–1.7, UK) was used to begin freezing according to the temperature drop gradient set by the computer program. Once the temperature reached −120 °C, the tubes were transferred to a liquid nitrogen tank at −196 °C.

Thawing and retransplantation of cryopreserved ovarian tissue

Round tissue pieces with a diameter of 3 mm were thawed 1 day before OTCT to measure the tissue activity and follicle count. The number of tissue pieces to be transplanted was determined according to the number of follicles, age, and purpose of the retransplantation. The cryopreserved tubes were kept at room temperature for 30 s and then placed in a water bath at 37 °C for 2 min, and the tissue pieces were then poured into a sterile Petri dish. The tissues were then sequentially placed into different concentrations of thawing solution, and the cryoprotectant in cells was replaced by water. The thawed ovarian tissue was transported to the operating room within 10 min and laparoscopically placed into a pelvic peritoneal pocket of ovarian fossa, with the medullary surface at the bottom to establish a good blood supply. In nine cases the tissues were autotransplanted into the right side, and in one patient into bilateral sides.

Assessment of follicle viability for quality control (viability test)

Follicle activity was assessed by Calcein-AM (Sigma) staining using a protocol published previously[15]. Calcein-AM is a cell-permeant dye that can be used to determine cell viability in most eukaryotic cells. In live cells, the non-fluorescent Calcein-AM is converted to green-fluorescent calcein, after acetoxymethyl ester hydrolysis by intracellular esterases[17]. All live follicles were observed and counted using fluorescence microscopy (excitation/emission 495/515 nm; Leica, Germany).

Monitoring of hormonal levels and ultrasound after OTCT

Hormonal levels such as follicle stimulating hormone (FSH) and estradiol (E2) were measured before OTC, before OTCT, and at 3, 4, and 6 months and 1, 2, and 3 years after OTCT. Pelvic ultrasound examination was performed to observe follicles from the transplanted ovary and regular monitoring of follicle growth was performed.

Statistical analysis

All data were analyzed using Statistical Package for the Social Sciences version 23.0 (SPSS, Chicago, IL, USA). Values are presented as mean ± standard deviation (range: minimum–maximum).

Ethical approval

The Ethical Committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University, approved the project (2017-KY-020-01). All patients provided signed consent.

Results

Characteristics of women undergoing ovarian retransplantation

Table 1 presents the disease type, date at OTC and OTCT, number of tissue pieces transplanted, transplantation position, and time between OTC and OTCT. After OTC, six cases underwent radiotherapy and chemotherapy, two underwent radiotherapy, and two underwent uterine and bilateral ovarian salpingectomy. All 10 patients developed POI and menopausal symptoms after cancer treatment, and a reduction in the modified Kupperman score[18] was observed from more than 15 (mean) before OTCT to less than 6 after OTCT. The scores have subsequently remained low for all patients. The most frequent complaints were hot flashes and sweating, often more than 10 times (score 'severe') or 3–9 times (score 'moderate') per day before OTCT, and all improved greatly after OTCT. Four patients started HRT in the time span between OTC and OTCT because of very severe menopausal symptoms, two without a uterus used E2-only (patches), one with a uterus used tibolone (without getting bleedings), and

Table 1. Characteristics of the 10 patients who underwent OTC and OTCT.

Number	Age at OTC (years)	Type of disease	Date of OTC	Number of OTC pieces	Date of OTCT	Number of OTCT pieces	Transplantation position
1	35	Cervical cancer	12 June 2015	7	9 September 2016	4	Peritoneal pocket of ovarian fossa (right)
2	40	Cervical cancer	14 September 2016	10	6 September 2017	6	Peritoneal pocket of ovarian fossa (right)
3	38	Cervical cancer	19 September 2016	14	6 September 2017	6	Peritoneal pocket of ovarian fossa (both)
4	37	Breast cancer	10 November 2016	13	16 April 2018	6	Peritoneal pocket of ovarian fossa (right)
5	27	Aplastic anemia	18 April 2017	25	6 September 2018	4	Peritoneal pocket of ovarian fossa (right)
6	31	Myelodysplastic syndrome	27 September 2016	23	6 September 2018	6	Peritoneal pocket of ovarian fossa (right)
7	27	Cervical cancer	15 March 2017	10	6 September 2018	3	Peritoneal pocket of ovarian fossa (right)
8	32	Rectal cancer	6 September 2017	16	16 April 2019	5	Peritoneal pocket of ovarian fossa (right)
9	36	Cervical cancer	3 April 2018	15	16 April 2019	5	Peritoneal pocket of ovarian fossa (right)
10	34	Endometrial cancer	1 November 2018	11	5 September 2019	4	Peritoneal pocket of ovarian fossa (right)

