

Evaluation of the Fast Freezing Method in Cryopreservation of Human Ovarian Tissue for Oocyte Viability

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Abstract— The evaluation of the fast freezing method as an option for fertility maintenance, after long-term human ovarian tissue cryopreservation.

Samples were obtained from premenopausal women, who have had children, who were undergoing gynecologic surgery. The samples were cryopreserved for six years. Subsequently, were thawed and examined for the presence of oocytes and were immunohistochemically evaluated.

Only 10% of the samples showed the presence of oocytes. That was not associated with age, body mass index, age at first birth or the number of offsprings, the underlying disorder or the side of the ovary removed.

Index Terms— cryopreservation, fast freezing, fertility, oocyte, ovarian tissue.

I. INTRODUCTION

Nowadays, female fertility maintenance has become a theme of great importance, because of the increasing number of women who may be in danger of losing their ability to reproduce, due to medical, occupational, economic or social reasons. Among them, malignancies have become a major medical cause of infertility in premenopausal women. The use of chemotherapy and radiotherapy has tremendously improved the survival of such patients. However, these therapies frequently affect ovarian function and fertility, and may even lead to premature ovarian failure. [1-2] The number of oocytes destroyed depends mainly on the type of therapy used, the dosage and the woman's age. To deal with the growing demands for fertility preservation in these women, several methods have been developed depending on patient's age, existing time limits, the type of malignancy and the risk of ovarian metastasis. [3-4] Current methods of fertility maintenance [5] include embryo, oocyte (mature / immature) or ovarian tissue/ovaries cryopreservation, follicles culture, gonadal shielding, ovarian suppression and

ovarian transposition. Combined strategies have also been tested.[6] All these methods should precede anticancer therapy initiation.

While sperm cryopreservation has been successfully used for several years, the cryopreservation of oocytes and ovarian tissue has progressed slowly. Current pregnancy rate per cryopreserved oocyte is around 2%, a percentage considerably lower than the one achieved with embryo cryopreservation and even lower than that of fresh oocytes. [7-8] Improvement of cryopreservation could increase pregnancy rate. Currently oocyte freezing is performed mainly by vitrification or slow freezing [9-10]. Vitrification is more efficient than slow freezing in case of immature oocyte freezing, because is an ultrarapid process which does not allow intracellular ice to form. [9-10] Immature oocytes seem to be more resistant to cryodamage due to their lower cell volume and lack of metaphase spindle, but the success rates are low due to insufficient in vitro maturation. [11] In this study, we aimed to evaluate the fast freezing method called vitrification as the method for prolonged human ovarian tissue cryopreservation.

II. PATIENTS AND METHODS

A. Patients

This prospective cohort study was conducted in the Department of Obstetrics and Gynecology, Division of Artificial Fertilization, Medical School, of Democritus University, Alexandroupoli, Greece. Ovarian tissue was obtained from twenty (20) consecutive premenopausal Caucasian females (age range 37-43 years) who had fibroids, adenomyosis or menometrorrhagia, and were subjected to laparoscopic ovarian removal for their condition under general anesthesia. All these patients preferred to have this done, instead of medical therapy or more extensive surgery i.e. hysterectomy etc. All women had given childbirth before, indicative of normal fertility. The study was approved by the Ethics Committee of Democritus University Hospital and was in accordance with the Declaration of Helsinki and the International Conference on Harmonization for Good Clinical Practice. Written informed consent was obtained from all the women.

B. Sample management

During surgery, ovarian tissue was obtained from at least one ovary for cryopreservation. Each sample was cut at least 2 cm long, was preserved in saline at 4°C and all hematic residues were removed with Ringers solution and retrograde flow. Also blood was obtained for hormonal measurement of FSH, LH, TSH, and progesterone. In order to determine the

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effect of long time freezing, we kept the tissue samples frozen for 6 years.

C. Materials

The following chemical and biological solutions and agents were used for tissue handling during the a) freezing and de freezing period and b) immunochemistry procedures:

a) Ringer solution, formaldehyde, 95% ethanol, medium Ferti Cult, Leibovitz L-15 medium +5% FCS, 40% ethylene glycol + 0.35 M sucrose+ 10% egg yolk solution, liquid nitrogen(-196 C), embryo freezing medium, PBS, Sucrose

b) paraffin, xylol, formol. hematoxylin, streptavidin, eosin, biotin, kit EnVision HRP, Mouse / Rabbit detection system (DAKO), Ki-67 mouse monoclonal antibody (Santa Cruz Biotechnology), Petri plates, scalpels No.22, laboratory oven, common lab refrigerators, plastic containers, microscope Nikon Eclipse 50i, liquid nitrogen bottle.

