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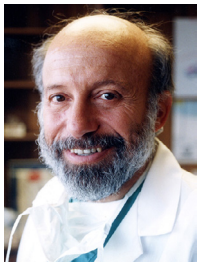
ARTICLE

In-vitro maturation and transplantation of cryopreserved ovary tissue: understanding ovarian longevity



BIOGRAPHY

Sherman Silber performed the first microsurgical vasectomy reversals, the first human testicle transplant and the first human ovary transplant. He developed testicular sperm extraction and microsurgical epididymal sperm aspiration and headed the clinical portion of the MIT (USA) team that discovered the *DAZ* gene. His latest research involves in-vitro gametogenesis in humans.

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KEY MESSAGE

Ovary tissue cryopreservation and orthotopic transplantation results in a 76% spontaneous pregnancy live baby rate. There has been no transmission of cancer. In-vitro maturation of oocytes from ovarian tissue is simple and robust. There is no need to delay cancer treatment for ovarian stimulation.

ABSTRACT

Research question: Is it possible to use experience gained from 24 years of frozen ovarian transplantation, and from recent experience with in-vitro gametogenesis to accomplish simple and robust in-vitro maturation (IVM) of oocytes from human ovarian tissue?

Design: A total of 119 female patients between age 2 and 35 years old underwent ovary cryopreservation (as well as in-vitro maturation of oocytes and IVM in the last 13 individuals) over a 24-year period. Up to 22 years later, 17 returned to have their ovary tissue thawed and transplanted back.

Results: Every woman had a return of ovarian function 5 months after transplant, similar to previous observations. As observed before, anti-Müllerian hormone (AMH) concentration rose as FSH fell 4 months later. The grafts continued to work up to 8 years. Of the 17, 13 (76%) became pregnant with intercourse at least once, resulting in 19 healthy live births, including six live births from three women who had had leukaemia. Of the harvested germinal vesicle oocytes, 35% developed with simple culture media into mature metaphase II oocytes.

Conclusions: The authors concluded the following. First, ovary tissue cryopreservation is a robust method for preserving fertility even for women with leukaemia, without a need to delay cancer treatment. Second, many mature oocytes can often be obtained from ovary tissue with simple media and no need for ovarian stimulation. Third, ovarian stimulation only be necessary for removing the oocyte from the ovary, which can also be accomplished by simple dissection at the time of ovary freezing. Finally, pressure and just eight 'core genes' control primordial follicle recruitment and development.

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KEYWORDS

Cancer and fertility
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Primordial follicle recruitment

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INTRODUCTION

Beginning in 1997 a series of 142 patients (an increase from 108 in a previous report) referred for ovary tissue cryopreservation (119 of whom went through the procedure) provided an opportunity to assess the long-term efficiency of this procedure for young women about to undergo sterilizing cancer treatment (*Silber, 2015*, *2016*; *Silber et al., 2018*). This paper is an update, 4 years later, of the authors' previous summary in 2017. It also presents new results with in-vitro maturation (IVM) from the preserved ovarian tissue (*Andersen et al., 2008*; *Candy et al., 2000*; *Deanesly, 1954*; *Demeestere et al., 2003*; *Dittrich et al., 2012*; *Donnez et al., 2004*, *2011*, *2013*; *Kagawa et al., 2009*; *Meirow et al., 2005*; *Mussett and Parrott, 1961*; *Poirot et al., 2012*; *Revel et al., 2011*; *Revelli et al., 2013*; *Sanchez et al., 2007*; *Silber and Gosden, 2007*; *Silber et al., 2005*, *2008a*, *2008b*, *2010*, *2012*; *Stern et al., 2013*; *Stoop et al., 2014*).

The interest in human ovary transplant began after Gosden and co-workers' report of successful pregnancies in sheep in 1994 (*Gosden et al., 1994*). However, there are only a small number of systematic reports from single centres (*Donnez and Dolmans, 2017*; *Donnez et al., 2013*; *Gellert et al., 2018*; *Lotz et al., 2019*; *Meirow et al., 2016*; *Pretalli et al., 2019*; *Shapira et al., 2018*, *2020*). Furthermore, there is no series from the USA except for that from the current authors (*Silber et al., 2018*). Moreover, there has been no systematic study of the mechanisms of ovarian function and longevity that can be obtained from this extensive experience.

The purpose of this report is to the update the results of the author's study in the USA and include what has been learned from in-vitro gametogenesis about IVM, i.e. obtaining competent metaphase II (MII) oocytes at the same time as cryopreservation of the ovarian tissue. Unique to this report are: (i) results from vitrification as well as slow freezing; (ii) the birth of six healthy infants from women with leukaemia; (iii) the relative simplicity within the series of IVM from ovary tissue; and (iv) the success rate from just one centre using the same techniques (*Donnez and Dolmans, 2017*; *Greve et al., 2012*; *Meirow et al., 2016*; *Shapira et al., 2018*, *2020*; *Stoop et al., 2014*). Here, the authors report the latest update of a single large series of cryopreserved transplants from one centre in the USA, carried out with the same technique assessed uniformly over long-term follow-up, and studied carefully to elucidate the mechanisms of ovarian longevity and failure. The authors also wished more recently to see if mature oocytes could be obtained easily *in vitro* from the removed ovarian tissue, prior to freezing, and thus avoid the need for hormonal stimulation.

