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FERTILITY PRESERVATION

Cryopreservation of euploid blastocysts obtained after fertilization of in vitro matured ovarian tissue oocytes: a case report

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Abstract

With the increased rate of stable remission after gonadotoxic cancer treatment, new methods of fertility preservation are required in order to provide the best possible care for oncological patients. Here, we report an original case of euploid blastocyst cryopreservation after in vitro maturation of ovarian tissue oocytes (OTO IVM). Thirty-three oocytes were obtained from the ovarian tissue after ovariectomy in the breast cancer patient. Six out of 12 matured oocytes fertilized successfully and 3 blastocysts were formed. Genetic investigation for mutations associated with this type of malignancy found that the patient is not a carrier. Preimplantation genetic testing was performed only for aneuploidies and found all 3 blastocysts to be euploid and suitable for embryo transfer. Our study showed that the ovarian tissue oocytes matured in vitro have the potential for euploid blastocyst formation after ICSI which could be screened for aneuploidies and inherited mutations and then be vitrified in order to provide the best fertility preservation strategy for women with cancer.

Keywords In vitro maturation (IVM), · Fertility preservation · Breast cancer · Preimplantation genetic screening · Blastocyst formation · Case report

Introduction

Among women of childbearing age, breast cancer is the most common malignancy. Though the radiation applied during the treatment of this group of patients does not have a strong gonadotoxic effect [1], the chemotherapy can impair fertility and can lead to premature menopause. In general, breast cancer survivors report more menopausal symptoms than women in a control group [2] and might experience temporary or permanent chemotherapy-related amenorrhea [1]. Even if the menstrual cycle remains after the cancer treatment, fertility may be compromised due to the damaged quality of oocytes [3].

Thus, it is strongly recommended that breast cancer patients consider a fertility preservation interventions a potentially gonadotoxic treatment. Embryo and oocyte vitrification after the controlled ovarian stimulation is considered the gold standard of fertility preservation methods [4]. However, this approach requires a 2-week delay of the main treatment and may carry potential risks for breast cancer patients associated with the raised estradiol levels [5, 6].

Another approach to fertility preservation suitable for women diagnosed with breast cancer is ovarian tissue cryopreservation. This method has recently become more widely used as it allows the preservation of fertility without the delay of oncological treatment and gives a chance for spontaneous conception and endocrine function restoration post-autotransplantation. It has also reached acceptable efficiency with cumulative clinical and live birth + ongoing pregnancy rates of 57.5% and 37.7%, respectively [7].

Ovarian tissue cryopreservation can be combined with in vitro maturation of immature ovarian tissue oocytes

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collected from the surplus tissue. These oocytes are present in a relatively large number [8] and can be vitrified or used for in vitro fertilization after IVM. The first case of OTO in vitro maturation was reported in 2003 [9], and since then, several groups had implemented this technique in their clinical practice [8, 10–12]. OTO IVM can be beneficial for many patients scheduled with ovariectomy as it can result in oocytes and/or embryos cryopreservation without controlled ovarian stimulation and any additional surgical manipulations. Moreover, it is the safest option of fertility preservation for patients with ovarian cancers as it allows avoiding malignant cell spillage into the peritoneal cavity [13]. The first embryo transfer of a 2-cell embryo following OTO IVM was reported in 2012, but the viable pregnancy was not achieved [14]. The first live birth was reported in 2014 after the transfer of a 2-day embryo [15], and 2 more pregnancies were achieved in the following year [12, 16]. However, preimplantation genetic testing has never been performed on embryos derived from OTO. In our opinion, it will be valuable to analyze the molecular karyotype of the developed embryos as the final stages of meiosis of OTO occur in vitro, and there might be a risk of the chromosomal missegregation due to the maturation conditions.

The case we present here demonstrates, for the first time, the potential of ovarian tissue oocytes matured, fertilized, and cultured in vitro to form euploid blastocysts. This approach not only allows cryopreserve embryos derived from OTO but also preimplantation genetic testing for aneuploidies and/or mutations in order to provide the most effective and safe option of fertility preservation for cancer patients.

