Implementation of ovarian tissue cryopreservation in Hong Kong

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ABSTRACT

Introduction: Worldwide, >130 babies have been born from ovarian tissue cryopreservation (OTC) and ovarian tissue transplantation (OTT). Ovarian tissue cryopreservation can improve quality of life among young female cancer survivors. Here, we assessed the feasibility of OTC and subsequent OTT in Hong Kong via xenografts in nude mice.

Methods: This pilot study was conducted in a university-affiliated tertiary hospital. Fifty-two ovarian tissues were collected from 12 patients aged 29 to 41 years during ovarian surgery, then engrafted into 34 nude mice. The efficacies of slow freezing and vitrification were directly compared. In Phase I, non-ovariectomised nude mice underwent ovarian tissue engraftment. In Phase II, ovariectomised nude mice underwent ovarian tissue engraftment, followed by gonadotrophin administration to promote folliculogenesis. Ovarian tissue viability was assessed by gross anatomical, histological, and immunohistochemical examinations before and after OTC. Follicular density and morphological integrity were also assessed.

Results: After OTC and OTT, grafted ovarian tissues remained viable in nude mice. Primordial follicles were observed in thawed and grafted ovarian tissues, indicating that the cryopreservation and transplantation protocols were both effective. The results were unaffected by gonadotrophin stimulation.

Conclusion: This study demonstrated the feasibility of OTC in Hong Kong as well as primordial follicle viability after OTC and OTT in nude mice. Ovarian tissue cryopreservation is ideal for patients who cannot undergo the ovarian stimulation necessary for oocyte or embryo freezing as well as prepubertal girls (all ineligible for oocyte freezing). Our findings support the clinical implementation of OTC and subsequent OTT in Hong Kong.

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New knowledge added by this study

- This study assessed the viability of ovarian tissue cryopreservation and subsequent ovarian tissue transplantation in Hong Kong via xenografts in nude mice.
- Grafted ovarian tissues remained viable after transplantation, regardless of protocol (slow freezing or vitrification).

Implications for clinical practice or policy

- Ovarian tissue cryopreservation is ideal for patients who cannot undergo the ovarian stimulation necessary for oocyte or embryo freezing, as well as prepubertal girls (all ineligible for oocyte freezing).
- These findings support the clinical implementation of ovarian tissue cryopreservation and subsequent ovarian tissue transplantation in Hong Kong.
- Further studies are needed to clarify optimal cryopreservation and engraftment protocols.

在香港推行卵巢組織冷凍保存

鍾佩樺、陳耀樑、宋藝、吳綺莉、羅思敏、吳嘉詠、梁寶華、王斯嘉、溫海明、李景曦、黃志超

引言：當前全世界已有130多名嬰兒通過卵巢組織冷凍保存和卵巢組織移植出生。卵巢組織冷凍保存有助提高年輕女性癌症倖存者的生活品質。本研究透過在裸鼠進行異種移植實驗，評估在香港進行卵巢組織冷凍保存及隨後的卵巢組織移植的可行性。

方法：這是一項在大學附屬醫院進行的試點研究。本研究使用來自12例接受了卵巢手術的29至41歲患者捐贈的共52份卵巢組織樣本進行實驗，這些組織經異種移植到34隻裸鼠中。研究直接比較卵巢組織慢凍和玻璃化冷凍保存的效能。在第一階段，卵巢組織異種移植到未切除卵巢的裸鼠體內，而在第二階段，卵巢組織異種移植到已切除卵巢的裸鼠體內，並給予促性腺激素以促進卵泡生成。這些組織在冷凍保存前和經冷凍保存後，通過肉眼、組織學分析和免疫組織化學檢測卵巢組織的存活性，同時評估卵泡密度和形態完整性。

結果：卵巢組織在冷凍保存和移植至裸鼠後仍可存活。在解凍和移植後的卵巢組織中仍可見原始卵泡，這表明兩種冷凍方案都是有效的，而注射促性腺激素後沒有影響結果。促性腺激素對於未能進行卵巢刺激用作卵子或胚胎凍存的患者來說，卵巢組織冷凍保存是理想選擇，尤其是適用於無法進行凍卵的前青春期少女。本研究結果支持應該在香港臨床推行卵巢組織冷凍保存及隨後的卵巢組織移植。

Introduction

A diagnosis of cancer is disheartening news for every patient in terms of both disease and treatment. Anticancer treatments for common cancers (eg, chemotherapy and radiotherapy) are gonadotoxic and detrimental to future fertility.[1] Advances in medical treatment have improved the 5-year survival rates of some cancers to >80% in children and adolescents.[2] However, most surviving patients experience illness-related infertility.[3] Infertility is also a concern for patients with severe endometriosis, patients with poor ovarian reserve, patients with benign medical conditions requiring chemotherapy, and transgender individuals undergoing gender-affirming surgery.[4] Fortunately, advancements in fertility preservation (FP) technologies offer these patients the opportunity to have biological offspring in the future. In Hong Kong, only 45.6% of clinicians,[5] 22.2% of medical students,[6] and 21.7% of the general public[7] are familiar with FP. Therefore, FP technologies require greater attention in Hong Kong. Both clinicians and the public should be aware where and how to seek help when patients are diagnosed with cancer and need to use FP technologies to preserve their fertility.