MATERIALS AND METHODS

Participants

Over a period from 1997 to 2020 (24 years), 119 female patients (out of 142 who were initially consulted) between age 1 and 42 years underwent ovary tissue freezing for fertility preservation by either slow freezing or vitrification, and the 13 most recent also underwent IVM with vitrification of the resultant MII oocytes. Of these 119 cases, 85 related to cancer, 8 were for threatened premature ovarian failure, 13 for social reasons, and 13 for a variety of conditions including Turner's syndrome, multiple sclerosis, endometriosis, aplastic anaemia, a daughter born with no ovary or massive bilateral ovarian teratoma. All patients underwent slow freezing prior to September 2007, and all subsequent participants underwent vitrification of their ovarian tissue.

The most recent 13 cases of ovary tissue freeze also underwent IVM of oocytes retrieved during the ovarian dissection. All patients were counselled in detail with the advice that the transplant might not ever be performed or might not function. All underwent international review body consent.

Cryopreservation and transplant surgery

The technique for slow freeze and thaw of ovarian tissue has not changed since the original description by Gosden and colleagues in 1994 (*Gosden et al., 1994*; *Silber et al., 2005*). Slow freezing was the approach initially used when this programme began in 1997, and it has been previously described in detail (*Silber et al., 2018*). The thaw for slow freezing has, however, not been well described in the literature. Thawing is performed rapidly (100°C/min) in a warm bath after first holding the cryovial in air for 30 seconds. Serial transfer to the thawing solutions of 1.0M cryoprotectants, 0.5M cryoprotectant, 0.2M sucrose and then standard media is undertaken for 5 min for each step. As there is usually redundant tissue under the cortex after thawing with slow-freeze cases, the tissue is trimmed under an operating microscope before transplantation, as previously described in great detail (*Donnez et al., 2004*; *Gosden et al., 1994*; *Newton et al., 1996*; *Silber et al., 2005*, *2015*). The purpose of this trimming is to avoid interference with revascularization, or diffusion of nutrients to the transplanted cortex, and thus prevent ischaemic damage.

Since 2007, the authors have exclusively used vitrification, because in-vitro viability analysis studies have shown no oocyte loss with vitrification, but a 40% oocyte loss with slow freezing (*Kagawa et al., 2009*; *Keros et al., 2009*; *Silber et al., 2010*). Nonetheless, slow freezing seems equally robust for ovarian tissue cryopreservation because the loss of half of a huge number of eggs (200,000) would not be likely to affect the clinical result. The authors' practice exclusively adopted vitrification in 2007 because it was simpler and more feasible for the series. The technique for vitrification of ovary tissue has been well described in the literature and has also not changed (*Kagawa et al., 2009*; *Keros et al., 2009*; *Silber et al., 2010*). The transplant technique has not changed either since the team's first fresh transplant in 2004, or the frozen transplants described in 2018 (*Silber et al., 2018*) (FIGURES 1 AND 2).

The pieces of thawed cortex are first quilted together with 9-0 monofilament nylon under an operating microscope and continuous pulsatile irrigation with ice cold media. This quilted ovarian cortex is then transplanted onto the residual ovary medulla after the oocyte-depleted cortex has been resected. Any haemorrhages on the medulla are controlled with micro-bipolar forceps and pressure stitches of 9-0 nylon to avoid micro-haematoma formation under the graft. The graft was placed orthotopically to allow natural conception from ovary retrieval by the fallopian tube.

In-vitro maturation

After the ovarian cortex had been dissected from the medulla (FIGURE 3) and divided into slices for cryopreservation, the 'spent' medium in which the

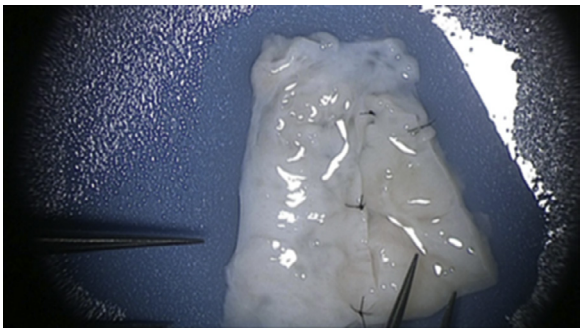

FIGURE 1 Ovary transplant tissue quilting. Reproduced from *Silber et al., (2018)*.

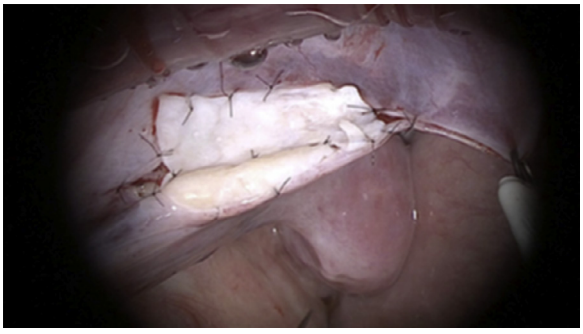

FIGURE 2 Ovary transplant. Reproduced from *Silber et al. (2018)*.