Materials and methods

Ovariectomy and ovarian tissue cryopreservation

The unilateral ovariectomy was performed laparoscopically. The ovary was transferred from the OR to the laboratory in the pre-warm HEPES buffer (Sydney IVF Gamete Buffer, Cook, Australia) within 15 min (Fig. 1a). Ovarian tissue was dissected with scalpels on the heated stage at a laminar flow bench. Fifteen pieces of cortical tissue were cryopreserved using the slow-freezing technique according to the protocol by Schmidt and coauthors [17].

Oocytes collection and IVM

Before ovarian tissue dissection, the ovary was examined on the presence of visible follicles at its surface. The cumulus-oocyte complexes (COCs) were retrieved from visible follicles by aspiration using a 21-gauge syringe connected to the 10-ml syringe. Then the ovary was cut into halves, and the cortical layer was dissected. The remaining fluid with ovarian tissue was examined on the presence of COCs. Cumulus-oocyte-complexes were cultured in groups in the IVM medium (Sage, CooperSurgical) supplemented with 0.75 IU/ml HP-hMG (Menopur, Ferring) and 10% HSA (FUJIFILM Irvine Scientific) for 48–72 h.

Fertilization and manipulations with embryos

Semen samples from the patient's husband were evaluated according to the World Health Organization guidelines. Fertilization was performed via ICSI. All oocytes were denuded after 48 h of IVM by SynVitro Hyadase (Origio, CooperSurgical). Embryos were cultured individually in G1 and G2 medium (Vitrolife, Gothenburg, Sweden). Trophectoderm biopsy was performed on day 7 with the assistance of the OCTAX laser. The three blastocysts were cryopreserved using the standard vitrification method (Kitazato Corporation, Japan).

Images were taken at the Nikon Eclipse Ti-U microscope.

Genetic testing

Testing of the patient for mutations associated with breast cancer

A detailed description is included in the Supplementary Material.

Preimplantation genetic testing for aneuploidies

Detection of aneuploidies was performed using the Ion ReproSeq PGS Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instruction. Aneuploidy haplotyping was done using Applied Biosystems® 3130 (4-capillary) Genetic Analyzer.

Results

Case description

A 30-year-old Caucasian woman, BMI 17, presented to the Medical Research Center for fertility preservation counseling. One month earlier, the patient was diagnosed with T1N1M0, HER2-positive luminal breast cancer and received a left-side mastectomy. The patient had polycystic ovarian morphology with an irregular cycle length of 28–36 days. On the preoperative assessment, the antral follicle count (AFC) was 60. The patient was not administrating any type of medication including hormonal contraceptives which might have affected the AFC.

The patient was provided with the medical council including the oncologist and the reproductive endocrinologist. In our Medical Research Center, we offer oocytes/embryos