Patients' age at OTC, 33.7 ± 4.423 years (range: 27–40); pieces of cryopreserved ovarian tissue, 14.4 ± 5.739 (range: 7–25); number of tissue pieces transplanted for the first time, 4.9 ± 1.101 (range: 3–6); time from OTC to OTCT, 15 ± 4.163 months (range: 10–23); time from OTCT to restoration of ovarian function 3.4 ± 1.265 months (range: 2–6); average time from OTCT to 15 December 2019, 17.7 months. OTC, ovarian tissue cryopreservation; OTCT, ovarian tissue cryopreservation transplantation.

one used E2 + sequential dydrogesterone (getting regular progestogen-induced withdrawal bleedings). The remaining six patients were given only traditional Chinese medicine because they were afraid to use HRT. For all four patients who used HRT, this treatment was stopped after OTCT. All 10 patients before and after OTCT took Chinese medicine, and all received the same type of Chinese medicine.

Fresh and frozen–thawed ovarian tissue

The follicle count per 3 mm of fresh ovarian tissue was 25.40 ± 23.646 (range: 1–75) and per 3 mm of frozen–thawed ovarian tissue was 31.10 ± 19.604 (range: 9–79), without significant difference ($p > 0.05$). There was no significant difference in the survival of the follicles in all fresh and frozen–thawed ovarian tissue. Typical photographs of follicles under a fluorescence microscope and a flat-light microscope are shown in Figure 1.

Menstrual recovery and ultrasound monitoring of follicles after OTCT

Of the 10 patients, three were hysterectomized due to gynecological cancer. Menstrual cycles returned 3–6 months after OTCT for all seven of the non-hysterectomized patients. All 10 patients were followed up with regular appointments after OTCT, and follicular growth could be observed by ultrasound in all patients. Only one of the 10 patients wished to become pregnant, which has not yet occurred. Typical photographs of ovaries and follicles under ultrasound are shown in Figure 2.

Changes in FSH and E2 before OTC and before/after OTCT

The FSH level (IU/L) before OTC was 4.44 ± 1.90 (range: 1.8–7.17), and the time span between OTC and OTCT was 65.42 ± 53.15 months (range: 15.05–174.88). The FSH level 3 months after OTCT was 17.78 ± 8.11 (range: 5.55–29.74) and 1 year after OTCT was 15.29 ± 11.35 (range: 5.14–36.84).

The E2 level (pg/ml) before OTC was 102.89 ± 57.11 (range: 11.80–183.00), and the time span between OTC and OTCT months was 36.65 ± 28.05 (range: 11.80–86.65). The E2 level 3 months after OTCT was 77.80 ± 56.53 (range: 11.80–171.43) and 1 year after OTCT was 113.16 ± 84.94 (range: 13.99–254.92).

Discussion

OTC and OTCT successful for all 10 patients

This report covers 10 Chinese women with eight different types of cancer, one patient with myelodysplastic syndrome and one case with aplastic anemia who underwent OTC and OTCT in the first specialized center in China and with follow-up of ovarian function and fertility. As indicators for the recovery of ovarian function after retransplantation we used:

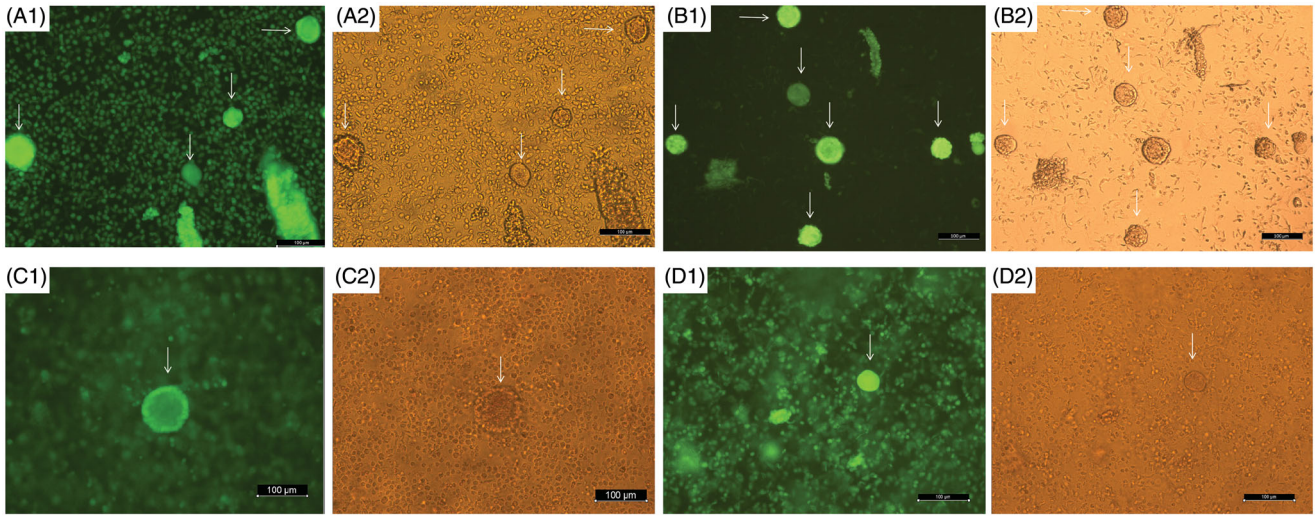

Figure 1. Typical photographs of follicles under the microscope. (A1/A2–D1/D2) Photographs of fresh tissue and frozen–thawed tissue of the same patient, respectively: (A1–D1) fluorescence microscope; (A2–D2) flat-light microscope. Follicles survive in all fresh and frozen–thawed ovarian tissue. White arrows represent follicles. Scale bar = 100 μm.

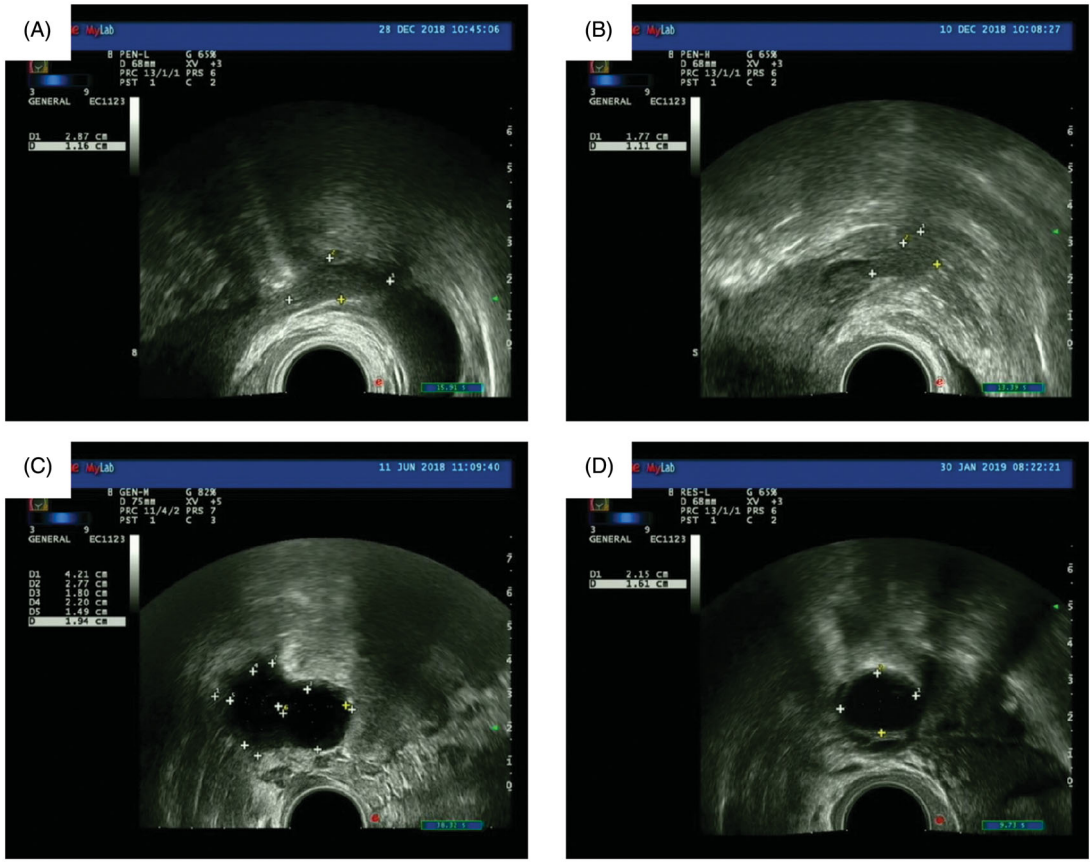

Figure 2. Typical photographs of ovaries and follicles under ultrasound. (A, B) Transplanted ovaries detected by ultrasound after ovarian tissue cryopreservation transplantation (OTCT). (C, D) Follicular development monitored by ultrasound after OTCT.