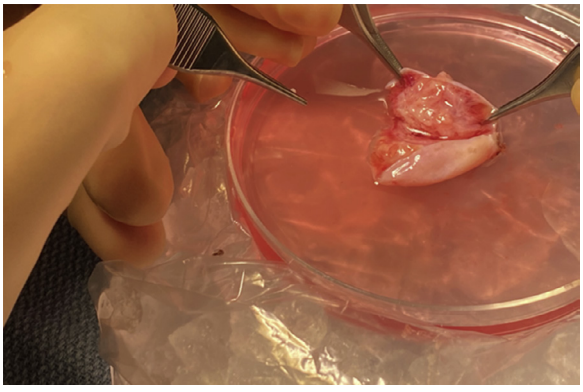

FIGURE 3 Dissection of ovarian cortex from the medulla.

dissection took place was examined for free, loose cumulus complexes, which usually contain immature germinal vesicle oocytes. The protocol of the Denmark group was followed closely for this dissection (*Nikiforov et al., 2020*). These cumulus complexes were then placed in culture with widely varying concentrations of FSH and human chorionic gonadotrophin (HCG) or LH, and the cumulus was then stripped at between 24 and 44 h. The reason that a variety of media and gonadotrophin concentrations were employed is based on the authors' previously published data from in-vitro gametogenesis in mice (*Hayashi et al., 2021*). The group wished to see whether IVM could proceed in various media because the oocytes might already be able *in vivo* to mature. Note that no prior hormonal stimulation was administered. Any oocytes that had advanced by then to mature MII stage were then vitrified in standard fashion (*Kuwayama et al., 2005*; *Nikiforov et al., 2020*; *Prasath et al., 2014*, *Segers et al., 2015*, *2020*; *Uzelac et al., 2015*).

For IVM culture, a variety of concentrations of FSH and HCG (LH) were employed. Those concentrations were: (i) 75 mIU/mL FSH with only 10 mIU/ mL HCG with ordinary cleavage media; (ii) 150 mIU/ml FSH with only 20 mIU/mL HCG with ordinary cleavage media; (iii) 75 mIU/mL FSH with 1000 mIU/mL HCG in cleavage media; (iv) 75 mIU/mL FSH with 1000 mIU/mL HCG in Sage, United States IVM media; and (v) 75 mIU/mL HCG in Sage IVM media. This variety of ordinary medium was used because of previous results with in-vitro gametogenesis demonstrating the stages of IVD (in vitro development), IVG, (in vitro gametogenesis) and IVM, postulating that many of these germinal vesicles would already have completed the full phase of IVD and IVG *in vivo* in the ovary. The authors suspected that no special IVM media would be needed and that almost any concentration of HCG would suffice.

Postoperative follow-up

All the women underwent monthly hormone monitoring and recording of the return of menstrual cycling for many years, from the time of the transplant to the time of writing this paper, occasionally missing a few months. All women were free to get pregnant via intercourse. IVF and hormonal stimulation were not used with any of the participants. All cases were approved by the IRB of St. Luke's Hospital in St. Louis, Missouri, USA (IRB No. 2016.003, approved 27 April 2021). Informed consent was obtained from all patients or their guardians according to the IRB. This is a prospective series.

RESULTS

All 17 transplant cases to date (as already reported for the first 13) showed a return of spontaneous menses from 4 to 5 months after transplantation, plus a return of FSH to normal concentrations, with regular cycling. As FSH returned to normal, anti-Müllerian hormone (AMH) rose to high concentrations, and then fell to very low ones after a further 4–8 months, as previously noted with the first 13 participants. All the grafts functioned from 2 years to as long as 8 years after surgery, and eight are still functioning at the time of writing. All recipients were between 18 and 31 years of age at the time of freezing, with a median age of 24 years (TABLE 1). Eleven of these 17 had undergone slow freezing, and 6 had undergone vitrification.

Thirteen transplants resulted in spontaneous pregnancy and the delivery of at least one live healthy infant (76%). In one case, five singletons so far have

TABLE 1 OVERALL RESULTS WITH FROZEN OVARY TISSUE TRANSPLANTS BEGINNING IN 1997 TO THE PRESENT, NOW UPDATED FROM 2017

Date of transplant	Age at transplantation (years)	Age at freeze (years)	Diagnosis	Pregnant	Live birth or ongoing	Time until pregnancy (days)	Miscarriages	Duration of ovarian function (months)
6 March 2007	26	24	Premature ovarian failure	Yes	Female	174	–	23 (ended)
13 January 2009	31	20	Hodgkin's lymphoma	Yes	Male	272	–	29 (ended)
9 June 2009	29	24	Premature ovarian failure	Yes	–	276	1	19 (ended)
17 June 2011	33	20	Hodgkin's lymphoma	No	–	–	–	38 (ended)
12 October 2012	33	31	Multiple sclerosis	Yes	Female	481	–	67 (ended)
29 March 2013	32	25	Premature ovarian failure	Yes	Female	243	–	26 (ended)
5 April 2013	33	30	Brain cancer	Yes	Male	665	–	61 (ended)
12 April 2013	25	18	Leukaemia	Yes	Male	502	–	93 (still functioning)
					Female	998	–	
					Male	157	–	
					Female	2082	–	
					Male	2497	–	
1 October 2013	29	28	Synovial sarcoma	No	–	–	–	69 (ended)
7 October 2013	39	24	Leukaemia	Yes	Female	1287	–	87 (still functioning)
21 July 2015	28	25	Leukaemia	No	–	–	–	65 (still functioning)
5 August 2015	32	21	Hodgkin's lymphoma	Yes	Female	343	–	65 (still functioning)
18 September 2014	36	20	Hodgkin's lymphoma	Yes	Female	473	–	72 (ended)
					Female (2)	908	–	
31 August 2018	32	26	Hodgkin's lymphoma	Yes	–	210	1	28 (still functioning)
					Female	292	–	
					–	799	1	
					Male	964	–	
9 December 2019	37	28	Synovial sarcoma	No	–	–	–	12 (still functioning)
27 February 2019	39	33	Large B-cell lymphoma	Yes	–	240	1	21 (still functioning: never stopped having periods)
					Male	645	–	
13 August 2019	36	23	Hodgkin's lymphoma	Yes	Female	210	–	17 (still functioning: never stopped having periods)