For patients who cannot undergo the ovarian stimulation necessary for oocyte or embryo freezing as well as prepubertal girls (all ineligible for oocyte freezing), ovarian tissue cryopreservation (OTC) and subsequent orthotopic or heterotopic ovarian tissue transplantation (OTT) are ideal options for FP after recovery.[8] Many European countries have provided OTC for patients with various medical reasons.[4] Although OTC and OTT are widely available in Belgium, Denmark, Spain, France,[9] Japan, Singapore,[10] the United States, India, Australia, the Philippines, Korea,[11] and some parts of China,[12] these FP technologies remain unavailable in Hong Kong. Thus far, >130 babies worldwide have been born via OTC and OTT,[13] and the American Society for Reproductive Medicine removed the 'experimental' designation for these technologies in 2019.[14] Ovarian tissue cryopreservation can be performed via slow freezing or vitrification. Slow freezing has been the standard treatment for ovarian tissues,[15] but there is increasing evidence to support the use of vitrification.[16,17] Nevertheless, controversies remain concerning subsequent oocyte viability and the preservation of morphological integrity after ovarian tissues have been processed using these two cryopreservation techniques.[18]

The development of OTC, which can improve quality of life among young female cancer survivors,[19] is urgently needed in Hong Kong. Here, we performed a pilot study to assess the feasibility of OTC and subsequent OTT in Hong Kong via xenografts in nude mice. In this study, we collected ovarian tissues, established both slow freezing and vitrification protocols, and evaluated tissue viability and follicle preservation after OTC and OTT in a nude mouse xenograft model. This mouse model provided important insights that will support the clinical implementation of OTC and OTT in Hong Kong. Our primary outcome was ovarian tissue viability after slow freezing or vitrification, as determined by histological analysis and immunohistochemistry. Our secondary outcomes were follicular density and the morphological integrity of grafted ovarian tissues.

Methods

This study was conducted between July 2019 and December 2021 at the Prince of Wales Hospital, a university-affiliated tertiary hospital in Hong Kong. All participants received a detailed explanation of the study, then provided written consent for inclusion. All researchers involved in the animal experiments were licensed by the Department of Health of the Hong Kong SAR Government.

Ovarian tissue collection

Ovarian tissues were collected from women or transgender individuals who underwent laparoscopic or open, unilateral or bilateral, ovarian cystectomy or salpingo-oophorectomy as treatment for benign ovarian cysts or tumours. During the operation, each patient underwent removal of a small section of

ovarian tissue or the whole ovary; each specimen of donated ovarian tissue was retrieved from a routine surgical specimen or directly removed during surgery. To prevent thermal injury during tissue removal, cold scissors were used and diathermy was avoided. The amount of donated tissue varied among patients, depending on their age and clinical condition. For example, larger volumes of ovarian tissue were often collected from patients undergoing oophorectomy. Each patient was assigned a unique identification number linked to an encrypted file containing the patient's data and demographic information; during analyses of tissue from each patient, the pathologist and research staff who conducted histology and immunohistochemistry analyses were blinded to the contents of the encrypted files.

Ovarian tissue cryopreservation

Tissues were transported to the laboratory in a standardised culture medium at 4°C and processed within 30 minutes after collection. After removing the medullary region, the ovarian tissue was frozen in accordance with a controlled-rate slow freezing machine protocol (Ovarian Tissue Cryopreservation Scientific Roundup; Planer, UK)[20] or a vitrification manual (Ova Cryo Kit Type M, VT301S; Kitazato Corporation, Japan).[21] The cortical region was cut into small fragments with a thickness of approximately 1 mm. Some collected ovarian tissues were very small and could not be sectioned for parallel slow freezing and vitrification fresh tissue controls; these small tissues were either frozen using the standard slow freezing method or vitrification.[22] Larger ovarian tissues (typically collected from transgender individuals during oophorectomy) were cut into smaller fragments prior to slow freezing and vitrification, or prior to use as fresh tissue controls. Before the xenograft procedure, fragments were removed from fresh tissue, thawed slow-frozen tissue, and thawed vitrified tissue; these fragments were subsequently compared with grafted tissues to identify any differences related to engraftment.

A subset of fresh ovarian tissue fragments was fixed and subjected to histological analysis. When a large amount of ovarian tissue was available from a single patient, we compared cryopreservation methods using tissue from that patient; we also compared the cryopreserved tissue with fresh tissue.

Slow freezing

Slow freezing was performed in accordance with a validated protocol.[23-25] Collected ovarian cortices were equilibrated at 4°C on a tilting shaker for 30 minutes in freezing solution (1.5 mol/L ethylene glycol and 0.1 mol/L sucrose in G-MOPS PLUS; Vitrolife, Sweden). After equilibration, the ovarian tissue pieces were placed into 1.8-mL cryogenic vials that had been pre-filled with 1 mL of freezing solution (two tissue pieces per vial). The cryogenic vials were then placed into an automated, computer-controlled freezing system (Kryo-360; Planer, UK).[20] The slow freezing protocol was performed in accordance with the method described by Dolmans et al[26] and the Planer Ovarian Tissue Cryopreservation Scientific Roundup.[20]

Vitrification

For vitrification of ovarian cortices, the Ovarian Tissue Vitrification Kit (Ova Cryo Kit Type M, VT301S; Kitazato Corporation, Japan)[21] was used. The collected ovarian cortices were cut into 1 × 1 × 1 cm^3 cubes using a surgical knife and a square measuring device provided in the kit. Vitrification was then performed in accordance with the kit manufacturer's protocol, and the ovarian tissues were stored in liquid nitrogen.

