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FERTILITY PRESERVATION

In vitro maturation (IVM) of oocytes recovered from ovariectomy specimens in the laboratory: a promising "ex vivo" method of oocyte cryopreservation resulting in the first report of an ongoing pregnancy in Europe

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Abstract
Purpose We present our center's experience with 34 consecutive cases who underwent in vitro maturation (IVM) of oocytes obtained from ovariectomy specimens and compare our data with updated literature data.
Methods Feasibility and efficiency of oocyte collection during ovarian tissue processing was assessed by the recovery rate, maturation rate, and embryological development after IVM.
Results On average, 14 immature oocytes were retrieved per patient during ovarian tissue processing in 33/34 patients. The overall maturation rate after IVM was 36 %. The maturation rate correlated with the age of the patient and the duration of IVM. Predominately, oocyte vitrification was performed. Eight couples preferred embryo cryopreservation. Here, a 65 % fertilization rate was obtained and at least one good-quality day 3 embryo was cryopreserved in 7/8 couples. The retrieval of oocytes ex vivo resulted in mature oocytes or embryos available for vitrification in 79 % of patients. One patient with ovarian insufficiency following therapeutic embolization of the left uterine and the right ovarian artery because of an arteriovenous malformation had an embryo transfer of one good-quality warmed embryo generated after IVM ex vivo, which resulted in an ongoing clinical pregnancy.
Conclusions IVM of oocytes obtained ex vivo during the processing of ovarian cortex prior to cryopreservation is a procedure with emerging promise for patients at risk for fertility loss, as illustrated by the reported pregnancy. However, more data are needed in order to estimate the overall success rate and safety of this novel approach.

Keywords Fertility preservation · In vitro maturation · Ovarian cortex cryopreservation · Oocyte · Embryo

Capsule In vitro maturation of oocytes obtained during ovarian tissue processing for cryopreservation holds promise for patients in need of fertility preservation as illustrated here with acceptable maturation rates, the capacity to form good-quality embryos, and an ongoing clinical pregnancy.

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Introduction

For many years, cancer survival rates have been steadily increasing urging the medical community to shift their focus to the quality of life of the growing population of cancer survivors. For women in their reproductive period of life, fertility preservation is an emerging discipline that thrives on the improved efficiency of cryotechnology and the advances in reproductive medicine. Female cancer patients can undergo ovarian stimulation with gonadotropins to have a number of oocytes or embryos vitrified for eventual later use, providing hope of an increased chance to have their genetically own children.

In general, ovarian stimulation followed by transvaginal oocyte retrieval requires almost 2 weeks, which in a subset of cancer patients causes an unacceptable delay to initiate cancer therapy. In these patients and in those with hormone-sensitive tumors or those of prepubertal age, ovarian tissue

cryopreservation (OTC) is the only available option if high-risk gonadotoxic cancer treatment is planned (reviewed by De Vos et al. [1]). Although OTC is still considered experimental [2], many researchers believe that this technology holds huge promise and 37 live births are reported to date [3].

In order to maximize the fertility preservation potential of surgically removed ovaries, Revel et al. [4] were the first to "rescue" immature oocytes derived from small antral follicles within ovariectomy specimens during tissue processing in the lab ("ex vivo"), thereby generating an additional source of oocytes for cryopreservation. Since the first report of this combined technique, 13 further publications [4–17] have documented this strategy, either as a case report or in a case series (summarized in Table 1). In these publications, collection of immature oocytes was attempted in patients ranging from 0 to 44 years old and COC were identified in approximately 87 % of patients, making this an interesting procedure to augment the number of oocytes available for cryopreservation. Interestingly, neither the phase of the menstrual cycle nor the use of oral contraceptives appears to compromise the retrieval of COC during ovarian tissue processing [11], rendering this procedure potentially applicable in all women in need of OTC.

Published in vitro maturation (IVM)-rates of COC collected ex vivo ranged from 3 % [14] to 100 % [10, 15]. From the 1583 immature oocytes collected and incubated in IVM culture media reported in literature, 351 reached the metaphase II (MII) stage (22 %). However, an important proportion of these immature oocytes originated from two reports with very low maturation rates (3 % [14] and 13 % [17]), possibly caused by differences in starting material and analysis method (ovaries transported for several hours, naked oocytes, absence of morphologic assessment of maturity). Because of the different scientific strategies in both studies, the data from these studies were omitted from our analysis, which resulted in an adjusted overall maturation rate in literature of 39 % for oocytes harvested ex vivo from ovaries collected for OTC (Table 1).

