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Assessment of follicle viability using fluorescence microscopy before and after ovarian thawing

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Summary

The incidence of young women diagnosed with cancer has been globally increasing. In many cases the surgical approach followed by chemotherapy, radiotherapy or hormonal therapy could lead to infertility or premature ovarian failure. Several options are available in order to preserve fertility and increase the future gestation rate. Among embryo cryopreservation and oocyte cryopreservation, ovarian tissue cryopreservation represents an ideal option, especially for premenopausal women and for those who cannot delay the start of chemotherapy. The purpose of this study was to examine the follicle viability using fluorescence microscope before and after ovarian thawing.

Key words: Cryopreservation; Chemotherapy; Radiotherapy; Ovarian thawing.

Introduction

Fertility preservation in premenopausal women undergoing cycles of chemotherapy or radiotherapy remains a big challenge for all practitioners. All these chemotherapeutic agents are extremely cytotoxic and could lead to decrease of ovarian reserves and premature ovarian failure [1]. Several options are growing continuously, some of them are more established and others, on the other hand, are still experimental, such as ovarian tissue cryopreservation. Ovarian tissue cryopreservation represents the optimal option for prepubertal girls and for women who cannot delay the presence of chemotherapy [2]. Approaching this method, we can use fragments of ovarian cortex, entire ovary with its vascular pedicle or isolated follicles [3].

During the ovarian tissue procedure the objective remains the harvest of numerous immature follicles detected in ovarian cortical fate. In this way a surface portion of ovarian ablation may contain thousands of immature follicles. The tissue is dissected into thin slices to facilitate penetration of the cryoprotectant material, and through the mechanism of freezing, the process ends. The ovarian tissue can be harvested laparoscopically, histologically examined, under ideal circumstances preserved, and finally thawed. Additionally, the ovarian viability before and after ovarian thawing, can be examined and analyzed using immunofluorescence microscopy. The slow freezing protocol represents the optimal option for ovarian thawing. The ovarian viability can be examined using the Live/Dead Viability/Cytotoxicity Kit [4]. Following specific protocol shown in Table 1, this kit in conjunction with the ovarian tissue, allows follicles to be visualized and counted depend on the calcein AM and ethidium homodimer (EthD-1) levels. Through logistic parameters many facts are feasible, such as the examination, analysis. and estimation of ovarian viability [5].

Strategic issues that should be considered are the ideal freezing protocol, the transplantation position, the follicle quality, the presence of metabolic trauma (survival of ovarian tissue, the metastatic transfer of cancer cells, and the production of mature oocytes in vitro) [6].

According to current bibliography, there are many patients considered as future candidates for ovarian tissue preservation. In detail, patients with breast cancer, lymphomas, osteosarcomas, and chronic autoimmune lesions are mentioned. The common ground of all these diseases remains premature ovarian failure and the loss of ovarian reserves [7].

Materials and Methods

The authors collected 17 patients-candidates for ovarian tissue thawing. The mean age was 32 years at the time of the ovarian thawing (26-38 years).

Table 2 shows all the measured hormones (FSH, LH, E2, and AMH) which were assessed before the ovarian thawing, in order to specify the decrease of the ovarian reserves (Table 2). All the patients were of reproductive age, with no family history, and with no previous signs of malignancy. All patients over 40 years with signs of ovarian malignancy were excluded. The protocol was approved by the Ethics Committee in Aretaieion Hospital in Athens.

The slow-freezing protocol is the standard ovarian tissue freezing protocol. According to the protocol used by Donnez, the vials are placed in the freezer according to the following schedule: (1) cooling from 0 ° C to -8°C at a rate of -2°C / minute, (2) “seal”

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Table 1. — *Treatment protocol of ovarian tissue.*

Live/Dead Viability/Cytotoxicity Kit for mammalian
- Cells (Cat. No. L-3224)
- Ex/Em (nm) calcein: 494/517 Green (live)
- Ethidium homodimer-1: 517/617 Red (dead)

The reagents of the kit are stored in -80°C protected from light and humidity.