Totals: 17 participants; 19 babies; 13 women pregnant with delivery (76%); 12 female and 6 male offspring; 6 vitrifications; 11 slow freezes.

resulted from just one participant from one transplant, two singletons from another woman, and in a third case, two singletons and one spontaneous set of twins from just one patient. There have thus been a total of 19 live, healthy infants without IVF in these 17 cases. There have been a total of 22 pregnancies, three of which miscarried before 3 months (14%) (TABLE 1). Ovarian function is still continuing in 3 of the 6 cases involving vitrified tissue and 5 of the 11 slow-freeze cases (TABLES 2 and 3).

Three of the transplants were for leukaemia, for which the oncologists involved gave approval. The basis for approval was that the ovary had been removed and frozen after the patient was in her first remission, before the bone marrow transplant, and that stains for residual cancer cells were negative (*Greve et al., 2012*). Two of these three resulted in spontaneous pregnancy, with the delivery of a total of six healthy infants (TABLE 4). There has been no recurrence of the leukaemia, and in fact no recurrence in any of the cases involving cancer. All three women who had leukaemia still have ovarian function. These were among the first cases of success in terms of leukaemia patients having babies from transplanting their frozen tissue, although the first such case was published in 2017 (*Silber et al., 2018*). One of the participants who had had leukaemia now had five live births from her transplanted ovary tissue.

The most recent 13 cases involved IVM of the cumulus complexes recovered in the media after cortico-medullary dissection. The number recovered varied widely – between 3 and 37 cumulus complexes depending on the age of the women and whether there had been any prior chemotherapy. Additional details are provided in TABLE 5. The rate of maturation to an MII oocyte (FIGURES 4 AND 5) varied from a low of 19% to a high of 56% (average 35%). The average values for other centres publishing on IVM has varied between 30% and 39% (*Andersen et al., 2008*;

TABLE 2 VITRIFIED OVARY TISSUE TRANSPLANTS, NOW UPDATED FROM 2017

Date of transplant	Age at transplantation (years)	Age at freeze (years)	Diagnosis	Pregnant	Live birth or ongoing	Time until pregnancy (days)	Miscarriages	Duration of ovarian function (months)
12 October 2012	33	31	Multiple sclerosis	Yes	Female	481	–	67 (ended)
5 April 2013	33	30	Brain cancer	Yes	Male	665	–	61 (ended)
1 October 2013	29	28	Synovial sarcoma	No	–	–	–	69 (ended)
21 July 2015	28	25	Leukaemia	No	–	–	–	65 (still functioning)
27 February 2019	39	33	Large B-cell lymphoma	Yes	–	240	1	21 (still functioning: never stopped having periods)
					Male	645	–	

Totals: 6 participants; 3 babies; 3 women pregnant; 1 miscarriage.

TABLE 3 SLOW-FREEZE OVARY TISSUE TRANSPLANT, NOW UPDATED FROM 2017

Date of transplant	Age at transplantation (years)	Age at freeze (years)	Diagnosis	Pregnant	Live birth or ongoing	Time until pregnancy (days)	Miscarriages	Duration of ovarian function (months)
6 March 2007	26	24	Premature ovarian failure	Yes	Female	174	–	23 (ended)
13 January 2009	31	20	Hodgkin's lymphoma	Yes	Male	272	–	29 (ended)
9 June 2009	29	24	Premature ovarian failure	Yes	–	276	1	19 (ended)
17 June 2011	33	20	Hodgkin's lymphoma	No	–	–	–	38 (ended)
29 March 2013	32	25	Premature ovarian failure	Yes	Female	243	–	26 (ended)
12 April 2013	25	18	Leukaemia	Yes	Male	502	–	93 (still functioning)
					Female	998	–	
					Male	157	–	
					Female	2082	–	
					Male	2497	–	
5 August 2015	32	21	Hodgkin's lymphoma	Yes	Female	343	–	65 (still functioning)
18 September 2014	36	20	Hodgkin's lymphoma	Yes	Female	473	–	72 (ended)
					Female (2)	908	–	
7 October 2013	39	24	Leukaemia	Yes	Female	1287	–	87 (still functioning)
31 August 2018	32	26	Hodgkin's lymphoma	Yes	–	210	1	28 (still functioning)
					Female	292	–	
					–	799	1	
					Male	964	–	
13 August 2019	36	23	Hodgkin's lymphoma	Yes	Female	210	–	17 (still functioning: never stopped having periods)

Totals: 11 participants; 19 babies; 10 women pregnant; 3 miscarriages.

TABLE 4 OVARY TISSUE FREEZE TRANSPLANTS IN LEUKAEMIA, NOW UPDATED FROM 2017

Date of transplant	Age at transplantation (years)	Age at freeze (years)	Diagnosis	Pregnant	Live birth or ongoing	Time until pregnancy (days)	Miscarriages	Duration of ovarian function (months)
12 April 2013	25	18	Acute myeloid leukaemia	Yes	Male	502	–	93 (still functioning)
					Female	998	–	
					Male	157	–	
					Female	2082	–	
					Male	2497	–	
7 October 2013	39	24	Acute lymphocytic leukaemia	Yes	Female	1287	–	87 (still functioning)
21 July 2015	28	25	Acute myeloid leukaemia	No	–	–	–	65 (still functioning)

Totals: 3 participants; 6 babies (67%); 2 women pregnant with delivery.

