Cryopreservation of ovarian cortex and vitrification of immature oocyte

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Abstract:

Background:

Unlike sperm production in men which is continuous, women are born with all their eggs and they do not produce any more. Technique to bank eggs would allow girls and women to have the same reproductive options as men when faced with a serious disease and the long term complications of chemotherapies that threatens to destroy their eggs.

Objective:

The aim of this study is to improve the efficiency of existing ovarian cryopreservation, by following the most recent method named needle immersed vitrification (NIV) beside the slow freezing method. In this review we discussed also the historical description of the methods used, freezing and vitrification of mature and immature oocytes, this include comparison of principles, procedures and results as reported in the literature.

Materials and Methods:

A total of 285 fragments of human ovarian tissues were isolated and cryopreserved from 36 women aging 26 – 55 years old in the Reproductive Unit, Lubeck-Germany. Ovarian tissue strips of 13- mm thick of ovarian cortical biopsies obtained from patients. The collected human ovarian cortex fragments were assigned to two different cryopreservation procedures, slow-freezing and NIV. On the otherhand, a total of 80 patients (100 cycles) with polycystic ovaries (PCO) were underwent *in vitro* maturation (IVM) treatment. The number of inspirated immature oocytes was 536. vitrification of immature oocytes were carried out.

Results:

Successful freezing and storing were performed on 285 fragments of ovarian cortex tissue from 36 patients by two cryopreservation protocols as mentioned before but these tissues do not thaw and use yet. Concerning the IVM, the percentage of maturation, fertilization, survival, embryo transfer, and pregnancy rates were 55.2%, 44.5%, 96.2%, 88.1%, and 10.6%, respectively.

Conclusion:

Recent advances in freezing technology, modifications of conventional protocols used and continuing optimization of vitrification have efficiently improved the method. These results may lead to increase the possibility of preserving fertility by cryopreservation of ovarian tissue. Ovarian tissue banking can offer hope for cancer patients who want to safe guard their fertility against sterilizing chemotherapy and radiotherapy. Immature oocytes can be successfully isolated from the oophorectomy specimen regardless of the day of menstrual cycle, and undergo IVM and cryopreservation.

Keywords: Cryopreservation, immature oocytes, ovarian cortex, vitrification.

Introduction:

ART includes all fertility treatments in which both eggs and sperm are handled. In general, ART procedures involve surgically removing eggs from a woman's ovaries, combining them with sperm in the laboratory, and returning them to the woman's body or donating them to another woman. They do NOT include treatments in which only sperm are handled (i.e. intrauterine or artificial insemination) or procedures in which a woman takes drugs only to stimulate egg production without the intention of having eggs retrieved. Because ART consists of several steps over an interval of approximately 2 weeks, an ART procedure is more appropriately considered a cycle of treatment rather than a procedure at a single point in time[1]. Unlike sperm production in men which is continuous, women are born with all their eggs and they do not produce any more. The natural process of each menstrual cycle consumes approximately 5001000-eggs until the supply is exhausted (about age 51,menopause). Men have been able to cryopreserve (freeze) their sperm for decades. A woman's chances of having a pregnancy and a live birth by using ART are influenced by many factors, some of which are patient-related and outside a clinic's control e.g., the woman's age, the cause of infertility[2]. However, women have not been able to freeze their eggs reliably because the eggs are hard to retrieve and unfertilized eggs have generally not survived freezing. With the advent of IVF, oocyte, embryo and even ovarian tissue freezing became possible[2]. For all these reasons, a technique to bank eggs would allow girls and women to have the same reproductive options as men when faced with a serious disease and the long term complications of chemotherapies that threatens to destroy their eggs[3].