Thawing of ovarian tissue for transplantation

Slow-frozen tissues were removed from liquid nitrogen and exposed to room temperature air for 5 seconds, then placed in 37°C water for 2 minutes. Subsequently, they were transferred to thawing solution 1 (0.75 mol/L ethylene glycol and 0.25 mol/L sucrose in G-MOPS PLUS) for 10 minutes, then to thawing solution 2 (0.25 mol/L sucrose in G-MOPS PLUS) for 10 minutes, and finally to a handling medium (G-MOPS) for 10 minutes. Vitrified ovarian tissue fragments were thawed using Ova Thawing Kit Type M (V302S; Kitazato Corporation, Japan), in accordance with the manufacturer's instructions.[21]

Ovarian tissue transplantation into nude mice

The nude mouse xenograft model is ideal for assessment of OTC and OTT outcomes. Ovarian xenografts in immunodeficient nude mice can be used to test follicular viability and development. This approach can reveal whether freezing and thawing cause damage to ovarian tissue; it can also demonstrate the ability of cryopreserved tissue to support the development of large antral follicles.[27] Considering the higher rate of immune leakiness in severe combined immunodeficient mice,[28] we used BALB/c athymic nude mice to validate our OTC and OTT protocols before clinical implementation. Thirty-four female BALB/c athymic nude mice (age, 4-6 weeks; Laboratory Animal Services Centre, The Chinese University of Hong Kong) were used for this study. To prevent fighting between engrafted mice, only three mice were housed in individually ventilated cages at 28°C under controlled sterile conditions, with a 12-hour light/dark cycle and free access to an autoclaved pelleted diet and water. Mice were anaesthetised by intraperitoneal injection of ketamine (75 mg/kg)/xylazine (10 mg/kg) (AlfaMedic

Limited, Hong Kong; manufactured in Holland). Ovarian tissues collected from patients were grafted onto nude mice. During ovarian tissue engraftment, the cortical surface was carefully oriented outward and tightly attached to the subcutaneous tissue or abdominal wall. There were two phases in our study, as described in the following sections (Fig 1).

Phase I: Analysis of ovarian tissue xenograft viability in non-ovariectomised nude mice

To maintain endogenous hormone secretion and avoid the risk of ovariectomy-related death, mice in this phase were not subjected to ovariectomy. One fresh, slow-frozen, or vitrified tissue of approximately 4 × 6 × 1 mm^3 was engrafted into the subcutaneous site on the neck of nine nude mice.[29] The mice were then sacrificed by intraperitoneal injection of overdose of the anaesthetic. One mouse engrafted with vitrified tissue, two engrafted with slow-frozen tissues and one engrafted with fresh tissue were sacrificed after 2 weeks. Two mice engrafted with vitrified tissues, one engrafted with slow-frozen tissue and two engrafted with fresh tissues were sacrificed after 5 weeks.

Phase II: Analysis of ovarian tissue xenograft viability, folliculogenesis, and ovulation in ovariectomised nude mice

To promote graft survival and growth, mice in this phase were subjected to ovariectomy. Fresh, slow-frozen, or vitrified tissues of approximately 4 × 6 × 1 mm^3 were either engrafted into the subcutaneous site on the neck of ovariectomised nude mice, or used for intraperitoneal engraftment in the left abdomen of those mice.[29] Mice in this phase were divided into a saline group and a treatment group after 2 or 6 weeks of engraftment. The presence of gonadotrophins can optimise graft establishment and stimulate follicle growth.[30] To promote folliculogenesis, mice in the treatment group underwent intraperitoneal injection (in the right abdomen) of 1 IU (100 μL) of follitropin alfa (GONAL-f; Merck Serono, Geneva, Switzerland) every other day for 5 to 8 weeks after 2 or 6 weeks' engraftment.[30] During the same period, mice in the saline group underwent intraperitoneal injection (in the right abdomen) of an equal volume of physiological saline every other day. Thirty-six hours before the mice were sacrificed, both groups of mice received a single dose of 10 international units of human chorionic gonadotrophin (Sigma-Aldrich, St Louis [MO], US) by injection to promote ovulation.

Grafted ovarian tissue viability

All grafted tissues were fixed in buffered formalin and embedded in paraffin wax, then sectioned and stained for analysis.

Histological analysis

Microscopic observations up to 400 times the original magnification (Leica DMIRB; Leica Microsystem, Wetzlar, Germany) of fresh and thawed ovarian tissues were performed after the tissues had been stained with haematoxylin and eosin (H&E). All follicles from the entire grafted tissue specimen on every slide were counted; section thickness and the presence/absence of a nucleolus were also considered.