TABLE 5 SUMMARY OF IN-VITRO MATURATION

Patient's age (years)	Reason for cryopreservation	Culture medium base used	FSH/HCG	Number of cumulus complexes	Metaphase II oocytes frozen	In-vitro maturation rate (%)	Total ovarian tissue freeze pieces
29	Large B-cell lymphoma of the cervix	Quinn's Cleavage	75 mIU/ml FSH + 10 mIU/ml HCG	25	12	48	21
35	Triple-negative breast cancer: *BRCA* positive	Quinn's Cleavage	75 mIU/ml FSH + 1000 mIU/ml HCG	11	1	9	17
41	Fertility Preservation (social)	Medicult IVM	75 mIU/ml FSH + 1000 mIU/ml HCG	3	2	67	17
18	Large B-cell lymphoma	Medicult IVM	75 mIU/ml FSH + 1000 mIU/ml HCG	10	2	20	20
18	Ewing's sacroma	Quinn's Cleavage	150 mIU/ml FSH + 20 mIU/ml HCG	34	10	29	21
32	Fertility preservation for Turner's syndrome: daughter	Quinn's Cleavage	75 mIU/ml FSH + 10 mIU/ml HCG	24	9	38	10
18	Breast cancer	Quinn's Cleavage	75 mIU/ml FSH + 10 mIU/ml HCG	8	2	25	16
33	Breast cancer	Quinn's Cleavage	75 mIU/ml FSH + 1000 mIU/ml HCG	26	5	19	10
25	Breast cancer	Sage IVM	75 mIU/ml FSH + 1000 mIU/ml HCG	14	5	36	10
14	Non-Hodgkin's lymphoma	Sage IVM	75 mIU/ml FSH + 100 mIU/ml HCG	37	14	38	20
19	Hodgkin's lymphoma	Quinn's Cleavage	75 mIU/ml FSH + 100 mIU/ml HCG	40	14	35	22
13	Rhabdomyosarcoma	Quinn's Cleavage	75 mIU/ml FSH + 100 mIU/ml HCG	25	14	56	16
22	Fertility preservation for premature ovarian failure: sister	Quinn's Cleavage	75 mIU/ml FSH + 100 mIU/ml HCG	44	17	38	22

Average in-vitro maturation rate, 35% of metaphase II oocytes vitrified; average number of ovary pieces vitrified, 17.

HCG, human chorionic gonadotrophin.

Hayashi et al., 2011, *2012*; *Hikabe et al., 2016*; *Nagamatsu et al., 2019*; *Nikiforov et al., 2020*; *Rosendahl et al., 2011*; *Schmidt et al., 2011*; *Segers et al., 2015*). Maturation of germinal vesicle to MII oocytes was detected between 24 and 48 h of exposure to the HCG-containing media. For most participants, the number of mature oocytes was what would be obtained from ovarian hyperstimulation, as is apparent from TABLE 5. Surprisingly, the success of IVM was not related to the media or to the concentration of gonadotrophin in the media. A variety of media and concentrations were intentionally used in light of now-established mechanisms of in-vitro oogenesis, to see if this understanding could be used for a simplification of IVM (Hamazaki et al., 2021a, 2021b; *Hayashi and Saitou, 2013*, *Hayashi et al., 2011*, *2012*; *Hikabe et al., 2016*; *Nagamatsu et al., 2019*).

DISCUSSION

Since the report by Gosden and colleagues with frozen ovary tissue autologous transplantation in sheep, and the first reported cases in 2005 in humans, with spontaneous pregnancy and healthy deliveries, there has been intense interest in preserving the fertility of cancer survivors (Anderson and Cameron, 2007; Bleyer, 1990; *Donnez et al., 2004*; *Gosden et al., 1994*; *Lee et al., 2006*; *Maheshwari et al., 2008*; *Meirow et al., 2005*; *Silber et al., 2005*; *Young et al., 1986*). The first ovary freezing for cancer patients at the current authors' centre was in 1997. Thawed tissue did not begin to be transplanted back until 10 years later, in 2007.

There have now been over several hundred infants born around the world from ovary tissue transplantation in cancer survivors, with no reports of the transmission of cancer except possibly one case from an ovarian cancer (*Donnez and Dolmans, 2017*; *Gellert et al., 2018*; *Lotz et al., 2019*; *Meirow et al., 2016*; *Pretalli et al., 2019*; *Rowell et al., 2020*; *Shapira et al., 2018*, *2020*; *Stern et al., 2013*). All patients at the current authors' centre were taken at no charge, which may explain why the groups was able to accumulate the only such series in the USA. This current report of relatively robust results in a small but well-studied series might generate more enthusiasm in the USA to help these patients. However, it is noteworthy that out of 119 women who had ovary tissue frozen, only 17 so far (14%) have requested to have the tissue thawed and transplanted back. It is often 10–20 years before they return, even though most have survived their cancer.