Cryopreservation of ovarian tissue: and the whole ovary is a new progress in ART. Transplantation of cryopreserved ovarian tissue was reported in 2004[4], in spite of the introduction of the concept of ovarian transplantation since 1906[5]. Despite its infancy to preserve fertility and lack of studies, ovarian tissue cryopreservation has several potential advantages, such as the presence of many primordial follicles with oocyte arrest in diplotene of prophase of the first meiotic division, primordial follicles being theoretically less cryosensitive than mature oocytes, and preservation of the endocrinal function of the ovary[6]. Several diseases, mainly cancers and their treatments threaten to destroy all the follicles in a womans ovaries. Other diseases rarely have a direct effect on the oocytes in the ovary. Chemotherapy or radiation used to treat cancer or some non-cancerous disorders have the unfortunate side effect of destroying the follicles in the ovary as well as the diseased cells[7]. Advances in chemotherapy and radiotherapy have increased the survival rate of cancer patients[8], amazingly up to 90% for young cancer patients[9]. Increased life span actually raises concerns on the quality of life after treatment for cancer. Having children may be difficult for cancer survivors as both radio- and chemotherapies have been proven to be gonadotoxic[10]. Recovery of ovarian function after anticancer treatment is very much affected by the loss of follicles due to chemo- or radiotherapy resulting in premature ovarian failure (POF) and consequently, infertility in many female cancer survivors. Fertility preservation before treatment for cancer is an important option nowadays to overcome infertility induced by cancer itself or by the treatment to cure cancer [6, 8, 11].

The ovary has hundreds of primordial follicles containing immature oocytes which are resist cryopreservation due to the absence of zona and cortical granules[12]. Ovarian cortex can be obtained from any female cancer patient irrespective of age and marital status with no procedural delays in cancer treatment. Amorim *et al*[13] have shown that steroidogenic and gametogenic functions are well preserved in cryopreserved ovarian tissue. All these advantages make ovarian tissue cryopreservation a better option than other means for female fertility preservation. There have been two human live births after orthotopic transplantation of cryopreserved ovarian tissue [4, 14]. Huang *et al*[15] reported that immature oocytes can be retrieved successfully from the visible antral follicles of excised ovarian tissue, matured *in vitro* and cryopreserved by vitrification. Oocyte viability rate in ovarian tissue before and after cryopreservation by vitrification was studied, first in bovine and later in humans, and a 97% survival rate of oocytes has been reported from the ovarian tissue[16]. It has been proven that slow freezing of oocytes followed by thawing would yield inferior results when compared to fresh oocytes[17,18] while, cryopreserved oocytes using vitrification will yield results comparable to fresh oocytes after thawing [15, 16, 19]. Thus, it would depend on the age of the women from whom the oocytes were harvested and frozen. On the other hand, Fasano *et al* [20] in their study showed that a significant number of immature oocytes can be collected from excised ovarian tissue whatever the menstrual cycle phases and the age of the patients, even for prepubertal girls. Gosden *et al* [21] reported the live births of young animals after autografting frozen-thawed ovarian tissue in sheep. Similar reports have been published in rats [22] and rabbits [23]. These successful attempts in animals prove that ovarian tissue cryopreservation is a feasible means of fertility preservation with a potential application for human beings.

It has been found that women with polycystic ovarian syndrome (PCOS) are characterized by abnormal endocrine parameters, anovulation, numerous antral follicles within their ovaries and frequently infertility[24]. Patients with PCOS are extremely sensitive to stimulation with exogenous gonadotrophin and are at increased risk of developing ovarian hyperstimulation syndrome (OHSS) when treated with gonadotrophins for assisted reproduction[25, 26]. Recovery of immature oocytes followed by in-vitro maturation (IVM) of these oocytes could be developed as a new method for the treatment of patients with infertility due to PCOS. Methods developed by Trounson for transvaginal ultrasound guided recovery of immature oocytes from the ovaries of patients with PCOS introduced IVM firmly into the clinical feild[27]. It has been noted that most of the follicles from patients with PCOS are not atretic and these oocytes appear to have developmental competence[28,29]. However, the maturation rate of immature oocytes retrieved from women with PCOS is lower than that of those retrieved from women with normal menstrual cycles[30,31].

Materials and Methods:

Patients and Ovarian Tissue Collection: Human ovarian tissues were collected from thirty six women at different ages ranging from 6 – 55 years old. Some of these patients [28] had undergone oophorectomy for their specific conditions, including endometrial cancer and breast

cancer. The other [8] samples were obtained from ovarian biopsy from patients who underwent ovary transposition because of cervix cancer. All the patients signed an informed consent form that was approved by Institutional Ethics Committee of German Medical Center, Department of Obestetrics and Gynecology, Medical University of Lubeck, which also approved the current study. Ovarian tissue cryopreservation begins with laproscopy. The surgeon obtained 13- mm thick, 3x10 mm strips of ovarian cortical biopsies from patients. The collected tissues was placed immediately into a sterile petri dish containing the L-15 medium supplemented with 10% FBS for the next procedure.