The available literature data show that COC can consistently be obtained during the OTC procedure. These oocytes are capable of maturing in vitro, can survive the process of vitrification and warming [13], and can undergo fertilization followed by embryo development [4, 11, 15]. Recently, the first live birth was reported after transfer of two frozen-thawed day 2 embryos obtained after IVM ex vivo in Singapore [15]. Here, we document our own experience with this strategy and the efficiency of oocyte collection during OTC to provide more evidence of the promise held by combining OTC and IVM. The combination of these two techniques has culminated in the report of the first clinical pregnancy from ex vivo harvested oocytes in Europe.

Materials and methods

Patient population

From January 2012 to October 2014, 34 patients were enrolled in the fertility preservation program of UZ Brussel and consented to undergo a combination of OTC and IVM ex vivo procedures (Table 2). This strategy was approved by the IRB of the hospital and all patients gave written informed consent. In 32 patients, unilateral ovariectomy was performed; one patient underwent ovarian biopsies from each ovary and one patient underwent bilateral ovariectomy as a preventative measure because of BRCA1 mutation carriership. The age of the patients ranged from 0 to 38 years, with 6 prepubertal girls (<12 years old, no menarche) and 28 adult women (17–38 years). None of the patients had undergone ovarian stimulation with gonadotropins immediately prior to ovariectomy. One patient received chemotherapy 1 year before OTC (patient no. 8); the other patients did not receive chemotherapy or radiotherapy before OTC.

Ovariectomy and ex vivo IVM

Unilateral ovariectomy was performed by laparoscopy. Ovarian tissue was transported in a sterile 0.9 % saline solution on ice to the IVF laboratory within 10 min. For three patients, ovarian tissue was surgically collected in another hospital and transported in a sterile saline solution on ice to our hospital within 3 h. On arrival in the lab, visible antral follicles were punctured with a scalpel to release the follicle fluid in the culture dish. Ovarian tissue was trimmed to 1–2 mm thickness in Leibovitz L-15 medium (Life Technologies, Merelbeke, Belgium) supplemented with 4 mg/ml HSA (Vitrolife, Göteborg, Sweden) and 100 IU/ml penicillin and 100 μg/ml streptomycin (penicillin/streptomycin mix, Life Technologies) as described elsewhere [18]. Immature oocytes, released from follicles that ruptured during the OTC process, were immediately collected by a second operator using stereo microscopy at 37 °C. COC were washed twice in Flushing medium (Medicult, Origio, Vreeland, Netherlands). All collected COC were surrounded by compact cumulus cells and were assumed to contain immature oocytes. Naked or partially denuded oocytes (>50 %) were not considered for IVM. COC were washed in LAG medium (IVM System, Medicult, Origio) and incubated in IVM medium (IVM System, Medicult, Origio) supplemented with 75 mIU/ml HP-hMG (Menopur, Ferring, Saint-Prex, Switzerland), 100 mIU/ml hCG (Pregnyl, Organon, MSD, Haarlem, Netherlands), and 10 mg/ml HSA (Vitrolife) for 30 or 40 h in a four-well dish with oil overlay (Ovoil, Vitrolife) in an incubator containing 6 % CO_2 in air at 37 °C.

Oocyte or embryo vitrification

After IVM, cumulus cells were removed by hyaluronidase exposure and manual denudation of the oocyte. Mature oocytes were used for vitrification or for insemination with partner's sperm by ICSI. All sperm samples were fresh ejaculates of good quality. Embryos were cultured in individual 25 μl droplets of sequential media formulations under oil (Ovoil, Vitrolife) up to day 3 after ICSI. Six cycles were performed in Quinn's Advantage series (SAGE, Origio) and two cycles in Origio sequential series (Origio). Sixteen to 18 h post-insemination, fertilization was assessed by the presence of two pronuclei. On day 2 of preimplantation development, cleavage divisions were recorded. Embryos reaching at least six cells on day 3 with fragmentation limited to 20 % or less were considered for cryopreservation. Day 3 embryos were categorized as excellent, good, moderate, and poor quality based on the cell number, degree of fragmentation, blastomere uniformity, multinucleation, and the presence of vacuoles and/or granularity. In summary, excellent embryos have ≥7 cells, <10 % fragmentation, and blastomere size according to the division pattern; good embryos have ≥6 cells and/or less than 50 % fragmentation; moderate embryos have ≥4 cells and/or <50 % fragmentation or <50 % of the cells multinucleated; and poor embryos display growth arrest (<4 cells) and/or severe fragmentation (>50 %) and/or severe multinucleation.