The unique features of this report include: (i) the robustness of results with this technique; (ii) the inclusion of both slow freezing and vitrification; (iii) some of the first successful results with three leukaemia patients, who delivered six healthy infants; (iv) a well-studied series from just one centre (the only such centre in the USA); and (v) the very recent addition of IVM from ovarian tissue to freeze MII oocytes at the same time as cortical tissue cryopreservation. The advantages of ovary cryopreservation over oocyte vitrification for cancer patients include no delay of cancer treatment, the avoidance of ovarian stimulation, and a resumption of endocrine function. As these results (and those reported by other groups) with IVM demonstrate, one might consider the option to dispense with ovarian stimulation, and go right to oophorectomy, with no delay in cancer treatment. Surprisingly, there might therefore possibly be no need for stimulation to obtain mature

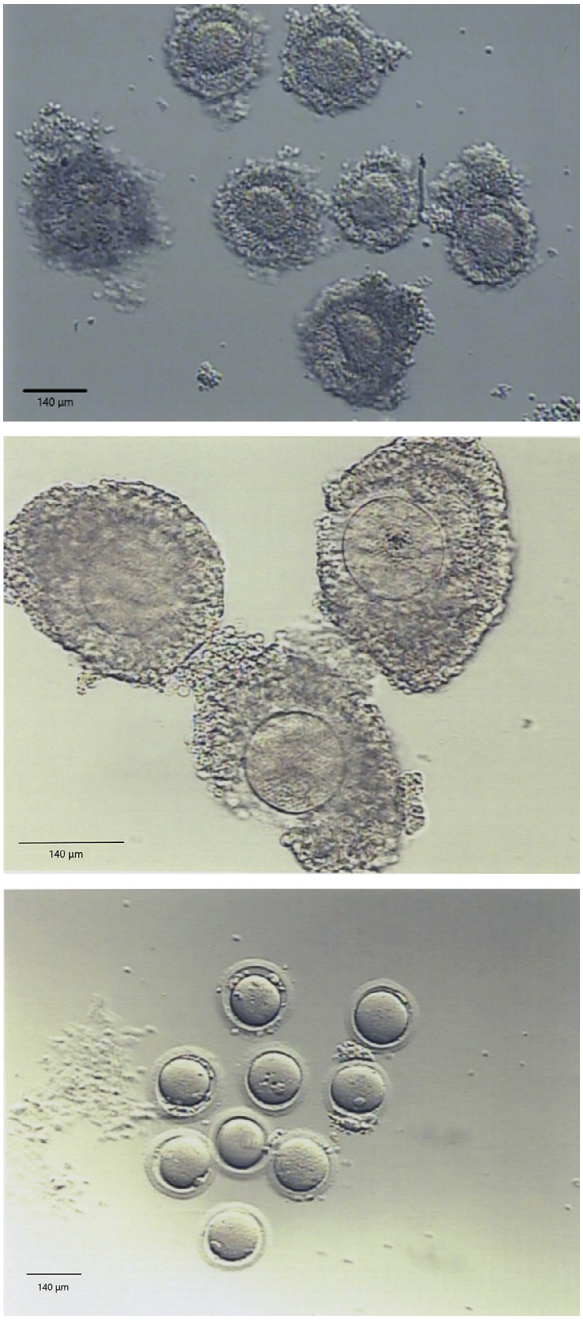

FIGURE 4 (A) Day 0: compact appearance of cumulus complexes at the time of dissection. (B) Day 1: spreading of cumulus complexes after 24 h in culture with human chorionic gonadotrophin (HCG) or LH. (C) Day 1: metaphase II oocytes after 24 h in culture with HCG or LH.

oocytes (*Nikiforov et al., 2020*; *Prasath et al., 2014*; *Segers et al., 2015*, *2020*; *Uzelac et al., 2015*). Mature oocytes may possibly be directly obtained from the excised ovarian tissue. Other groups have preceded these current efforts at IVM from excised ovarian tissue and achieved pregnancy with healthy infants (*De Vos et al., 2021*; *Prasath et al., 2014*, *Segers et al., 2015*, *2020*; *Uzelac et al., 2015*; *Vesztergom et al., 2021*).

It might at first seem puzzling why IVM from ovarian tissue suddenly seems so easy at many different centres, when it has been difficult in the past. There are several potential reasons for this. First, this group was not trying to mature primordial follicles (although that may become possible in the future with the 'core genes') (*Hamazaki et al., 2021*). Culturing germinal vesicle oocytes that have already become meiotically competent by in-vivo IVD and IVG would not be expected to be difficult. In addition, it is far easier to obtain many germinal vesicle oocytes with cortical dissection rather than with a needle.

This study and the results of in-vitro gametogenesis reveal the limited role of the ovulatory cycle and ovarian stimulation in oocyte development other than to remove the oocyte from the ovary (*Andersen et al., 2008*; *Hayashi et al., 2011*, 2013, 2013; *Hikabe et al., 2016*; *Nikiforov et al., 2020*; *Rosendahl et al., 2011*; *Schmidt et al., 2011*). Intrinsic tissue pressure along with eight 'core genes' has been shown with in-vitro gametogenesis to be the initiating mechanism at work to control primordial follicle recruitment and development to antral follicle status (*Hikabe et al., 2016*; *Nagamatsu et al., 2019*; *Nikiforov et al., 2020*; *Prasath et al., 2014*; *Segers et al., 2015*, *2020*; *Uzelac et al., 2015*; Winkler-Crepaz et al., 2016; *Woodruff and Shea, 2011*; *Xiao et al., 2015*). Likewise an in-vivo cortical tissue pressure gradient may be a major regulator of primordial follicle recruitment and ovarian longevity (*Silber, 2015*; *Silber et al., 2015*). Primordial follicle arrest in the highly compact ovarian cortex is thought to be a possible key to saving the oocyte from disappearing after the fetal initiation of meiosis and the continuation all the way through meiosis and subsequent apoptosis (*Hayashi et al., 2011*, *2012*, 2013; *Hikabe et al., 2016*; *Nagamatsu et al., 2019*). It may also be a key to the gradual recruitment every month of a limited number of oocytes in the adult to develop over 4 months into gonadotrophin-sensitive antral and graafian follicles, which spares the resting oocytes from sudden total depletion (*Woodruff and Shea, 2011*). This pressure theory is supported clinically by the changes in AMH and FSH observed and previously reported and discussed in the current group's fresh and frozen ovary tissue transplants (*Silber et al., 2015*, 2016, *2018*).