Human specimen preparation: The collected tissues were transferred to the laboratory, the tissues was cut into strips of 2x2 mm in size in fresh L-15 medium supplemented with 10% FBS. The total samples isolated from all patients were 285 strips. All were confirmed as showing no ovarian metastasis by an independent pathologist before data analysis.

Cryopreservation procedures: Human ovarian cortex fragments were assigned to two different cryopreservation procedures: slow-freezing and needle immersed vitrification as described above. Before cryopreservation, one or two pieces of human ovarian cortex from each patient were fixed for morphological and ultrastructure assessment of immature follicules.

Patients and *In Vitro* Maturation procedure: A total of 80 patients (100 cycle) who underwent IVM treatment. Only those patients who had polycystic ovaries were recruited. Patients ages were range between 26- 38 year. They were attended our clinic centre at Lubeck University. Oocyte recovery was performed 36 h after hCG injection. Invitro maturation were carried out according to the procedure mentioned by[40]. Mature oocytes were inseminated by ICSI and then cryopreserved at 2pn stage by vitrification method[39] followed by thawing and embryo transfer on day 2 after thawing.

Technique of Ovarian Cortex cryopreservation is summarized through the two different cryopreservation procedures used for human ovarian cortex fragments as below.

1-Slow-freezing protocol for ovarian cortex tissue cryopreservation:

A slow-freezing protocol put forward by Gosden *et al* [21]. According to the protocol, 2–3pieces of human ovarian cortex fragments place in a 1.8 ml cryovial (Nunc, Roskilde, Denmark) containing 1 ml of Leibovitz medium (L-15) supplemented with 0.1 M sucrose (Sigma-Aldrich, St Louis, MO, USA), 10% Fetal Bovine Serum (FBS) and 1.5 M dimethyl sulfoxide (DMSO, Sigma-Aldrich). After a 30 min exposure to the cryoprotectant solution at 4°C, the cryovials load in the programmable freezer (Biomed Freezer Kryo 10, Series II, Planer, UK). Slow-cooling protocol starts temperature of 4°C. The vials cool at a rate of 2°C/min to -7°C and hold for 5 min, seeding use manually and maintain at -7°C for another 10 min. Then the temperature cools to -40°C at a rate of -0.3°C/min and further cool to -140°C at -10°C/min. Finally, the vials transfer to liquid nitrogen for storage. For thawing, the cryovials remove from liquid nitrogen, hold in air for 20 s and transfer to a water bath (37°C) for 20–30 s. The contents of cryovials deplete into the L-15 medium supplemented with 0.1 M sucrose, 10% FBS and 1.0 M DMSO for 5 min and washe in a stepwise manner (1.0 M DMSO, 0.1 M sucrose, 0.5 M DMSO, 0.1 M sucrose, 0.1 M sucrose) for 5 min each. The conventional slow-cooling method certainly has resulted in the delivery of several babies[14,16, 32,33].

2-Needle Immersed Vitrification (NIV) protocol for ovarian cortex tissue cryopreservation:

The ovarian tissues dehydrated by using a two-step regimen an equilibration solution consisting of 7.5% (v/v) Ethylene Glycol (EG) and 7.5% (v/v) DMSO in Dulbeccos Phosphate Buffered Saline (DPBS) supplement with 20% FBS for 10 min at room temperature and a vitrification solution consisting of 15% EG, 15% DMSO and 0.5 M sucrose for 2 min. This vitrification solution was used successfully in recent publications on blastocyst, oocyte and mouse ovarian vitrification[34,35,36]. The long needle can hold several ovarian tissue samples in a row, and thus all the samples can be exposed to cryoprotectants and then immerse into liquid nitrogen under the same conditions. This can maximize the cooling rate and simplify the vitrification process. To vitrify human ovarian cortex fragments, 4–5 pieces of ovarian tissue strips helds in a row by a needle in the L-15 medium supplemented with 10% FBS. After two steps of dehydration procedure, the ovarian tissues carried by the needles, held by the forceps, underwent the following procedure they placed on an aseptic absorbent gauze to remove the remaining vitrification solution. Then, they plunged in liquid nitrogen directly and finally, they put into liquid nitrogen-filled cryovials and stored in liquid nitrogen. For thawing, the solid drops or the needles holding ovarian tissues take out of the vial using forceps and quickly immersed into 1 M sucrose solution, which has been pre-warmed at 37°C for 5 min. They serially transfered into 0.5 and 0.25 M sucrose solution for 5 min each and incubate in DPBS supplemented with 20% FBS for 20 min at 37°C with 5% CO2.