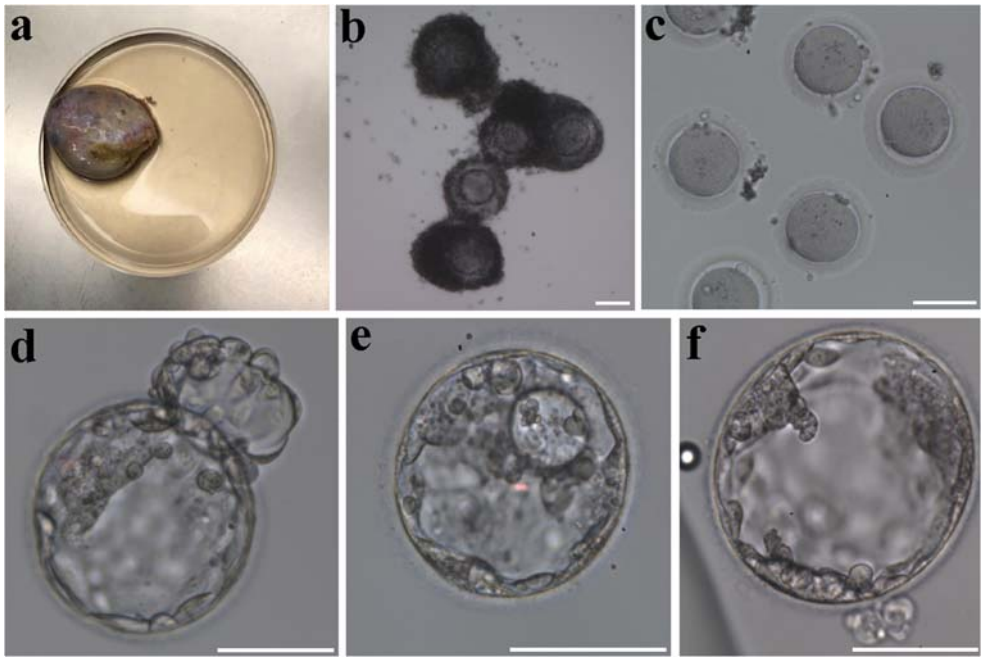

Fig. 1 **a** The ovary 15 min post ovariectomy. **b** Cumulus-oocyte complexes obtained from the ovarian tissue. **c** Mature MII oocytes after 48 h of IVM. (**d**–**f**) Blastocyst developed on day 7 of embryo culture. Scale bar, 100 μm

cryopreservation after controlled ovarian stimulation (COS) and ovarian tissue cryopreservation combined with OTO IVM for fertility preservation. The oncologist strongly advised against COS since the patient's cancer was ER-positive with the 7 Allred score and the chemotherapy treatment was urgent. It was decided to proceed with the unilateral ovariectomy in order to combine cryopreservation of the cortical fragments with in vitro maturation of immature oocytes from the ovarian tissue. The ovariectomy was performed on the 22nd day of the patient's menstrual cycle. The left ovary was selected for ovariectomy and the right ovary was left in situ.

In vitro studies

One COC was retrieved from a visible follicle by syringe aspiration and 32 COCs were detected in the fluid left after dissection of the ovarian tissue (Fig. 1b). Among 33 COCs, 13 oocytes were surrounded by a thick layer of cumulus, 9 were surrounded by a thin layer of cumulus cells, 6 were partially denuded, and 5 were found denuded (Fig. 2a–d). COCs' with different cumulus morphology were cultured together in groups of 8–9 COCs of per well in a 4-well dish. Twenty-two COCs demonstrated cumulus expansion after 2 days of in vitro culture. After 48 h of culture in the IVM medium, 11 oocytes reached the MII stage (Fig. 1c), and after 24 more hours, 1 more oocyte became mature. Four oocytes arrested at the MI stage, 2 at GV, 10 degenerated, and 2 empty zona pellucidas were identified. Interestingly, 3 in vitro matured COCs resulted in the formation of the parthenogenetically activated cleavage stage embryos (Fig. 2e). The overall maturation rate for this patient was 36.4% (12/33).

All mature oocytes were fertilized via ICSI using the husband's sperm which was characterized as normozoospermia (volume was 4 ml, concentration of spermatozoa 15 million/ml, progressive motility 41%). Table 1 summarizes the developmental and fertilization potential of obtained ovarian tissue oocytes. The oocyte which matured after 72 h did not fertilize, and 6 out of 11 oocytes which reached MII stage after 2 days of IVM displayed normal fertilization (2pn, 2pb), 3 had abnormal fertilization (3pn), 1 did not fertilize, and 1 degenerated after the ICSI procedure. Thus, the fertilization rate in this program was 50% (6/12). The embryos had 3–5 blastomeres on day 2 and 4–8 blastomeres on day 3 (Table 2). The cleavage rate was 100% (6/6). On the 5th day of embryo culture, 1 embryo was at the early blastocyst stage, 4 embryos were at the morula stage, and 1 embryo arrested. On the 6th day of development, 2 more embryos formed non-expanding blastocysts and the remaining 2 arrested. It was decided to prolong the culture until the 7th day in order to achieve blastocyst expansion. On the 7th day, 3 blastocysts were viable, and the trophectoderm biopsy was performed (Table 2). According to the Gardner classification, the scores were: Embryo №1: 5AA (Fig. 1d); Embryo №2: 3CC (Fig. 1e); and Embryo №3: 5BB (Fig. 1f). All 3 blastocysts were cryopreserved. Fifty percent (3/6) of embryos reached the blastocyst stage in this program.