laboratory endocrine parameters before and after OTCT (recovery of ovarian function comparing pre/post transplantation especially can be seen by monitoring FSH and E2); survival and growth of ovarian follicles; restoration of menstrual cycle; and disappearance of menopausal symptoms. According to international literature[1,5,9,12–14] and our first guidelines in China[4], retransplantation can be assessed as 'successful' if the recovery of ovarian function can be seen after 3–6 months according to these indicators. Based on these criteria, all 10 patients recovered within 2–4 months. OTC/OTCT could therefore prevent iatrogenic POI in all 10 patients.

However, we cannot yet make conclusions regarding fertility using this method in our center, since only one of the 10 patients wished to conceive, and she has not yet become pregnant. Three other women could become pregnant if

desired, and the remaining six were hysterectomized due to cervical or endometrial cancer or received previous high-dose radiotherapy. Primarily, all 10 women requested OTC/OTCT to restore ovarian endocrine function after treatment for their disease.

Selection criteria for cryopreservation

The first criterion is a high risk of POI due to the primary disease, and this applied to all of our 10 patients. None of our patients developed POI or premature ovarian failure prior to treatment, which would prohibit OTC/OTCT. We did expect that after anticancer treatment the risk of POI/premature ovarian failure would be less than 50% or the survival rate less than 50%, which would also not recommend OTC/OTCT[19].

Another criterion for OTC is age. The upper age limit is often stated as 35 years, but could be around 38 years for women with high ovarian reserve[4,19]. We therefore decided to use cryopreservation for four of our 10 patients who were older than 35 years. Patients with ovarian cancer or with a high risk of ovarian metastasis should be excluded. OTC is the only option for prepubertal girls with cancer[8,9,19], which, however, were not included in our first 10 cases, because the time from OTC to OTCT is longer in prepubertal girls. This technique is also the only available option for those in whom chemo/radiotherapy cannot be delayed[4], which applied to our patients.

Ovarian tissue transportation

In our center, ovarian tissue is usually transported to the cryobank on the day of biopsy and is routinely cryopreserved on the next day. Tissue from the 10 patients in this study was sent from the operation room to the cryobank within 2 h and was cryopreserved on the same or next day. In Germany[8], most tissue is transported to a centralized cryobank, such as Düsseldorf or Erlangen, which mostly requires overnight transportation. However, this does not seem to have a negative effect on the viability of the frozen–thawed ovarian tissue and outcomes after retransplantation[8]. Women who have indication for OTC/OTCT can undergo ovarian tissue removal in their local hospital, but should have their tissue cryopreserved in a centralized cryobank because these techniques require high expertise.

Cryopreservation: slow freezing versus vitrification

At present, SF is considered worldwide as the gold standard for OTC[5], and most of the reported live births after OTCT have been successful after SF[1,9,13]. In our center, the ovarian tissue was successfully cryopreserved by SF. Vitrification (VT) constitutes an alternative but controversial method[20,21]. The advantage of VT is to save time, it does not require a special freezer, and, more importantly, it can be performed without formation of harmful ice crystals. However, VT requires a higher concentration of cryoprotectant than SF, and therefore the risk of damage to follicles and stromal cells is higher[22]. Until today, only two live births have been reported after VT and two after VT followed by *in vitro* activation of the human ovarian cortex[23,24].

Timing of retransplantation

The time of retransplantation depends on the patient's primary disease, clinical recovery, low risk of recurrence, and desire for an early pregnancy[25]. There must be interdisciplinary communication with the oncologist to determine whether the patient is suitable for retransplantation. Menopausal symptoms appeared in all 10 patients in this study after disease treatment (including bilateral oophorectomy, chemotherapy, radiotherapy, and bone marrow transplantation). Retransplantation can be considered at least 3–6 months after finishing chemo/radiotherapy[5]. For our patients, the time between OTC and OTCT was 15 ± 4.163 months (range: 10–23).