Technique of *in vitro* maturation and vitrification of immature oocytes: The patients received a specialized consultation, as is offered to all patients with PCOS managed by the unit. A transvaginal ultrasound and hormonal measurements [FSH, LH, estradiol (E2), prolactin, delta -4- androstenedione,17-hydroxyprogesterone] are routine quides in our centre. The first ultrasound scan was scheduled for the third day of the cycle, as well as determination of E2, LH and progesterone levels and measurement of endometrial thickness. These investigations were repeated around the sixth to eighth day of the cycle to exclude the development of a dominant follicle. The patient received 10 000 IU of hCG Chorionique Endo (Organon, France) s.c. when follicle size reached 7 mm, before selection of the largest follicle [37]. All patients received hCG according to Chian *et al.* [38, 39] who have demonstrated in IVM cycles that hCG priming increases both the percentage and rate of immature oocyte maturation.

Immature Oocyte retrieval: Oocyte recovery was performed 36 h after hCG injection. During the collection, patients received a mild i.v. sedation with propofol (Driprivanw; AstraZeneca, France). Transvaginal ultrasonographically guided oocyte collection was done using a specially designed 19-Gauge single-lumen aspiration needle (K-OPS-7035-Wood; Cook, France). The aspiration pressure was set at 7.5 kPa. Follicular aspirates containing cumulus–oocyte complexes were collected in 15 ml Nucleon (Nunc A/S, Denmark) tubes containing prewarmed 3 ml sodium heparinate 2 IU/ml (Sanofi–Synthelabo, France). Follicular aspirates were not washed on a filter but tubes were spread onto sterile polystyrene culture dishes of 60 mm diameter Nucleone (Nunc A/S). The cumulus–oocyte complexes were isolated under a stereomicroscope and then washed once in the culture medium, Universal IVF Medium (MediCult, Denmark),

warmed to 37ºC in a thermostatically controlled incubator under an atmosphere enriched to 5% CO2.

In vitro maturation procedure was done as described by Chan and Tan,(39).Matured oocytes were inseminated by ICSI using the partner's spermatozoa. ICSI was performed at least 1 h after observing first polar body (PB) extrusion as suggested by Hyun *et al.*[40]. Fertilization was assessed 17–19 h after insemination for the appearance of two distinct pronuclei and two PBs. Vitrification of 2PN stage was performed as mentioned before. After thawing vitrified oocytes the zygotes were cultured in Embryo Maintenance Medium (Cooper Surgical). Embryonic development was assessed on Day 2 (41–43 h) and on Day 3 (65–67 h) after insemination according to the regularity of blastomeres, the percentage and pattern of anucleate fragments, and all dysmorphic characteristics of the embryos. The best quality embryos were transferred on Day 2 or Day 3 after ICSI.

This results showed a successful freezing and storing of some ovarian cortex tissues from 36 patients aging 6 -55 years with two different cryopreservation protocols in our reproductive unit at Lubeck Germany as mentioned before. The total number of fragments 285. These tissues do not thaw and use yet because our patients are still under treatment therapy (table 1).

On the otherhand, Table 2 represents some results which have shown that, there were no significant differences between the three cryopreservation methods compared with fresh for. Table (3) represents some author's results about ovarian cortex cryopresrevation.

Results of IVM and vitrification of immature oocytes are represented in table (4). After 100 cycle from 80 patients, the number of immatured oocytes isolated was 536. The percentage of maturation was 55.2%. The fertilization rate was 44.5%. Moreover, survival rate of vitrified oocytes, embryo transfer and pregnancy rates were 96.2%, 88.1% and 10.6% respectively.