Immunohistochemical assessment of stromal tissue viability

Stromal tissue viability was determined by assessing the morphologies of stromal cells on H&E-stained sections. Viability was defined as the presence of spindle cells with consistent cellularity; an intact nuclear membrane; the absence of pyknotic figures, apoptosis, or necrosis; and the absence of fibrosis or calcification. Viability was confirmed by immunohistochemical analyses using antibodies to cluster of differentiation 10 (CD10) and oestrogen receptors. Anti-CD10 antibody (clone NCL-CD10-270; Novocastra, Newcastle upon Tyne, UK) was used at a dilution of 1:50 with an incubation time of 30 minutes at a sustained temperature of 37°C. Anti–oestrogen receptor antibody (RM-9101; Thermo Fisher Scientific, Fremont [CA], US) was used at a dilution of 1:150 with an incubation time of 32 minutes at a sustained temperature of 37°C. Antigen retrieval was performed using ethylenediaminetetraacetic acid and microwave. Antibody detection was performed using Roche Diagnostics OptiView DAB IHC Detection Kit (Thermo Fisher Scientific, Waltham [MA], US). Immunohistochemical staining (original magnification × 400) was semi-quantitative and based on signal intensity (absent, weak, moderate, and strong). The presence of at least weak staining intensity in ovarian stromal tissue was regarded as a positive result.

Follicular density and quality after freezing, thawing, and transplantation

All follicles from the entire grafted tissue specimen on every H&E-stained slide were counted on multiple levels within thick sections (≥20 μm). The digital images were annotated on QuPath,[31] obtaining the two-dimensional area of the slide and number of follicles. Follicular density was calculated by established methods described previously.[32,33] Ovarian follicles were classified as primordial, primary, or secondary follicles according to morphological assessment of H&E-stained sections.[33] Evaluation of grafted follicle quality was based on basement membrane integrity, cellular density, presence or absence of pyknotic bodies, and oocyte integrity. Only morphologically normal

(ie, viable) follicles were counted. The results of gross anatomical examinations were confirmed by histological assessments. Gross tissue integrity was defined as the presence of a distinct vascularised tissue fragment that exhibited firmness and perfusion. Microscopic findings indicating viability were the presence of an intact nuclear membrane and the absence of necrosis, apoptosis, and pyknotic nuclei.

Results

In total, 52 ovarian tissues were collected from 12 patients aged 29 to 41 years. Ovarian tissues from different patients were cut into several pieces according to tissue size. These tissues were treated by vitrification or slow freezing, then engrafted into 34 mice as shown in Figure 1. Tables 1 and 2 only show data from patients with follicles to facilitate readability. Although there were nine tissues from four patients (Patients 1, 6, 7, and 10) in Phase I, Table 1 only shows data from the two patients with follicles (Patients 1 and 7). In Phase II, there were 25 tissues from six patients (Patients 1, 5, 8, 9, 11, and 12), but Table 2 only shows data from the four patients with follicles. In total, 18 control tissues were collected from 11 patients (Patients 1, 2, 4, 5, 6, 7, 8, 10, 11, 12, and 13). One patient (Patient 5) provided sufficient ovarian tissue for a comparison of cryopreservation methods using tissue from a single patient. Additionally, we compared fresh and slow-frozen tissues from Patient 5, and compared fresh and vitrified tissues from Patients 1, 5, and 12.

Graft recovery rate and macroscopic assessment

In Phase I, all xenografts were successfully retrieved from the experimental mice. Macroscopic

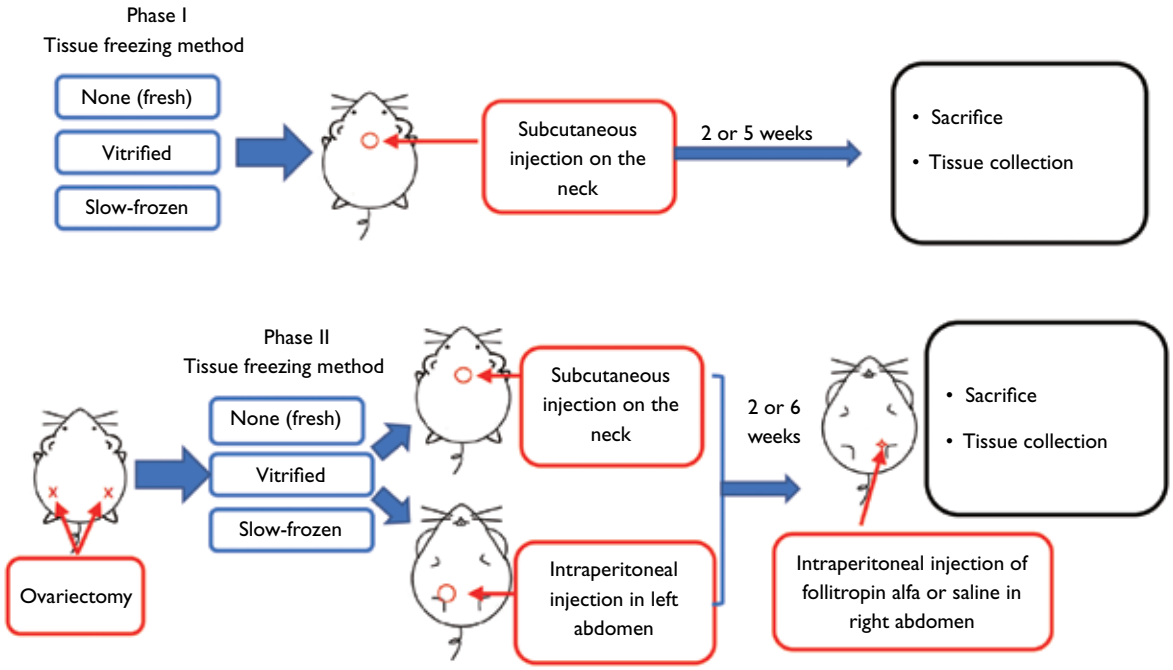

FIG 1. Flowchart depicting Phase I and Phase II studies. In Phase I, tissues were collected from four patients and engrafted into nine mice. In Phase II, tissues were collected from six patients and engrafted into 25 mice