D. Sample preparation-Fast Freezing Cryopreservation Method

The stages of ovarian tissue fast freezing performed were [12]: i) cut of the ovarian tissue in cylindrical portions 0,8 X 0,8 X 0,8 mm using scalpel No.22 and forceps under the stereomicroscope, followed by placement at room temperature within one hour in Leibovitz L-15 medium +5% FCS, ii) fixation of some pieces of the ovarian tissue pieces with formaldehyde in order to assess follicular presence, iii) saturation of the ovarian tissue particles (OTPs) at 40% ethylene glycol + 0.35 M sucrose+ 10% egg yolk solution for 6 minutes, iv) exposal of the OTPs to vitrification solutions and in vortex for 1 min, at 5Hz to 40 C, v) covering the particles with 1 cm of cryo protectant (Embryo Freezing Medium) and placement in plastic containers vi) plunge of the tissue samples directly in liquid nitrogen (LN2) at -196 C for freezing. The plastic containers remained in LN2 for six years.

E.Recovery/ Thawing Protocol of Human ovarian tissue

The thawing process [12] comprised of the following steps: i) thawing for 30 seconds at room temperature, ii) water bath at 37 0 C for 2 minutes, iii) gradual tissue washing to achieve cryoprotective removal with the use of medium gradually decreasing in DMSO (1,5 M, 1 M, 0.5 M.) with 20% calf serum and 0.1 M sucrose for 10 minutes each, iv) triple rinsing (about 10 minutes) with medium (PBS +15% FCS) of the tissues and transportation on plates with fresh nutrient fluid and serum.

F. Immunohistochemistry Method

The technique followed, was the one used by the Microbiological-Biochemical Department, in Medical School of Democritus University, in Alexandroupoli, Greece. For histopathological assessment and evaluation of each tissue section hematoxylin and eosin stains were used and microscopic examination was conducted by light microscope Nikon Eclipse 50i. The last and most crucial stage included the biotin - streptavidin immunohistochemistry technique for which the kit EnVision HRP, Mouse / Rabbit detection system (DAKO) was used and the Ki-67 mouse monoclonal antibody of Santa Cruz Biotechnology Company was used as an immunohistochemical indicator.

G. Statistical analysis

Data for continuous variables are presented as mean \pm

standard deviation (S.D.). Data for categorical variables are presented as number and/or percentage. Mann-Whitney U-test was used to test for the differences in continuous variables between two groups. Chi-square test was used for the differences in categorical variables between two groups. A two-sided p-value of less than 0.05 was considered statistically significant in all the above tests. Statistical analysis was performed using SPSS for Windows version 17.0 (SPSS, Chicago, IL, USA).

III. RESULTS

Twenty Caucasian premenopausal women [mean age 39.7 \pm 2.1 years (age range 37-43 years); BMI 26.7 \pm 4.1 kg/m²] with fibroids (n=11), adenomyosis (n=5) or menometrorrhagia (n=4) were included in this study. Ovarian tissue was obtained from the right ovary in ten from the left in eight and from both ovaries in two women. In all cases the hormone profile and the histological examination of the samples at baseline were normal. (FSH 3-20 m U/ml, LH 2-15 m U/ml, TSH 0,48-4,98 μ l U/ml progesterone 0,1-3,9 nmol/ml). Six years after vitrification only in 10% of the samples were observed oocytes (namely in two out of the twenty) (Figure 1 A & B). In 18 samples the ovarian tissue did not reveal any oocytes and there were considered abnormal. Individual patients' characteristics are detailed in Table I. There was no difference in the age (p=0.379), BMI (p=0.758) and age of 1st child birth (p=0.853) between women with normal and abnormal tissue biopsy (Table II). Furthermore, there was no difference in the cause of ovarian surgery (p=0.457), the side of the ovary removed (p=0.829) and the number of children (p=0.708) between the two groups.

IV. DISCUSSION

Several cryopreservation techniques have been developed in an attempt to maintain fertility in female patients. [13] In this study we found that only a small proportion of ovarian tissue samples subjected to fast freezing cryopreservation preserved their oocytes. The presence of functioning oocytes could not be predicted by any clinical parameter evaluated.