Oocytes and embryos were vitrified using the Vitrification kit media (Irvine Scientific, Tilburg, Netherlands) and high-security straws (VHS Kit, CryoBiosystem, L'Aigle, France) with a protocol adjusted for oocytes or embryos.

Results are presented as mean values±SEM in tables and text.

Results

OTC and ex vivo IVM

In two patients, no ovarian cortex tissue was available for cryopreservation (Table 2). For one patient who suffered from an immature teratoma of the tube, a small volume of macroscopically normal tissue was processed to search for COC and afterwards sent to the pathology department for further investigation. The second patient, a 34-year-old carrier of a deleterious BRCA1 mutation, underwent removal of both ovaries and preferred not to have ovarian cortex cryopreserved. This patient opted for an embryo cryopreservation approach and had undergone six cycles of controlled ovarian stimulation (COS) prior to ovariectomy.

On average, 14 ovarian cortex fragments were cryopreserved per patient in 34 patients, with a maximum of 28 fragments.

In total, 501 COC were retrieved. A wide range of COC numbers were identified (0–58 COC) with a mean of 14.7±2.2 COC per patient. In only one patient, COC were not detected. This 31-year old patient suffered from a borderline ovarian epithelial tumor. Only a small fragment of her ovary was identified as macroscopically normal tissue by the attending pathologist and was used for OTC; the limited amount of material for OTC may explain the inability to identify COC.

Maturation rate

The overall maturation rate was 36.1±4.3 % (Table 2). In five patients, no mature oocytes were obtained after IVM, partly due to a limited amount of COC (range 1–-10) available for these patients. Strikingly, in both of our two youngest patients of 9 months and 4 years old, COC were found but no mature oocytes were obtained after IVM. In four older prepubertal children, mature oocytes were obtained. If subdivided by age, a maturation rate of 18 %±7 % was obtained for prepubertal girls (n=6) and 40 %±5 % for adults (n=27).

IVM of ex vivo harvested COC was performed with the protocol used for a regular IVM oocyte pick up in PCOS patients. Between January 2012 and October 2014, the IVM incubation time was changed in our PCOS clinic, where a 40-h maturation period resulted in the highest maturation rate and 28-h maturation resulted in improved embryo quality (unpublished results). A 30-h maturation period was found to give the best balance between maturation rate and embryological outcome, and is currently the standard IVM incubation period in our center. In our adult patient population IVM was performed during 40 h for 9 patients resulting in a maturation rate of 50±8 %, compared to 35±6 % maturation after 28–30 h IVM in 18 patients.

Most patients (25 out of 34) preferred to cryopreserve mature oocytes. For 9 patients, who were in a stable relationship at the time of ovariectomy, the couple preferred to fertilize the oocytes with partner's sperm followed by cryopreservation of embryos.

Embryo cryopreservation

Within the group of women who preferred embryo cryopreservation (Table 3), one patient had 0/2 oocytes matured after IVM. This is the aforementioned patient in whom ovarian tissue cryopreservation could not be performed due to an ovarian immature teratoma. Fertility preservation could not be offered for this patient.

Table 1 An overview of the literature regarding oocytes retrieved from extracorporeal whole ovary tissue and subsequent IVM