TABLE 1. Follicular density of viable grafted tissues in Phase I analysis: two of four patients with primordial follicles

Patient No.	Age, y	Diagnosis	Freezing method	Primordial follicle density, No./mm³	Primary follicle density, No./mm³	Secondary follicle density, No./mm³
1	39	Endometrial carcinoma	None (fresh)	4.85	0.55	0
1	39	Endometrial carcinoma	Vitrification	1.20	0	0
7	41	Gender dysphoria	Slow freezing	0.93	0	0
7	41	Gender dysphoria	Slow freezing	0.83	0	0
7	41	Gender dysphoria	Slow freezing	1.28	0	0

TABLE 2. Follicular density of viable grafted tissues in Phase II analysis: five of six patients with primordial follicles

Patient No.	Age, y	Diagnosis	Freezing method	Group	Primordial follicle density, No./mm^3	Primary follicle density, No./mm^3	Secondary follicle density, No./mm^3
5	29	Gender dysphoria	None (fresh)	Control	3.87	0	0
5	29	Gender dysphoria	Slow freezing	Saline	2.56	0	0
5	29	Gender dysphoria	Slow freezing	Saline	5.29	0.94	0
5	29	Gender dysphoria	Slow freezing	Treatment	1.93	0.42	0
5	29	Gender dysphoria	Slow freezing	Saline	0.61	0	0
5	29	Gender dysphoria	Vitrification	Saline	1.99	0	0
5	29	Gender dysphoria	Vitrification	Treatment	1.97	0.64	0
8	37	Polycystic ovary syndrome	None (fresh)	Treatment	5.04	0	0
8	37	Polycystic ovary syndrome	None (fresh)	Control	1.88	0.26	0
9	37	Gender dysphoria	Slow freezing	Treatment	1.46	0	0.33
9	37	Gender dysphoria	Slow freezing	Saline	0.48	0	0
12	31	Gender dysphoria	None (fresh)	Control	5.92	0.16	0.03
12	31	Gender dysphoria	Vitrification	Saline	0.74	0	0
12	31	Gender dysphoria	Vitrification	Treatment	1.10	1.61	0

observations of fresh and thawed tissues did not show substantial differences between cryopreservation methods in terms of tissue integrity or morphology (Fig 2a). Microscopic findings showed the presence of viable nuclei and the absence of necrosis, apoptosis, and pyknotic nuclei.

In Phase II, all xenografts were successfully retrieved from the experimental mice, with the exception of two calcified tissues. Most subcutaneous sites contained soft tissue fragments that were completely encased in membranes; the graft–murine tissue interface was vascularised (Fig 2b). Intraperitoneal sites contained soft tissue fragments with small vessels visible on the graft surface; the fragments were attached to surrounding tissue, and some grafts were encased in abdominal adipose tissue (Fig 2c).

Analysis of stromal tissue morphology

Immunohistochemical staining showed that all retrieved grafts had maintained viability, with the exception of two calcified tissues (Fig 3). Haematoxylin and eosin staining, CD10 staining, and oestrogen receptor staining showed no between-group differences (fresh vs slow-frozen, fresh vs vitrified, and slow-frozen vs vitrified). Moreover, stromal tissue viability did not differ between the treatment and saline groups in Phase II.

Follicular histology and density

Retrieved grafts were embedded with paraffin and sectioned at a thickness of 4 or 30 μm. Microscopy analysis revealed primordial, primary, and secondary follicles (Fig 4). Tables 1 and 2 show the follicular densities of retrieved grafts from Phases I and II, respectively. Primordial follicles were observed in fresh and cryopreserved grafts from the same patient (Patient 5), regardless of cryopreservation method (slow freezing or vitrification) or gonadotrophin injection status.

Discussion

Summary of main findings

Our pilot study demonstrated the feasibility of OTC with subsequent OTT in Hong Kong via xenografts of fresh and cryopreserved ovarian tissues in nude mice. Most grafted ovarian tissues remained viable after engraftment, as demonstrated by CD10 and oestrogen receptor staining results in stromal tissue, along with the presence of viable nuclei and the absence of necrosis, apoptosis, and pyknotic nuclei. Regardless of cryopreservation method, primordial follicles were observed in thawed ovarian tissues after engraftment; thus, both cryopreservation methods are feasible and effective. There were no differences in folliculogenesis after gonadotrophin injection. Overall, these findings validate our protocol for surgical collection of ovarian tissue, cryopreservation via slow freezing or vitrification, and subsequent tissue engraftment into mice; this protocol successfully generated primordial follicles in the xenografts. To our knowledge, this type of