Genetic analysis

The patient has a positive family history: a first-degree relative had breast cancer diagnosed at the age of 65 years without detected inherited mutations; a second-degree relative had colon cancer at the age of 55 years, mutations were not checked.

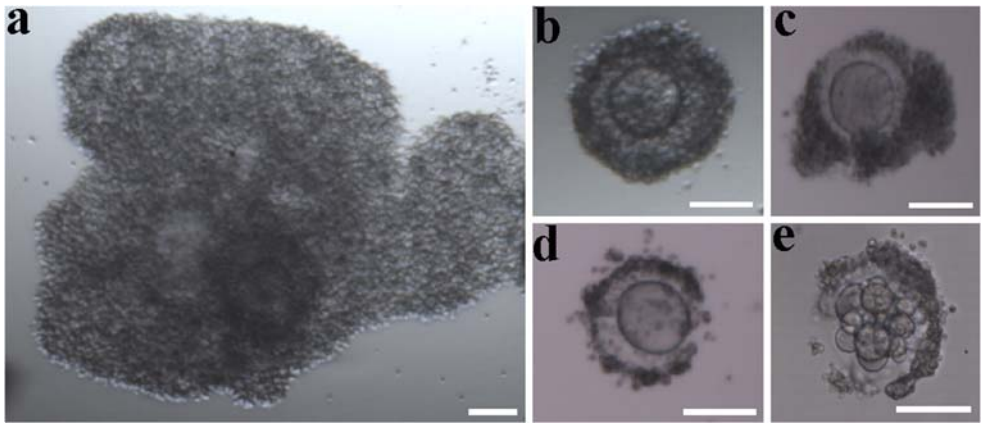

Fig. 2 **a** COC with a thick layer of cumulus cells. **b** COC with a thin layer of cumulus cells. **c** Partially denuded oocyte. **d** Denuded oocyte. **e** Parthenogenetically activated cleavage stage embryo. Scale bar, 100 μm

The patient had a moderate risk of cancer since all relatives had oncopathology diagnosed after 50 years.

In this study, we did not reveal pathogenic mutations in BRCA1/2 genes and other genes of the reparation system. The detected nucleotide variants are presented in the table in the Supplementary Material (Table S1).

The average coverage was ×714, the proportion of correctly mapped readings was 99.5%, and the share of target regions with coverage above ×100 was 96.5%. A total of two missense mutations, one synonymous substitution, and two mutations in the splicing regions were found. Among the nucleotide variants detected, there were no pathogenic mutations; a variant of uncertain significance was detected in the CTNNA4 gene. However, according to the HGMD database, this mutation is characterized as a nucleotide variant with a possible risk of developing Alzheimer's disease.

Table 1 Developmental and fertilization results of ovarian tissue oocytes

No. of COCs retrieved	33
No. of COCs with cumulus expansion	22/33
No. of GV oocytes	2/33
No. of GVBD oocytes	4/33
No. of degenerated oocytes	10/33
No. of empty zona pellucidas	2/33
No. of parthenogenetically activated cleavage embryos	2/33
No. of oocytes matured in 48 h	11/33
No. of oocytes matured in 72 h	1/33
Maturation rate (%)	12/33 (36.4%)
Fertilization rate (%)	6/12 (50%)
No. of abnormally fertilized oocytes (3pn)	3/12
No. of unfertilized oocytes	2/12
No. of degenerated after ICSI oocytes	1/12
Cleavage rate (%)	6/6 (100%)
Blastocyst formation rate (%)	3/6 (50%)

COCs cumulus-oocyte complexes; *GV* germinal vesicle stage; *GVBD* germinalvesicle breakdown stage; *3pn* three pronuclei zygote

Thus, it was decided to test the obtained embryos only for aneuploidies. We used whole-genome assay in order to screen all embryo chromosomes. The results of PGT-A were: Embryo №1: seq(1–22)×2,(X,Y)×1; Embryo №2: seq(1–22,X)×2; and Embryo №3: seq(1–22)×2,(X,Y)×1. As a result, all 3 blastocysts were euploid and suitable for transfer.