Ovarian tissue retransplantation sites

Retransplantation should be performed as fast as possible after transport of the frozen–thawed ovarian tissue to the operating room. This was within 20 min in the present study. The tissue was placed into a pelvic peritoneal pocket of ovarian fossa, as this has a good blood supply. In nine cases the tissue was transplanted into the right side, and in one case into both sides. Surgery for retransplantation was in accordance with the literature[8], which reports that 90% of women's frozen–thawed ovarian tissue was transplanted into a peritoneal pocket and 10% into both a peritoneal pocket and into the ovary[26]. Transplantation into or onto the remaining ovaries or into a peritoneal pocket in the pelvic peritoneum (orthotopic retransplantation) may provide the ability to achieve a natural pregnancy.

Heterotopic retransplantation, such as into the subcutaneous tissue of the forearm or abdomen, has been recommended in patients with severe pelvic adhesions, distorted pelvic anatomy, and poor pelvic vasculature due to radiotherapy[27]. Only a few live births after heterotopic retransplantation of frozen–thawed ovarian tissue have been reported. However, one center reported four live births from the same patient[28,29]. Thus, orthotopic or heterotopic retransplantation have their own characteristics and need further research.

Risk of cancer relapse or ovarian cancer after retransplantation

In our 10 cases, no relapse of the primary cancer or development of ovarian cancer in the graft has yet been observed after OTCT. Ovarian metastasis has been reported in 0.7–2.5% of patients with squamous cell carcinoma and in 0–6.8% of patients with adenocarcinoma of the cervix[30]. Four of our patients who underwent OTCT had squamous cell carcinoma. The risk of ovarian metastasis with endometrial cancer ranges from 1.9% (FIGO stage I tumors) to 41.7% (FIGO stage I–III tumors)[31] – our patient had FIGO stage I. OTCT

appears to be reliable in patients with low-stage breast cancers[5,9] – the breast cancer of our patient was of type $ER^{-}PR^{-}Her2^{3+}$, T1cN2M0. Experimental studies[32,33] utilizing molecular analyses and xenografting assessment have proved that leukemia and ovarian malignancies, which are not included in the now more than 300 cryopreserved tissues stored in our cryobank, have the greatest risk of ovarian metastasis.

Follow-up of ovarian function

The average time required for ovarian function to be restored after OTCT in our study was 3.4 months, which is consistent with international data[34]. Ovarian tissue activity has been reported for up to 11 years after OTCT[34]. The endocrine function of the ovary is very important for women's health and life quality. It is beneficial for preventing chronic disease, such as cardiovascular diseases and osteoporosis, and prevents the development of menopausal symptoms, which was the main aim for nine of our 10 patients. For many women, OTC is the last chance to restore fertility with future retransplantation[13]. In our center, only one patient had a strong desire to become pregnant. A natural pregnancy can be achieved if there are no other infertility factors. Cycle monitoring with management of follicle and timed sexual intercourse can increase the possibility of conception. For patients with other infertility factors, such as tubal infertility and male infertility, assisted reproductive technology must be used[35]. In the follow-up, all 10 of our patients undergo a 3-monthly assessment of hormone levels and an ultrasound examination.

Limitations

Of the 10 patients, six were unable to give birth, and only one patient currently wishes to become pregnant. Therefore, we cannot assess the quality of our fertility protection method in terms of pregnancy success and no babies have yet been born after OTCT technology in China. Many patients with a wish to conceive will undergo transplantation in the future. However, it should be not be forgotten that, independent of the wish to conceive, OTCT is the best method for certain patients to prevent iatrogenic POI with all its hazardous sequelae.

Summary and conclusion

OTC/OTCT is a relatively young technique in assisted reproductive technology but is no longer considered experimental. In Europe and other developed countries, it has become an effective routine fertility preservation method. Our center, China's first ovarian tissue cryobank, has cryopreserved tissues from more than 300 patients. We recently reported the first retransplantation in China in this journal, and now present the first 10 cases, all successful with respect to preventing iatrogenic POI. Since only one patient wishes to conceive but has not become pregnant, China does not yet have a baby from this technique. We will perform many more retransplantations in the near future, which will add to the global data and will allow confirmation of the advantages of this technique for certain patients in preventing POI and hopefully also for conception.

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Potential conflict of interest The authors disclose no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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