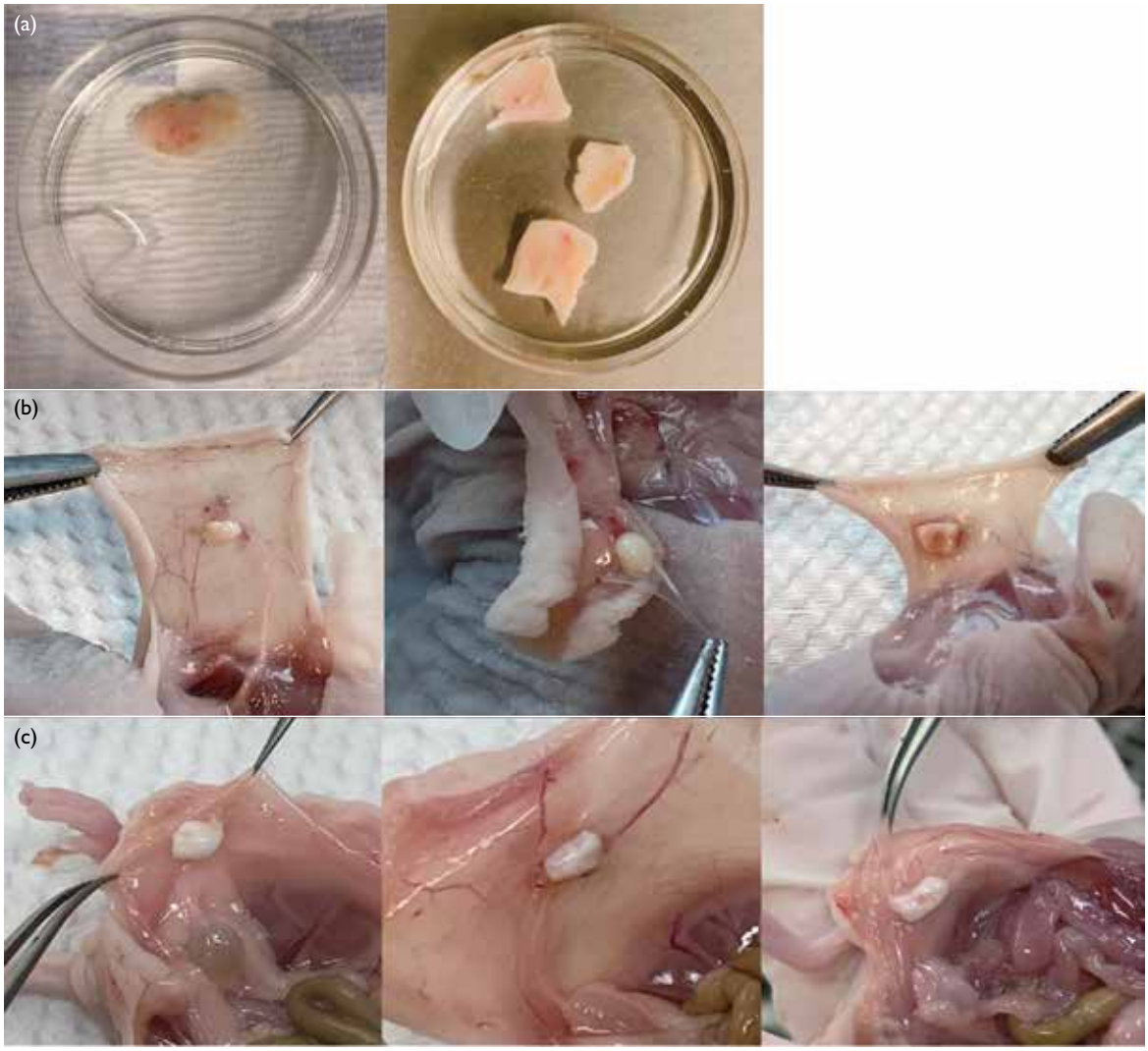

FIG 2. Macroscopic observations of fresh, thawed, and grafted tissues. (a) Fresh tissue (left) and thawed slow-frozen tissue (right). (b) Grafted slow-frozen ovarian tissues at subcutaneous sites on the neck in three BALB/c athymic nude mice. (c) Grafted fresh tissues (left and middle) and vitrified ovarian tissue (right) at intraperitoneal sites in three BALB/c athymic nude mice. Note that angiogenesis was observed around each xenograft

protocol was not previously validated in Hong Kong.

The benefits of OTC and OTT are not limited to gynaecology patients; they are also useful for patients in other specialties[4] (eg, medicine, oncology, and paediatrics), including adolescents,[34] as well as women who cannot undergo ovarian stimulation. To our knowledge, this is the first study to demonstrate the feasibility of OTC and OTT in Hong Kong; our findings support the clinical implementation of these technologies at medical centres in Hong Kong.

Current condition and success rate of ovarian tissue cryopreservation

Ovarian tissue cryopreservation has become an accepted FP technology in many fertility centres since the removal of its experimental designation.[11,14] Notably, OTC allows the preservation of thousands of primordial follicles in a single procedure; compared with mature oocytes, preserved primordial follicles are more resistant to cryodamage.[35] This technology is also appropriate for patients who cannot undergo ovulation stimulation because they require urgent chemotherapy or must avoid the enhancement of a hormone-sensitive malignancy[36]; it is also the only available FP technology for prepubertal girls.[36] Furthermore, OTC allows natural conception; several spontaneous pregnancies have been reported after successful orthotopic autotransplantation.[9,37] In some instances, both fertility and gonadal function are restored.[34] According to a meta-analysis,[38] endocrine function was restored in 63.9% of patients; the combined rate of pregnancies and live births