Each method or technique of cryopreservation has advantages and disadvantages related to current success rate, required delay in cancer treatment, sperm requirement, and risk of reintroducing cancer cells. To date, vitrification is most commonly used, as it achieves faster cooling rate and higher cryoprotectant concentration compared to slow-freezing [12] along with similar or better outcomes, namely preservation of ovarian tissue morphologic integrity, similar oocyte survival rate, and improved granulosa cell survival and stroma integrity. [14-19]

In our study, 10% of the samples had normal appearing oocytes which may be capable for fertilization after six years of cryopreservation, but we did not evaluate their functionality. We recruited only women with history of successful pregnancy/-ies to increase the possibility that the ovarian tissue was capable of fertilization at baseline. We picked patients with "advanced age" in order to determine the effect of age in oocyte freezing. We cannot be sure which methodological variables might have affected the viability of the samples. The duration of cryopreservation might have played a role. [20-21] However, it has been reported that after five years of slow cooling cryopreservation at -20o C, ovarian

function was restored within five months after auto-transplantation, with re-establishment of spontaneous menstrual bleeding and raise of estradiol levels.[22] Similar results, including successful pregnancies, have been reported after 3.75 years of slow freezing cryopreservation and auto-transplantation in cancer patients [23-24] and after 8 years in a patient with b-thalassemia [25] All the above studies have used the slow freezing technique in contrast with us and this might have also played a role in the discrepancy of our results with theirs. Finally, the cryoprotectant we used might have affected the outcome, as several studies demonstrate cryoprotectants toxicity [26] especially of the dimethyl sulfoxide, and others that compared different cryoprotectants, concluded that dimethyl sulfoxide is superior than ethylene glycol,[27] or that propanediol and raffinose combined with glutamine and taurine, can safely used in slow freezing protocols. [24] Finally, the use of sucrose, propandiol, ethylene glycol, and dimethyl sulfoxide as cryoprotectants [8,23,25] in slow freezing protocols demonstrated high cryoprotective results.

We were not able to identify any clinical parameter that could predict the outcome with our method. Neither BMI nor age at sample acquisition, age at birth of the first offspring, the number of off springs, the underlying disorder that led to surgery or the side of the ovary removed seemed to differ between samples with preserved oocytes or not.

Our study has certain limitations. First of all, the number of samples was small; a larger scale of samples would provide safer statistical results. Second, we were not able due to ethical reason to obtain biopsies from perfectly normal women to use as a control group. Third, despite the documented earlier fertility, the advanced reproductive age of the participants at baseline could have affected their fertility maintenance and that was a point of interest in our research.

IV. CONCLUSIONS

In conclusion, our data suggest that ovarian tissue cryopreservation by fast freezing for six years results in fertility preservation in only a small proportion of the stored samples, therefore, it cannot be proposed instead of other methods already used e.g. embryo freezing. [28] However, it is encouraging that fertility potential is maintained to some extent even after such a long cryopreservation period. It is important to improve current methods and/or the development of new ones for effective cryopreservation imperative.

FUTURE PERSPECTIVE

The optimization of ovarian tissue cryopreservation protocols and the necessity for long-term preservation of a woman's reproductive ability, will be the challenge in research of cryopreservation. It is therefore very likely in the next decade the existing data to be satisfactory and all these methods to be considered no longer as experimental and inadequate.

SUMMARY POINTS

Fast freezing for six years results in only 10% of the ovarian tissue samples having oocytes present which suggest significant lowering of ovarian function. This was not

associated with age, body mass index, age at first birth or the number of offsprings, the underlying disorder or the side of the ovary removed. Since we had no way of otherwise checking the functional capacity of these samples we cannot determine their fertility potential but we can only say that there seem to be a significant decrease observed oocytes. Therefore, fast freezing ovarian tissue cryopreservation cannot be proposed instead of embryo freezing.

ETHICAL CONDUCT OF RESEARCH

The study was approved by the Ethics Committee of Democritus University Hospital and was in accordance with the Declaration of Helsinki and the International Conference on Harmonization for Good Clinical Practice. Written informed consent was obtained from all the women.

FIGURE LEGEND

Fig. 1 A & B. Two ovarian tissue samples with living and capable for fertilization follicles following ovarian tissue fast freezing cryopreservation and thawing restoration six years later.