Perhaps the most striking scientific postulate about ovarian function resulting from this series is the high rate of maturation of germinal vesicle oocytes from ovary tissue to MII in less than 2 days, with no ovarian stimulation. The success of IVM did not correlate in this series with the specifics of the media used or the concentration of gonadotrophin. This might have

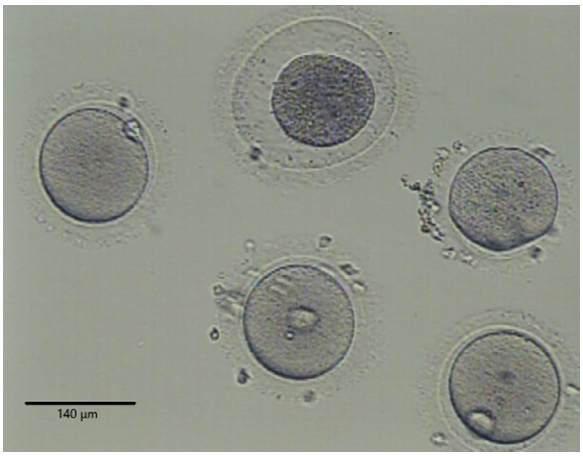

FIGURE 5 Three normal metaphase II oocytes, one metaphase I oocyte and one degenerated oocyte, resulting from germinal vesicle oocytes from cultured ovarian tissue, on day 2.

been expected from the early work of Edwards and of Cha and colleagues, as well as Hayashi's group (*Cha et al., 1991*; *Edwards, 1962*; *Hayashi et al., 2011*, *2012*, 2013). The success of IVM appears to be intrinsic to the cumulus, in which the germinal vesicle oocyte has already *in vivo* developed meiotic competence, and because the germinal vesicle oocytes have already achieved meiotic competence, by in-vivo exposure to the eight 'core genes', and in-vivo FSH (*Hamazaki et al., 2021*).

On average the ovarian cortex of a young woman contains about 200,000 oocytes. Every month about 1000 are recruited from 'resting' follicles in the cortex, and these require 4–5 months thereafter to become sensitive to gonadotrophins and enter the ovulatory cycle. In-vitro gametogenesis studies in mice have termed this phase 'IVD', i.e. in-vitro differentiation to germinal vesicle stage. This phase of "PPT" (primordial to primary follicle transition) can proceed *in vitro* with merely eight 'core genes'. In-vitro gametogenesis uncovers three distinct phases of oocyte development: IVD, IVG and IVM. IVD represents the non-gonadotrophin-sensitive growth from either recruited oocytes or pluripotent stem cell to germinal vesicle oocytes, which, as in antral follicles *in vivo*, are now sensitive to gonadotrophin. This takes 3 weeks in mice, but closer to 3 or 4 months in humans (*Donnez et al., 2004*; *Hayashi et al., 2012*, 2013; *Silber, 2015*; *Silber et al., 2015*, 2016). IVD *in vivo* is a constantly occurring process, as also is IVG. There is a continuous exposure of IVG-ready oocytes in the intact in-vivo ovary to FSH. This process usually takes about 10–12 days in both mice and humans. Oocytes that have completed IVG and are not exposed to HCG *in vitro*, or to the ovulatory LH surge *in vivo*, gradually deteriorate. It is the population of oocytes retrieved from the dissected ovarian cortex that have recently completed IVG that mature readily with exposure to HCG in a variety of concentrations (as *in vivo*) in ordinary culture media.

The phase referred to as "IVG", i.e. gonadotrophin (FSH)-induced meiotic competence of germinal vesicle oocytes, usually requires 9–12 days, similar to ovarian stimulation in human IVF. The unstimulated ovary has already been exposed to FSH *in vivo*. Many (possibly as many as 30–50%) of the 30 or more germinal vesicle oocytes (in cumulus complexes) recovered from the ovarian dissection have already gone through the "IVD" and "IVG" phases *in vivo*, and therefore are meiotically competent, having already had adequate exposure to endogenous FSH. Therefore just 1–2 days of exposure to LH or HCG is all that is needed for these specific germinal vesicle oocytes to develop to mature MII oocytes. Furthermore, the eight 'core genes' are all that are needed to convert stem cells to oocytes. However, these oocytes are not fully competent. Full competence requires culture with fetal granulosa cells, which can also be produced from stem cells using a more complex culture system (Yoshino et al., 2021).

It is easy to collect many germinal vesicle oocytes from these tiny follicles when you have the ovary in hand instead of using a needle, and their intrinsic 30–40% meiotic competence is universal. So why do we even need ovarian stimulation, or even the normal ovulatory cycle? The normal ovulation cycle is not needed for meiotic competence. Ovarian stimulation for IVF and even for the normal ovulatory cycle is only required for easy oocyte retrieval or just to allow oocytes to exit the ovary.