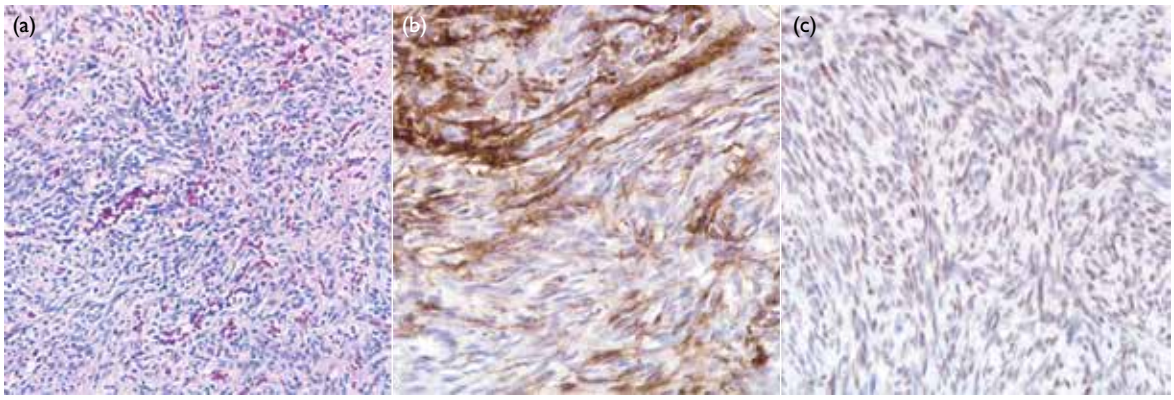

FIG 3. Haematoxylin and eosin (H&E) and immunohistochemical staining of ovarian stromal tissues in xenografts retrieved from nude mice (400 ×). (a) H&E staining; (b) cluster of differentiation 10 staining; (c) oestrogen receptor staining

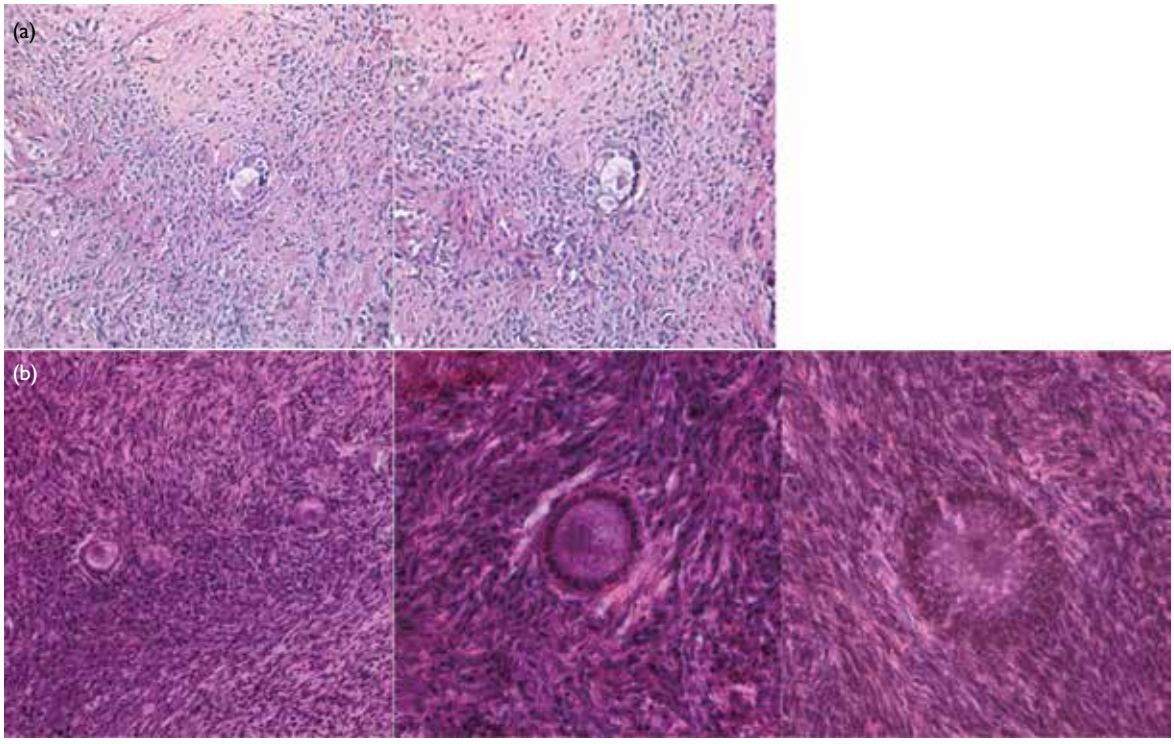

FIG 4. Microscopic observation of different stages of follicles in the grafted tissues on nude mice (400 ×). (a) Haematoxylin and eosin (H&E) staining of primordial follicles in 4-μm sections; (b) H&E staining of primordial (left), primary (middle), and secondary (right) follicles in 30-μm sections

was 28.4%. Dolmans et al[9] also reported that 26% of women became pregnant and gave birth to one or two infants after the transplantation of frozen-thawed ovarian tissue; the live birth rate was 30.6%.