Oncological treatment and perspectives for the subsequent pregnancy

After fertility preservation, the patient started the treatment consisting of 8 cycles of chemotherapy. The aromatase inhibitor anastrozole (Arimidex) was prescribed to the patient for long-term therapy of 5–7 years. Embryo transfer is advised to be scheduled not earlier than 5 years after the start of the treatment.

Discussion

We describe here a case of fertility preservation for a patient diagnosed with breast cancer. After unilateral ovariectomy, our intervention resulted in the ovarian cortex cryopreservation and vitrification of 3 euploid blastocysts.

Table 2 Embryo development

	Day 2	Day 3	Day 5	Day 6	Day 7	Destiny
1	4b	5b	1	2BB	5AA	b + v
2	3b	4b	M	1	3CC	b + v
3	5b	6a	M	2BB	5BB	b + v
4	4a	8a	M	M	–	Discarded
5	3a	4a	M	M	–	Discarded
6	4a	5b	0	–	–	Discarded

(b + v) biopsy and vitrification; *M* morula; *0* arrested embryo; *score on day 2 and 3:* number of blastomeres: a – regular blastomeres, no fragmentation; b – fragmentation < 20% or irregular blastomeres

It was reported by several clinical centers that oocytes obtained from the ovaries ex vivo have the potential for maturation [9, 13, 18]. Nevertheless, IVM of oocytes recovered from ovariectomy specimens is very often looked upon as a secondary method of fertility preservation complimentary to the ovarian tissue cryopreservation [19, 20]. This might be due to the little data available on the competence of such oocytes to development and viable embryos formation. Several clinics cryopreserved ovarian tissue oocytes and embryos [8, 12, 13, 19–21], yet not many gametes and embryos were thawed. A 64% survival rate was reported for the vitrified MII oocytes based on the live-dead assay [8] and the 100% survival rate for the thawed embryos [20]. The fertilization rate for in vitro matured OTO was reported to be 43.5–65% [11, 12] that is lower than the benchmark value for fertilization rate after ICSI in regular COS cycles which is 80% [22]. Only three pregnancies have been reported after OTO IVM, two of which resulted in live births [12, 15, 16]. Nothing is known about the molecular karyotype of these children. Absence of data available on the blastocyst formation was stated as the major limitation of the IVM research which hinders to provide patients with the relevant information [23]. Our case extends the knowledge to the potential of ovarian tissue oocytes matured in vitro, and, for the first time, demonstrates that euploid blastocysts can be obtained during this program.

We believe that OTO IVM might serve as the primary intervention when ovarian tissue transplantation is contradicted due to the high risk of the presence of the malignant cells in the frozen ovarian cortex. For instance, it has been shown that samples of ovarian tissue from borderline ovarian tumors, leukemias, non-Hodgkin's, and Hodgkin lymphomas can contain tumor cells [24–27]. The risk of relapses after ovarian tissue transplantation for breast cancer patients is also a concern. Though there is still little data on this matter and it is impossible to determine accurately the risks, several cases of relapses have been reported after OTT [28, 29]. One of these cases in a patient with breast cancer occurred during pregnancy and led to pregnancy termination [30]. The relapse rate of breast cancer patients who underwent ovarian tissue retransplantation was similar to the group who did not yet have the procedure (7% in both groups) [29], and no histologic or immunohistochemical evidence of malignant cells in cryopreserved ovarian tissue biopsies was detected in early-stage breast cancer patients [31]. However, the frequency of ovarian metastases in breast cancer patients of reproductive age is around 20–25% [32], and cryopreserved ovarian tissue from advanced-stage breast cancer patients might contain cells expressing the breast tumor marker, MGB2 gene [33]. Breast cancer patients should be treated with greater caution in order to provide the safety of fertility restoration programs.

We performed PGT-A using the NGS platform in order to provide the best chances of achieving pregnancy and delivering a healthy baby as breast cancer survivors are considered at potential risk for perinatal complications [3], and some of them have to resort to the surrogate gestation. In this case, the patient was also screened for the mutations associated with breast cancer and did not reveal these pathogenic mutations. Thus, PGT-M was not required.