	Transport from OK to lab	Transport from OK to lab	Collection method	In vitro maturation	[O2]	Duration	Strategy	Age (years)	No. of patients	No. of patients with COC	No. of COC
Revel et al., 2003	Leibovitz's L-15	Ice	Antral follicle puncture+medullar follicles	P-1 (Irvine)+SSS	5 %	24+24 h	Oocyte or embryo slow freezing	14–31	9	7	32
Isachenko et al., 2004	DPBS+10 % HSA	37 °C	Antral follicle puncture	IVF medium (IVF Science)+FSH+ hCG+E2+ITS+ patient's serum	20 %	36 h	Oocyte slow freezing	26–33	2	2	18
Huang et al., 2007	Leibovitz's L-15	4 °C	Antral follicle puncture	IVM medium (Cooper Surgical)+FSH+LH	20 %	24+24 h	Oocyte vitrification	43	1	1	4
Huang et al., 2008	Leibovitz's L-15	4 °C	Antral follicle puncture+medullar follicles	IVM medium (SAGE)+ FSH+LH	20 %	24+24 h	Oocyte vitrification	18–38	4	4	11
Huang et al., 2008b	Leibovitz's L-15	4 °C	Antral follicles puncture+medullar follicles	IVM medium (SAGE)+ FSH+LH	20 %	24+24 h	Oocyte vitrification	16	1	1	11
Revel et al., 2009	Leibovitz's L-15	Ice	Antral follicle puncture+medullar follicles	P1 (Irvine)+SSS or IVM medium (Sage) or homemade IVM medium	5 %	24+24 h	Oocyte slow freezing	5–20	19	17	179
Gonzalez et al., 2011	Leibovitz's L-15	Cold	Antral follicle puncture	IVM medium (Origio)+ FSH+hCG	20 %	24+24 h	Oocyte vitrification	28	1	1	2
Fasano et al., 2011	Leibovitz's L-15	4 °C	Antral follicle puncture+medullar follicles	IVM medium (SAGE)+ FSH+LH	20 %	24+24 h	Oocyte or embryo vitrification	8–35	57	42	266
Escriba et al., 2012	DMEM	25 °C	Antral follicle excision+medullar follicles	CCM (Vitrolife)	20 %	24+24 h	Artificial activation	15–38	33	30	108
Imesch et al., 2013	IVM medium (Sage)	NA	Antral follicle puncture	IVM medium (SAGE)+ FSH+LH	20 %	24+24 h	Oocyte vitrification+ activation	18–41	7	7	63
Wilken-Jensen et al.,2013	N.A.	37° 5–10′ or 0° 2–5 h	Antral follicle puncture+medullar follicles	LAG+IVM medium (Origio)+FSH/LH/ EGF/FF in different compositions	7 %	24+24 h	Time lapse imaging of IVM	0–38	69	61	682
Prasath et al., 2013	HTF	NA	Antral follicle puncture	IVM medium (Origio)+FSH+hCG	NA	24 h	Embryo slow freezing	21	1	1	4
Takae et al., 2014	Saline solution	37 °C	Antral follicle puncture+medullar follicles	IVM medium (Origio)+FSH+SSS	5 %	24+24 h	Oocyte cryopreservation?	25–41	27	25	226
Shirasawa et al., 2013	PBS	Warm	Antral follicle puncture	M199+SSS+FSH	5 %	24+24 h	Oocyte fixation	35–44	8	8	87

Table 1 (continued)

	No. of COC for IVM	% maturation	% survival (vitrification)	No. of patients for ICSI/AA	No. of MII for ICSI/AA	% fertilization	No. of embryos vitrified	Conclusions
Revel et al., 2003	10	50 %		3	5	100	3	Clinical case report
Isachenko et al., 2004	18	56 %						Clinical case report
Huang et al., 2007	4	75 %						Clinical case report
Huang et al., 2008	11	79 %						Clinical case report
Huang et al., 2008b	11	73 %						Clinical case report: mosaic Turner syndrome
Revel et al., 2009	133	34 %						Retrospective clinical study: oocyte retrieval possible from 5 years onwards
Gonzalez et al., 2011	2	100 %						Clinical case report
Fasano et al., 2011	235	31 %		3	10	–	5	Retrospective clinical study: oocyte retrieval possible regardless menstrual phase, use oral contraceptives or age
Escriba et al., 2012	108	36 %				41[b]		Research study: oocyte retrieval possible from follicles > and <6 mm, regardless menstrual phase
Imesch et al., 2013	63	62 %	62 %			75[b]		Research study: in vitro maturation, oocyte vitrification and artifical activation
Wilken-Jensen et al.,2013	682	3 %[a]						Research study: oocyte retrieval possible after transport, after chemo/radio therapy, but compromizes maturation rate.
Prasath et al., 2013	4	100 %		1	4	–	3	Clinical case report: live birth
Takae et al., 2014	215	50 %						Retrospective clinical study: oocyte retrieval is correlated with AMH, retrieval regardless menstrual phase
Shirasawa et al., 2013	87	13 %						Research study: some spindle and chromatin abnormalities