Barriers to clinical implementation of ovarian tissue cryopreservation

Despite its advantages, there are multiple barriers to the clinical implementation of OTC. Effective use of this technology involves two surgical procedures: the initial removal of ovarian tissue (prior to cryopreservation) and a future transplantation procedure, which may cause surgical and ethical problems (particularly in prepubertal patients).[39] The technology also requires expertise that is not available in some parts of Asia. A Japanese group reported a live birth in 2015[40]; another successful live birth was reported by a Chinese group in 2021,[41]

involving the cryopreserved ovarian tissue bank established by the Beijing Obstetrics and Gynecology Hospital.[12] However, OTC is not widely available in Hong Kong. Reproductive health centres in Hong Kong may lack sufficient surgical expertise and/or an optimal cryopreservation environment.[11] Thus, there is a need to reduce the obstacles to clinical implementation of OTC. From our experience, in terms of laboratory requirements, the protocol, equipment, and consumables can be incorporated into most assisted reproductive technology units. However, practical education is needed regarding OTC, including tissue management (eg, tissue thinning during removal of the medulla) and specific aspects of cryopreservation. Proper records of success measures (eg, freeze-thaw outcomes and graft survival rate) are essential; these data should be carefully documented in laboratory records. Additional equipment is also needed for the clinical implementation of OTC as a routine service because the harvesting surgery may be performed on an urgent basis that differs from the routine assisted reproductive technology laboratory programme.[11] While planning for this study, we found that there have been inconsistencies in terms of selection criteria, cryopreservation methods, laboratory management of harvested tissue, and the transplantation technique itself. Although we found no differences in the morphological integrity of ovarian tissue after cryopreservation via slow freezing or vitrification, further studies with larger numbers of patients are needed to confirm the feasibility of follicular stimulation in vivo.

Current status of ovarian tissue transplantation

Human OTT remains unavailable in Hong Kong. Notably, our analysis of tissue engraftment was conducted in a mouse model. After the clinical implementation of OTC in Hong Kong, OTT involving autotransplantation could be established as a routine service. Ovarian autotransplantation is performed when a patient has fully recovered from disease, but this approach may carry a small risk of reintroducing malignant cells in patients with cancer.[42] The results of some studies have suggested that the risk of reintroducing malignant cells could be minimised by meticulous examination of representative biopsy samples via histology, immunohistochemistry, and molecular biology techniques.[43] Moreover, optical coherence tomography can be used to assess malignant cells in thawed ovarian tissue before transplantation.[44]

Limitations

There were several limitations in this study. First, tissue engraftment was not conducted in humans because of ethical concerns; thus, we analysed tissue engraftment in a nude mouse xenograft model, which was the best available model. Second, some ovarian tissues were collected from transgender individuals who had undergone testosterone replacement therapy, which might have affected the hormonal milieu of the ovarian tissue.[45] Third, we only retrieved small fragments of ovarian tissue (~1 cm) from random locations in the ovaries of included patients; this may have led to sampling error if the sampled cortical layers did not contain primordial follicles. Fourth, the small number of included patients hindered our ability to compare the effects of cryopreservation methods on tissue from a single patient. Moreover, the small sample size might have reduced the strength of the findings. Finally, there are no standardised protocols for freezing, gonadotrophin stimulation, or transplantation in nude mouse xenograft models. However, our study demonstrated the feasibility of OTC in our centre. Further randomised controlled trials are needed to confirm our findings.

Future trends

We plan to conduct a randomised controlled trial of the two cryopreservation methods used in this study to determine which is best for clinical implementation. From the experience of the Danish group Rosendahl et al[24] on OTC, they suggested that before applying the technique to humans, each laboratory should thoroughly test and validate the OTC method. In the future, implantation of artificial ovaries or the engraftment of human ovarian tissue into mice may enable fertility restoration without the potential reintroduction of malignant cells. These approaches may be particularly useful in women with a high risk of blood-borne leukaemia or cancers with a high risk of ovarian metastasis, as well as women who cannot undergo autotransplantation.[46]

Conclusion

Our study demonstrated the feasibility and viability of OTC with subsequent OTT in Hong Kong via xenografts in nude mice. These findings support the clinical implementation of OTC and subsequent OTT in Hong Kong, particularly for prepubertal young girls and for women who cannot undergo the ovarian stimulation necessary for oocyte or embryo freezing. Further studies are needed to clarify optimal cryopreservation and engraftment protocols.

Author contributions

Concept or design: JPW Chung, DYL Chan.
Acquisition of data: All authors.
Analysis or interpretation of data: JPW Chung, DYL Chan, Y Song, MBW Leung, JJX Li, CC Wang.
Drafting of the manuscript: All authors.
Critical revision of the manuscript for important intellectual content: JPW Chung, DYL Chan, CC Wang.

All authors had full access to the data, contributed to the study, approved the final version for publication, and take responsibility for its accuracy and integrity.

Conflicts of interest

As an editor of the journal, JPW Chung was not involved in the peer review process of the article. All other authors have no conflicts of interest to disclose.

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Ethics approval

The research was approved by the Institutional Review Board of the Joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethics Committee (Ref No.: 2019.356) and overseen by an independent data and safety monitoring committee. The trial was registered with the World Health Organization Primary Registry–Chinese Clinical Trials Registry (Trial No.: ChiCTR2100041611). The experimental animal protocol was approved by The Chinese University of Hong Kong Animal Experimentation Ethics Committee (Ref No.: 19-214-MIS). All participants received a detailed explanation of the study and provided written consent for inclusion. All researchers involved in animal experiments were licensed by the Department of Health of the Hong Kong SAR Government.

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