Results:

Table I: Ovarian tissue cryopresrevation from 36 patients at different ages (6-55 years), cryopreserved biopsy numbers and methods used (Slow freezing and Vitrification).

Slow Freezing / Leibo+3M DMSO			Vitrification / NIV		
Patient #	Patient Age	Cryo Biopsy #	Patient #	Patient Age	Cryo Biopsy #
1	17	20	1	14	5
2	23	12	2	55	4
3	18	5	3	11	10
4	24	8	4	6	5
5	32	10	5	25	12
6	18	10	6	36	10
7	8	8	7	24	9
8	36	9	8	39	6
9	32	9	9	33	5
10	30	10	10	33	5
11	38	10	11	36	5
12	22	8	12	34	8
13	25	5	13	19	18
14	19	10	14	22	8
15	7	5	15	16	12
16	21	6			
17	25	2			
18	24	4			
19	25	5			
20	12	2			
21	33	5			

Total number of patients were 21 and 15 for slow freezing and NIV respectively. Mean age for the patients 6-55. The total number of biopsy were 163 and 122 for slow freezing and NIV respectively.

Table 2. Percentage of morphologically normal human primordial follicles and mouse follicles (various stages) in groups of frozen–thawed ovarian cortex and fresh tissues.

Groups	Human	Mouse		
	Primordial follicles	Primordial follicles	Primary follicles	Secondary follicles
Slow-freezing	82.93±2.31a	83.63±1.41b	74.00±1.85c1	43.5±3.29d1
Vitrification	81.34±3.72a	82.63±2.39b	73.25±2.31c2	42.75±2.12d2
NIV	83.16±2.70a	84.38±3.34b	81.75±1.83c1,c2	68.25±1.93d1,d2
Fresh	90.70±2.50	92.80±1.30	90.40±1.52	86.4±1.4

Percentage data expressed as mean+SD; NIV, needle immersed vitrification.
a,b No difference among the three groups
c1,c2 NIV versus slow-freezing and dropping vitrification group: both P, 0.001.
d1,d2 NIV versus slow-freezing and dropping vitrification group: both P, 0.001.

Table 3: Review on the historical data of Ovarian cryopreservation (diagnosis, nature of tissue, reimplantation side and outcome results).

FIRST AUTHOR, YEAR	WHERE	DIAGNOSIS	AGE at cryo	FRESH or CRYO-PRE-SERVED	SLICES or WHOLE	REIMPLANTA-TION SIDE	OUTCOME
Donnez, 2004 (4)	Belgium	Stage IV Hodg-kin's lymphoma	25	Frozen/ thawed	Slices	Orthotopic: ovarian fossa peritoneum	Spontaneous pregnancy: live birth
Donnez, 2011-a (41)	Belgium	Neuroectodermic tumour	17	Frozen/ thawed	Slices	Orthotopic: ovary	Spontaneous pregnancy: live birth
Meirow, 2005, (14) 2007 (42)	Israel	Non Hodgkin's lymphoma	28	Frozen/ thawed	Slices	Orthotopic: ovary	Mild ovarian stimulation - IVF: live birth
Demeestere, 2007(33) 2010 (43)	Belgium	Stage IV Hodg-kin's lymphoma	24	Frozen/ thawed	Slices	Orthotopic (+ heterotopic)	Spontaneous pregnancy: live birth in 2007 live birth in 2009
Andersen, 2008 (32)	Den-mark	Hodgkin's lymphoma	27	Frozen/ thawed	Slices	Orthotopic: ovary	Ovarian stimulation - IVF: live birth
Andersen, 2008 (32) Ernst, 2010(44)	Den-mark	Ewing sarcoma	27	Frozen/ thawed	Slices	Orthotopic: ovary	Mild ovarian stimulation - IVF: 1 live birth. Spontane-ous pregnancy: 1 live birth
A- Silber, 2010 (45) B-Donnez, 2011 b(46)	USA	Stage IIIb Hodg-kin's lymphoma	20	Frozen/ thawed	Slices	Orthotopic	A- Ongoing pregnancies B -live birth
Silber, 2010 (45)	USA	Premature ovar-ian failure	24	Frozen/ thawed	Slices	Orthotopic	1 live birth + 1 live birth (twins)
Piver, 2009 (47)	France	Microscopic polyangiitis	27	Frozen/ thawed	Slices	Orthotopic	IVF: live birth
Sanchez-Serrano, 2010 (48)	Spain	Breast cancer	36	Frozen/ thawed	Slices	Orthotopic	Ovarian stimulation, IVF: 2 live births (twins)
Revel, 2011 (49)	Israel	Thalassemia major	19	Frozen/ thawed	Slices	Orthotopic	IVF: live birth
Roux, 2010 (50)	France	Homozygous sicule cell anemia	20	Frozen/ thawed		Orthotopic: ovary and few strips deposited in the peritoneal window	Spontaneous pregnancy: live birth