Literature overview of published research regarding oocytes retrieved from extracorporeal whole ovary tissue for cortex cryopreservation

NA not available, *DPBS* Dulbecco's phosphate buffered saline, *DMEM* Dulbecco's modified Eagle's medium, *HTF* Human Tubal fluid, *P-1* preimplantation stage 1, *SSS* serum substitute supplement, *IVF* in vitro fertilization, *FSH* follicle-stimulating hormone, *hCG* human chorionic gonadotropin, *E2* estradiol, *ITS* insulin-transferrin-selenium, *LH* luteinizing hormone, *CCM* blastocyst culture medium, *EGF* epidermal growth factor, FF: Follicle Fluid, M199: Medium 199

[a] Includes naked oocytes in IVM

[b] Artificial activation, not fertilization

Table 2 Patient characteristics and outcome parameters regarding OTC and IVM of oocytes retrieved during tissue processing for OTC and subsequent IVM in our center

Patient no.	Age at OTC	Phase menstural cycle at OTC	Indication	No. cortical strips for OTC	IVM duration (h)	No. COC	No. MII	Maturation rate (%)	Vitrification
1	0		Neuroblastoma	8	30	6	0	0	O
2	4		SCT for chronical myelomonocytic leukemia	5	30	9	0	0	O
3	7		SCT for hemophagocytic lymphohistiocytosis	8	40	15	2	13	O
4	8		SCT for severe aplastic anemia	10	30	21	8	38	O
5	10		McCune-Albright syndrome	20	30	58	17	29	O
6	11		Hodgkin lymphoma	10	30	18	5	28	O
7	17	NA	SCT for acute lymphoblastic leukemia	21	40	24	7	29	O
8	19	F	Hodgkin lymphoma	14	40	26	11	42	O
9	21	F	Synovial sarcoma	10	40	39	22	56	O
10	24	NA	Malignant neoplasm of breast	27	28	35	7	20	O
11	24	L	Malignant neoplasm of rectum	22	30	10	4	40	O
12	26	F	Malignant neoplasm of breast	14	40	20	12	60	E
13	26	NA	Arteriovenous malformation	26	30	13	6	46	E
14	27	NA	Minimal deviation adenocarcinoma of cervix	28[a]	30	15	4	27	O
15	27	F	Systemic lupus erythematosus	10	40	2	2	100	E
16	28	NA	Malignant neoplasm of cervix	16	30	1	0	0	O
17	28	L	Malignant neoplasm of breast	16	30	8	4	50	O
18	29	L	Malignant neoplasm of breast	10	28	10	0	0	O
19	29	F	Malignant neoplasm of breast	10	28	12	4	33	O
20	29	F	Malignant neoplasm of breast	17	28	18	6	33	E
21	30	L	Malignant neoplasm of breast	21	30	10	4	40	O
22	30	F	Malignant neoplansm of breast	8	30	3	1	33	O
23	31	F	Malignant neoplasm of breast	10	30	3	2	67	O
24	31	F	Malignant neoplasm of breast	20	40	21	4	19	E
25	31	F	Malignant neoplasm of endometrium	9[a,b]	28	19	7	37	O
26	31	NA	Ovarian borderline tumor	2[a]	30	0			O
27	31	F	Immature teratoma of tuba	0	30	2	0	0	E
28	32	F	Malignant neoplasm of breast	20	40	39	10	46	O
29	34	NA	Preventive bilateral ovariectomy for BRCA	0[c]	30	6	6	100	E
30	35	L	Malignant neoplasm of breast	14	40	7	2	29	E
31	36	F	Malignant neoplasm of breast	14	28	8	3	38	E
32	36	NA	Malignant neoplasm of breast	15	30	4	1	25	O
33	36	NA	Malignant neoplasm of breast	25	30	12	5	42	O
34	38	L	Malignant neoplasm of breast	17	40	7	5	71	O
Mean				14		14.7	5.4	36.1	
SEM				1.3		2.2	0.9	4.3	

Overview of the patients in which OTC was combined with ex vivo collection of COC for IVM to obtain mature oocytes (MII). After IVM, vitrification was performed of the mature oocytes (O) or of good quality embryos (E) after ICSI with partner's sperm