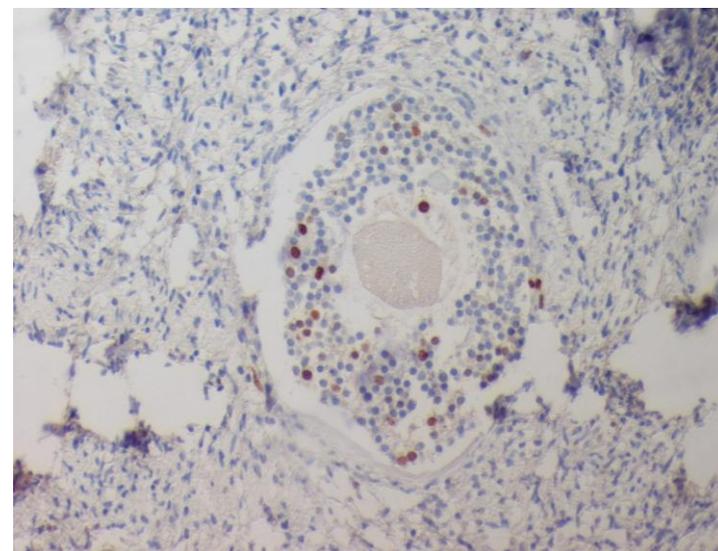
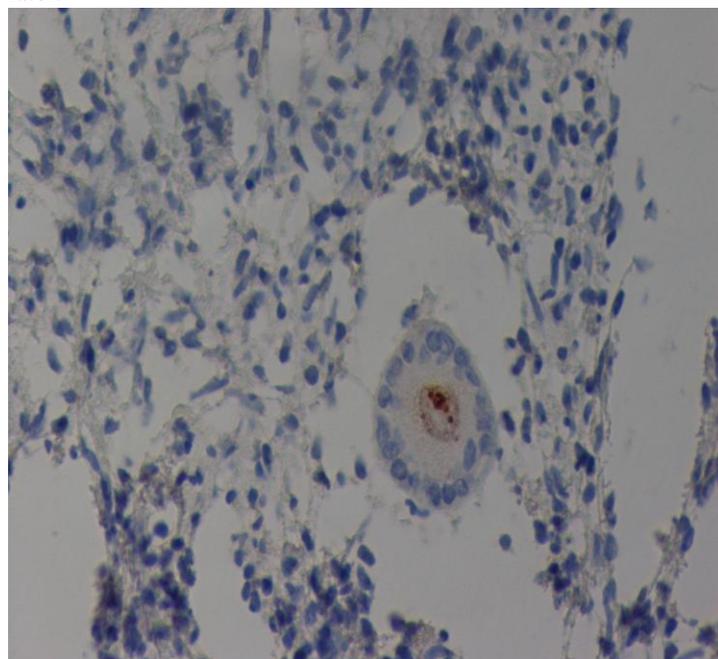


Table I. Individual patients' characteristics (demographics, underlying disorder, ovary removed and ovarian tissue post-cryopreservation).

A/A	Age (years)	BMI (Kg/m ²)	Age at 1 st child birth (years)	No of children	Underlying disorder	Ovary removed	Ovarian tissue post-cryopreservation
1	38	26.37	34	2	fibroids	left	abnormal
2	38	26.52	32	1	fibroids	left	abnormal
3	37	32.25	29	2	adenomyosis	right	abnormal
4	39	23.85	31	2	menometrorrhagia	left	normal
5	37	23.87	33	2	fibroids	right	abnormal
6	39	29.11	35	1	fibroids	right	abnormal
7	40	28.99	37	1	menometrorrhagia	right	abnormal
8	41	28.91	26	3	fibroids	left	abnormal
9	41	37.23	35	1	adenomyosis	right	abnormal
10	37	23.65	31	2	menometrorrhagia	left	abnormal
11	42	24.21	29	3	adenomyosis	right	abnormal
12	40	33.41	25	4	fibroids	left	abnormal
13	39	25.17	32	2	fibroids	right	abnormal
14	39	28.53	30	2	fibroids	right	abnormal
15	43	23.58	35	1	fibroids	both	abnormal
16	38	24.19	29	3	menometrorrhagia	right	abnormal
17	37	20.44	30	2	adenomyosis	left	abnormal
18	43	27.97	33	3	fibroids	right	normal
19	43	23.86	24	3	fibroids	both	abnormal
20	42	22.68	39	1	adenomyosis	right	abnormal

Abbreviations:BMI, body mass index

Table II. Comparison of age, BMI and age at 1st child birth between women with normal and abnormal ovarian tissue samples after long-term cryopreservation.

	Women with normal ovarian tissue (n=2)	Women with abnormal ovarian tissue (n=18)	p-value
Age (years)	41.0 ± 2.8	39.5 ± 2.1	0.379
BMI (Kg/m ²)	25.9 ± 2.9	26.8 ± 4.3	0.758
age at 1 st child birth (years)	32.0 ± 1.4	31.4 ± 4.1	0.853

Abbreviations:BMI, body mass index

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