Table 4: Outcome results from IVM cycles and vitrification of immature oocytes from PCO patients.

Patient No	Cycle No	Immature Oocyte No	Mature Oo-cyte No (%)	Fertilization Rate No (%)	Survival Rate After Thwaing No (%)	Embryo Transfer Rate No (%)	Pregnancy Rate No (%)
80	100	536	296 (55.2)	132 (44.5)	127 (96.2)	112 (88.1)	12 (10.6)

Discussions:

The recent experiments, human ovarian tissue yielded a similarly high post-warming viability using the same vitrification method. Ultrasound also revealed normal follicular growth in the grafted ovary. Most importantly, the transplanted vitrified–warmed bovine ovarian tissues were, in all the cases, histologically normal[51].There was no apparent loss of oocyte viability caused by the vitrification. Moreover, even the transplantation of this cortical tissue resulted in no histologically apparent ischaemic oocyte loss either. Histological analysis of ovarian tissue cryopreserved with the slow-cooling method has demonstrated subtle aberrations compared with fresh tissue[52].

Based on these findings in the cow, it was presumed that the cryotissue method used in these experiments could be applied to human ovarian tissue, the structure of which is similar to that in cattle. So human ovarian tissues were vitrified using the same approach. The viability of oocytes in human ovarian tissues vitrified with ultra-rapid Cryotissue method was also very high (90%). There was no significant difference between fresh and frozen tissue even though the donors of the ovaries were patients with ovarian cancer of a rather advanced age (38–40 years old). Histopathological analysis of post-vitrification human ovarian tissues demonstrated normal tissue structure after vitrification, similar to the results obtained from the latest experiments [51]. No devitrification occurred during warming, and excellent viability was demonstrated, indicating that this may now be an acceptable method for clinical trials.

Recent medical advances have dramatically increased the cure rate for cancer in young women of reproductive age, resulting in more than 90% of all young cancer patients to be cured [53, 54]. One of the adverse effects of cancer treatment, however, is infertility brought about by damage to the ovaries caused by chemo and radiotherapies. Almost all female patients with haematological cancer who undergo bone marrow transplants lose their ovarian function and become menopausal, regardless of age, and are thus rendered sterile. Approximately 1% of the total female population between 20–39 years of age in Japan are cancer survivors whose fertility has been destroyed by their treatment [55]. The solution to this dilemma would be to freeze the eggs or ovarian tissue of these patients before the initiation of cancer treatment. Vitrifying ovarian tissues by direct immersion into liquid nitrogen using a minimal volume of cryoprotectant could maximize the cooling rate and reduce toxicity of the vitrification solution with less-concentrated cryoprotectants. Moreover, the NIV method is relatively simple, convenient to manipulate and time-saving. Held by an acupunctureneedle, the ovarian tissue pieces can be exposed to cryoprotectants synchronously. So, NIV can facilitate vitrification process especially when a large number of ovarian cortex fragments require to be cryopreserved.

In the past 10 years, several groups have reported high survival rates (89.2% to 100%) and successful live births using vitrification methods. In fact, since the first report in 1999, vitrification of oocytes has resulted in a lot of live births worldwide [2, 56, 35, 57, 19].

In conclusion, immature oocytes can be successfully isolated from the oophorectomy specimen regardless of the day of menstrual cycle, and undergo IVM and cryopreservation. Patients with ovarian tumors who wish to preserve their fertility may benefit from this additional and novel possibility for fertility preservation.

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