F follicular phase, *L* luteal phase, *NA* information not available, *SCT* stem cell transplantation, *BRCA* breast cancer susceptibility gene

[a] Ovarian tissue was removed in a different hospital and transported to UZ-Brussel within 3 h on ice

[b] Two biopsies were taken

[c] Two ovaries were removed. If not stated otherwise one ovary was removed for OTC

In eight couples, mature oocytes were available for ICSI and an average fertilization rate of 65±11 % was obtained. In one of these couples, two COC were found during OTC, both oocytes matured, but fertilization failed. For seven couples, at least one embryo was vitrified, with an average of 2.9 embryos cryopreserved. The quality of the embryos classified as

excellent, good, moderate, or poor is presented in Table 3. Excellent embryos were obtained in the majority of the patients.

IVM ex vivo combined with OTC for patients undergoing fertility preservation resulted in mature oocytes or embryos available for vitrification and ovarian tissue freezing in 27 out of 34 patients (79 %).

Frozen embryo transfers

So far, none of the patients in this study have requested oocyte warming in order to achieve a pregnancy. One couple recently requested the transfer of vitrified-warmed embryos. This patient was nulliparous and 26 years old and had an AMH of 2.3 ng/ml at the time of diagnosis. She had undergone unilateral ovariectomy because of a benign pelvic arteriovenous malformation. This was treated by intravascular coiling of the ovarian artery and interventional radiological treatment. Imminent ovarian insufficiency was noted after interventional radiological treatment as illustrated by an AMH serum concentration of 0.04 ng/ml.

At the time of ovariectomy, the ex vivo harvesting procedure resulted in 13 immature oocytes, of which 6 matured after IVM, 3 oocytes were successfully fertilized, and 3 embryos were cryopreserved on day 3 (two of excellent and one of moderate quality).

In her first frozen-warmed embryo transfer cycle (FET), 6/8 cells survived in the warmed embryo, but no pregnancy was obtained. In her second FET, 9/10 cells survived in the warmed embryo and the patient became pregnant. At the time of writing this manuscript, the patient had an ongoing clinical pregnancy beyond 20 weeks of gestation.

Discussion

The option of ovarian tissue cryopreservation for fertility preservation has to be considered very carefully and a risk and benefit analysis for each individual patient should be made [19]. Future transplantation of ovarian tissue is currently not possible for a number of cancer types due to inferred high risks of reintroducing malignant cells (review Rosendahl [20]). In patients with, e.g., leukemia, ovarian tissue grafting is not recommended [21], although the tumor-inducing potential of a small numbers of leukemic cells within grafted ovarian tissue is unknown [22]. For this group of patients, isolated follicles embedded within an artificial ovary [23] or in vitro follicle growth will be required to safely restore fertility, but these approaches are not yet clinically available [24] and their (epi)genetic safety will need further scrutiny. When ovarian tissue transplantation can be performed, the "take home baby" rate remains uncertain due to underreporting. Donnez et al. [3] combined results of three centers to calculate a conception rate of 20/80 patients after ovarian tissue transplantation. Spontaneous conception after transplantation has been described and pregnancies after ovarian stimulation with IVF have also been reported, but with low efficiency [25, 26]. Hence, ovarian tissue cryopreservation holds a promise, but no guarantee. The collection of immature oocytes ex vivo during ovarian tissue preparation, followed by in vitro maturation and

Table 3 Embryological outcome of oocytes retrieved during tissue processing for OTC and subsequent IVM in our center

	In vitro maturation			Fertilization		Embryo quality on day 3					
Patient no.	No. of COC	No. of MII	Maturation rate (%)	No. of fertilized oocytes	Fertilization rate (%)	No. of vitrified embryos	Utilization rate (%)	Excellent	Good	Moderate	Poor
12	20	12	60	9	75	5	56	3	2	3	1
13	13	6	46	3	50	3	100	2	0	1	0
15	2	2	100	0	0						
20	18	6	33	5	83	1	20	1	0	1	3
24	21	4	19	3	75	2	67	2	0	0	1
27	2	0	0								
29	6	6	100	5	83	5	100	5			
30	7	2	29	1	50	1	100			1	
31	8	3	38	3	100	3	100	2	1		
Mean	10.8	4.6	47	3.5	65	2.9	68				
SEM	2.5	1.2	11	1.0	11	0.6	14				

Preimplantation development after IVM of oocytes harvested during OTC. Fertilization rate is defined as visible 2PN or early cleavage division on day 1 after ICSI. Utilization rate is defined as embryos vitrified per fertilized oocyte. Embryo quality of all fertilized oocytes are presented in categories: excellent, good, moderate, and poor

oocyte/embryo vitrification can result in pregnancies after embryo transfer. This additional source of oocytes is particularly important when transplantation of ovarian tissue is contraindicated.