Without the ovarian cortex, which induces the formation of primordial follicles, fetal oocytes would continue in meiosis and be completely depleted by birth (*Donnez and Dolmans, 2017*; *Donnez et al., 2013*; *Greve et al., 2012*; *Hayashi et al., 2011*, *2012*, 2013; *Hikabe et al., 2016*; *Jensen et al., 2015*; *Nesbit et al., 1980*; *Ortea et al., 1981*; *Silber et al., 2015*). Nagamatsu and colleagues demonstrated that dense cortical tissue pressure caused oocyte nuclei to rotate, and this held the primordial follicles in arrest (*Nagamatsu et al., 2019*). As they encountered less tissue pressure internally, the rotation stopped, and the primordial follicles were then recruited (*Nagamatsu et al., 2019*).

The dense fibrous tissue of the ovarian cortex not only controls follicle development, but also represents a relatively inhospitable location for cancer cells. A perhaps surprising correlation is that prepubertal boys with leukaemia usually have metastasis to the testis, but not to the tunica albuginea of the testis (*Greve et al., 2012*; *Nesbit et al., 1980*; *Ortega et al., 1981*). As the tunica albuginea of the testis is the same as the ovarian cortex, much can be learned by studying leukaemia in the male testes. After initial chemotherapy for leukaemia, when the female patient is in temporary remission, no viable leukaemia cells are detected in her ovarian cortex (*Greve et al., 2012*). The three leukaemia patients in the current series had their ovary removed and cryopreserved while they were in remission before their bone marrow transplant. There has been no recurrence of cancer in these or any of these transplants.

The recruitment of otherwise 'locked' primordial follicles is regulated by a reduction in tissue pressure, but also specifically requires eight 'core genes' to accomplish this *in vitro* and *in vivo* (*Hamazaki et al., 2021*; *Lind et al., 2018*; *Thomas-Teinturier et al., 2013*; *Wallace and Kelsey, 2010*; Winkler-Crepaz et al., 2016; *Yasui et al., 2012*; *Zhai et al.,*

2012). These eight 'core genes' can also recruit stem cells or IPS (induced pluripotent stem cell) cells to transform all the way to MII oocyte-like cells, but recruitment from primordial follicles (from ovarian tissue) results in normal, competent oocytes. Therefore with IVM it is quite easily possible to obtain normal MII oocytes from ovarian tissue, as this report and others demonstrate (*Nikiforov et al., 2020*; *Prasath et al., 2014*; *Segers et al., 2015*, *2020*; *Uzelac et al., 2015*). Of course, the only way to be certain of the functional competence of these MII oocytes is if live births can be obtained. But several reports have already shown this to be the case (*De Vos et al., 2021*; *Nikiforov et al., 2020*; *Prasath et al., 2014*; *Segers et al., 2015*, *2020*; *Uzelac et al., 2015*). This paper is in that sense not original in that others have demonstrated IVM from ovarian tissue. What is perhaps a new suggestion is the relative ease with which this can be achieved using ordinary media, and that this would be expected from this group's previously published studies of in-vitro gametogenesis from stem cells in mice.

The high success rates here with pregnancy and healthy babies after frozen ovary tissue transplantation are most likely aided by having tissue only from younger women with no prior history of infertility. Nonetheless, the live baby rate for these otherwise sterile cancer survivors and the obvious effectiveness of standard slow freezing (despite a previously demonstrated high oocyte loss compared with vitrification) for ovarian tissue cryopreservation testifies to its robustness and simplicity (*Aydin et al., 2010*; *Gosden et al., 1989*; *Hayashi et al., 2011*, *2012*, 2013; *Kaaijk et al., 1999*; *Lass et al., 1997*; *Lind et al., 2018*; *Ortega et al., 1981*; *Saiduddin et al., 1970*; *Silber, 2015*; *Silber et al., 2015*). One may be able to obtain as many mature oocytes from IVM using this tissue as one would from ovarian stimulation. Furthermore, following the lead of Greve and co-workers, this approach could even be used for patients with leukaemia (*Greve et al., 2012*). Most leukaemia patients present with severe illness and require initial chemotherapy to go temporarily into remission before having their bone marrow transplant. This is likely to reduce the risk of transmission of leukaemia cells back to the woman after thawed ovarian tissue is transplanted back. The already recruited, developing follicles will be destroyed by this initial chemotherapy. However, the primordial follicles will be resistant (Meirow et al., 2015). Thus, the only option for leukaemia patients may be ovarian freezing and transplant.

Ovarian tissue cryopreservation thus appears (24 years later) to be a robust method for preserving fertility, without any need to delay cancer or other treatment because of the time required for ovarian stimulation. Simple cortical tissue pressure (associated with primordial follicle nuclear rotation) has been found to be a key regulator of primordial follicle arrest, recruitment and ovarian longevity in humans, similar to mice (*Nagmatsu et al., 2019*). However, eight 'core genes' are also necessary to allow the primordial follicles to escape arrest and develop over 4 months to meiotically competent germinal vesicle oocytes. The ability of ovarian tissue germinal vesicle oocytes to undergo normal IVM indicates that the normal ovulatory cycle and ovarian stimulation is not necessary for for oocyte maturation. Their only purpose may be just to grow a follicle for mono-ovulation or for egg retrieval, i.e. to get the mature oocyte out of the ovary.

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