In all previous reports, embryos obtained from OTO were cultured till the cleavage stage. We decided to prolong embryo culture and to perform trophectoderm biopsy as it is less harmful to embryos than blastomere biopsy [34]; it allows to detect embryo mosaicism [35] and is more cost-effective as only viable and potentially more competent embryos are tested [36]. Since more embryos are suitable for biopsy at the cleavage stage than at the blastocyst stage, a higher aneuploidy rate is observed in the blastomere biopsies [34].

In our study, all 3 blastocysts were euploid. The expanded blastocysts were formed only on the 7th day of embryo culture, which indicates delayed development. Based only on one case of blastocyst formation from OT oocytes, it is impossible to determine the cause of a slow developmental pace. Nevertheless, it has been reported for the standard ICSI-PGT-A cycles that around 6% of all embryos suitable for trophectoderm biopsy are formed only on day 7 [37]. Euploid day 7 blastocysts have a high implantation potential, yet lower live birth rate due to pregnancy losses [37].

Studies performed on embryos derived from IVM oocytes collected by the transvaginal aspiration show no differences in aneuploidy rates compared to IVF embryos [38, 39]. No obvious increase in the incidence of chromosomal abnormalities was detected for rescue IVM embryos as well [40]. This might indicate that oocytes that have a potential for fertilization and embryo formation after IVM do not differ by molecular karyotype from the oocytes obtained in conventional COS cycles.

In this report, only 36.4% of oocytes successfully matured in vitro, and only 3 out of 33 oocytes gave rise to the euploid blastocysts. Our findings are consistent with previous larger studies showing the 29–36% maturation rate of OTOs [8, 11, 41]. This indicator is much lower than the maturation rate of COCs obtained by transvaginal aspiration which can reach 65–85% [42–44]. The decreased maturation potential of OTO might be due to the fact that during ovarian tissue preparation COCs from all follicles are recovered even from very small ones (0.5–2 mm) [45], while follicles of better quality and bigger sizes are selected during the transvaginal pick-ups [11]. For this reason, duration of maturation is usually longer for OTO than for conventional aspiration and varies from 36 to 48 h [8, 12, 20].

Patients' characteristics such as age, ovarian reserve, or cancer type might affect the oocyte competence to maturation and development. For instance, it had been demonstrated that the maturation rate of OTO in patients below 20 years of age (55%) was significantly higher than that of older patients aged 20–30 years and above 30 years (29% and 26%, respectively) [8]. Interestingly, an 82% maturation rate was reported for an older (43 years of age) PCOS patient which might be due to

the patient's good ovarian reserve [46]. Furthermore, the maturation potential of OTO might be associated with the suboptimal in vitro culture conditions. Better maturation and developmental rates might be achieved by the implementation of improved IVM systems such as CAPA (IVM with a prematuration "Capacitation" step) or SPOM (simulated physiological oocyte maturation) which promote synchronization of nuclear and cytoplasmic maturation [47, 48].

The clear limitation of our report is that we present here a single case. Also implantation potential of described embryos remains unknown as the patient is not advised to have an embryo transfer within 5 years after the start of her oncological treatment. Wider studies investigating the clinical predictors for successful IVM programs of ovarian tissue oocytes are needed in order to introduce OTO IVM method into the routine practice.

In conclusion, the present study demonstrates that it is possible to obtain euploid embryos from ovarian tissue immature oocytes. To the best of our knowledge, this is the first report of cryopreservation of euploid blastocysts formed after fertilization of in vitro matured OTO. Moreover, this case demonstrates that it is important to explore all fertility preservation options for patients diagnosed with cancer and combine several methods in one pre-cancer treatment plan. While autotransplantation of ovarian tissue fragments can grant patients endocrine function restoration, genetically tested embryos from OTO can provide an additional intervention in order to maximize the chance for future offspring.

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Author's roles AK, TN and GT developed the IVM procedure. AA supervised the IVM procedures. NM, TN and EB were responsible for the diagnostic evaluation and clinical management of the couple. AK and EK were responsible for the laboratory procedures. AE performed preimplantation genetic screening. OB performed testing for mutations associated with breast cancer. MG created the custom panel of the genes. AK wrote the paper. OB and TN edited the paper.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Informed consent A written informed consent was obtained from the patient.

Ethical approval This study was approved by the Ethics Committee of our institution (protocol №11 from 13.12.2018).

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