Ovarian tissue cryopreservation can be performed for prepubertal children as well as for adults. These populations are very divergent in terms of ovarian constitution and hormonal profile. Gonadotropin-independent recruitment of follicular growth until the early antral stages makes it theoretically possible to retrieve competent oocytes from ovaries of prepubertal children. In this study, oocytes were found during OTC processing for prepubertal children, but their maturation potential appeared to be compromised (18 %), compared to oocytes from adult ovaries (40 %). Similar low maturation rates of oocytes from prepubertal ovaries of 24 and 33 % were reported by Revel et al. [5] and Fasano et al. [11]. In the two youngest patients of our study, aged 9 months and 4 years old, no single oocyte matured after IVM. Wilken-Jensen [14] reported that no maturation was achieved after IVM of oocytes harvested ex vivo in a 3-year-old girl. Until now, the youngest age at which mature oocytes have been obtained after IVM of ex vivo oocytes was 5 years of age [5]. Compared to adult ovarian tissue, prepubertal ovarian tissue appears to contain a relatively larger population of abnormal follicles. In addition, follicle development and oocyte growth in culture was compromised [27]. It has been hypothesized that the prepubertal ovary needs a "maturation phase" in childhood to gain optimal follicle function around the age of 25 [27, 28]. Hence, it is possible that the oocytes harvested from ovarian tissue of young children might lack the capacity to resume meiosis.

In our study, a maturation rate of 40 % was obtained in adult women. According to the literature, an overall oocyte maturation rate of 39 % is obtained with respect to oocytes harvested ex vivo. This maturation rate is slightly lower than what is observed in non-hCG-triggered IVM cycles in women with PCOS in the fertility clinic after minimal stimulation with HP-hMG (46 % maturation rate, 211 cycles performed using the same IVM protocol as in IVM of ex vivo retrieved oocytes, unpublished results). It is also lower than the in vitro maturation rates achieved after transvaginal oocyte retrieval in cancer patients requesting fertility preservation (48 to 79 % [29]). In IVM cycles for reproductive treatment, oocytes from follicles with a diameter of 6–12 mm are transvaginally retrieved [30]. However, oocytes released during the processing of ovarian tissue are presumably originating also from follicles with smaller diameters, which are more likely to contain a less competent oocyte. However, oocytes from follicles <6 mm have also been reported to result in healthy offspring [31]. Similarly, oocytes derived from follicles <6 or ≥6 mm during OTC processing were both able to mature [12]. Hence, the retrieval of oocytes from small antral follicles can be a valid source of oocytes for fertility preservation.

While maturation rate is a highly amenable parameter to investigate after IVM of ex vivo retrieved oocytes, less information is available about the developmental competence of these oocytes. In our study, 74 % of the adult women opted for oocyte vitrification. In the remaining nine patients who requested embryo cryopreservation, a fertilization rate of 63 % was obtained using ICSI. From 49 % of the fertilized oocytes, embryos of sufficient quality for cryopreservation were formed, which is comparable to the 50–75 % utilization rate reported for oocytes harvested ex vivo [4, 11, 15]. For comparison, a 70 % fertilization and 43 % embryo utilization rate was obtained using the same IVM protocol for PCOS patients in our fertility clinic. It can be concluded that oocytes collected during an OTC procedure are capable of developing into embryos of good morphological quality, but with lower maturation and fertilization rates when compared to IVM after transvaginal oocyte retrieval in the clinic for infertile patients with PCOS.

The introduction of a successful oocyte vitrification program in the general ART practice brought new perspectives for fertility preservation. Oocyte vitrification has proven to be a safe technique [32] with a high post-warming survival rate (90 % [33]). When immature oocytes are vitrified, their maturation potential appears to be decreased after vitrification, which favors the approach of IVM after retrieval and subsequent vitrification of mature oocytes. This was observed in both stimulated [34, 35] and unstimulated IVM cycles [36]. Additionally, oocyte survival was decreased in in vitro matured (67.5 %) compared to in vivo matured oocytes (81.4 %; [37]). To date, only a few live births have been reported from cryopreserved IVM oocytes [38–40]. In a study reporting on 39 oocytes retrieved during the OTC process and matured in-vitro, Imesh et al. [13] reported a survival rate of 61.5 % after vitrification/warming. No live birth has been reported from in vitro matured and subsequently vitrified oocytes that had been recovered ex vivo.

Many aspects regarding the emerging technique of IVM of ex vivo retrieved oocytes remain unaddressed. Transport and dissection of the ovary creates a time interval during which oocytes, while still within their follicle, are deprived from circulating blood flow (oxygen, nutrients, metabolites,…) and, depending on the laboratory's protocol, exposed to cold temperature and pH fluctuations. All these factors may disturb normal physiology and impair the intrinsic oocyte's potential. Transport and dissection of ovarian cortex in cold temperature has been reported in seven publications (Table 1); in four publications, the ovary was kept warm until the oocytes were harvested for IVM. Chilling impairs spindle and cytoskeleton ultrastructure of the GV oocyte [41, 42] and should preferably be avoided

before oocyte IVM, although for tissue preservation, chilling is often recommended.

Theoretically, long-term storage of vitrified oocytes in liquid nitrogen should be relatively safe without any obvious undesirable biological and metabolic changes in the cells. However, research regarding long-term storage extending a 5-year period is scarce [43]. There has been one report of a live twin birth after IVF of oocytes that had been cryopreserved for almost 12 years after using a slow freezing method [44].

The hormonal environment of the ovary at the time of ovariectomy is another topic for further scrutiny. Retrieval of immature oocytes during OTC processing and maturation rates after subsequent IVM culture have been shown not to be influenced by the menstrual phase [11, 12, 16] similar to IVM after transvaginal egg retrieval in unstimulated cycles for fertility preservation [45]. Minimal stimulation with gonadotropins before ovariectomy might stimulate follicles to grow and more competent oocytes could potentially be obtained. COS followed by hCG maturation trigger and oocyte retrieval 34–36 h later, combined with ovarian tissue biopsy after oocyte retrieval has been described [46], however with a negative impact on the quality of ovarian tissue for cryopreservation [47].

In our own experience with patients undergoing OTC with IVM of oocytes retrieved ex vivo, only one couple requested transfer of their cryopreserved embryos generated with this approach. The patient had an arteriovenous malformation of the pelvis and underwent therapeutic embolization of the left uterine artery and the right ovarian artery because of a substantial risk of hemorrhage during pregnancy. Because of the risk of damage to the vascular supply of the right ovary, this ovary was surgically removed and cryopreserved. IVM of oocytes harvested ex vivo resulted in cryopreservation of three cleavage-stage embryos of good morphological quality. Three months after the embolization procedure, a single embryo transfer was scheduled for this patient. Embryo survival was good, but implantation was only obtained after the second single embryo transfer. At the time of writing, the patient has an ongoing clinical pregnancy beyond 20 weeks' gestation. To the best of our knowledge, this is the first report of an ongoing clinical pregnancy generated with an oocyte harvested during the procedure of OTC in Europe. The first live birth from an oocyte harvested during OTC was reported by Prasath et al. [15] in Singapore, where transfer of two warmed day 2 embryos was performed. It is evident that the children born from this novel technique will need to be closely monitored. However, studies investigating the health of children born after IVM after transvaginal oocyte retrieval are reassuring with regard to birth weight, congenital anomalies, growth pattern, and development [48, 49].

Conclusion

Based on the available literature data and our own experience, this report attempted to highlight the potential, the benefits, and the pitfalls from the novel approach in fertility preservation of harvesting oocytes during OTC. Current data indicate that immature oocytes can be retrieved during OTC and that a subset of these oocytes are able to mature in vitro; fertilization and embryo culture can result in good-quality cleavage stage embryos that can be cryopreserved. We report an ongoing pregnancy obtained after transfer of a vitrified/warmed day 3 embryo, generated with an oocyte retrieved ex vivo and matured in vitro. The data available so far highlight that oocytes collected ex vivo from the ovary constitute an additional source of oocytes for patients who need fertility preservation.

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