Protocol:
- The reagents were removed from the freezer and allowed to warm to room temperature.
- Tissue samples were placed in wells of a 24-well plate and washed in PBS three times.
- During washing the dye solution was prepared in phosphate-buffered saline (PBS) as follows:
  - Calcein stock C = 4 mM, use at 2 mm (1:2000 dilution).
  - Ethidium homodimer-1 (EthD-1) stock C = 2 mM, use at 4 mm (1:500 dilution).
- Incubated each sample in 2 ml volume, then 1 ml of calcein and 4 ml of EthD-1 were added per sample. Vortexed vigorously to ensure thorough mixing.
  *Note that aqueous solutions of calcein are susceptible to hydrolysis, so they should be used only on the same day.
- After last wash, dye solution was added to the sample. In parallel, a sample with PBS alone without the dye was incubated, as a negative control for the autofluorescence of the tissue.
- Incubated for 30 minutes in a 37°C incubator.
- At the end of the incubation, tissue is washed 3x with PBS.
- Tissue is placed on a slide with PBS and observed unfixed under a confocal microscope.

Table 2. — *Details of patients regarding ovarian tissue thawing.*

Age	Disease	FSH (IU/l)	LH (IU/l)	AMH (pg/ml)	E2 (pg/ml)
26	Sarcoma	6.9	4.8	2.6	35
37	Breast cancer	9.4	6.2	1.1	42
23	Morbus Hodgkin	5.2	3.4	1.9	51
35	Breast cancer	8.3	9.1	0.9	26
38	Endometrial cancer	7.2	6.9	1.6	63
24	Sarcoma	5.8	6.9	3.4	32
34	Breast cancer	7.1	4.9	1.2	45
25	Morbus Hodgkin	3.4	4.5	2.2	29
24	Sarcoma	5.8	4.9	2.9	20
23	Breast cancer	4.5	2.9	1.8	27
32	Breast cancer	6.8	6.2	2.7	53
37	Breast cancer	9.4	6.9	0.8	19
37	Endometrial cancer	9.5	6.2	1	57
27	Ovarian borderline	8.3	6.2	1.3	67
25	Ovarian borderline	6.9	4.5	1.9	38
38	Endometrial cancer	9.4	6.3	1.2	56
38	Breast cancer	4.1	6.7	3.4	39

(seeding) vials by hand using pliers have cooled before in liquid nitrogen, (3) cooling at -40°C at a rate of **-30°C** / minute, (4) cooling to -196°C at a rate of -30°C / minute and (5) direct placement into liquid nitrogen (-196°C) for storage.

After the protocol treatment, with the Live/Dead Viability/Cy-

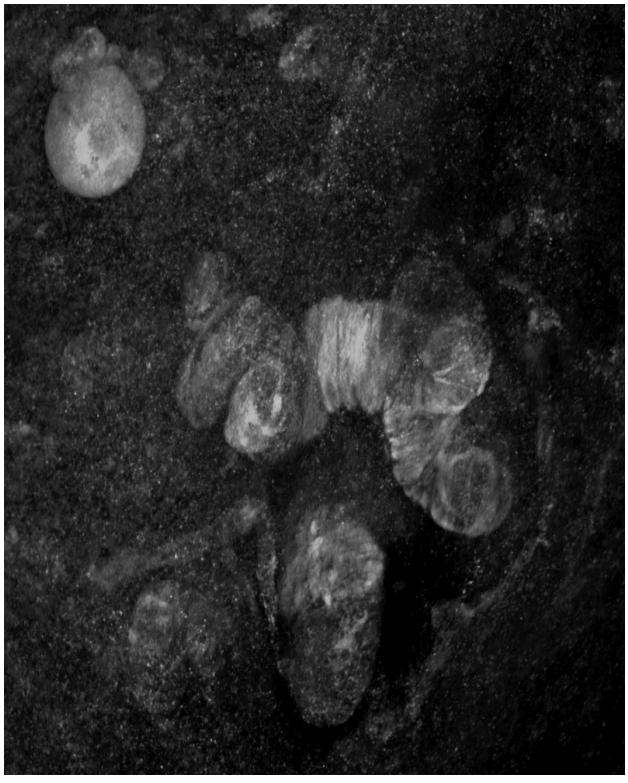

Figure 1. — Ovarian tissue examination through immunofluorescence microscope.

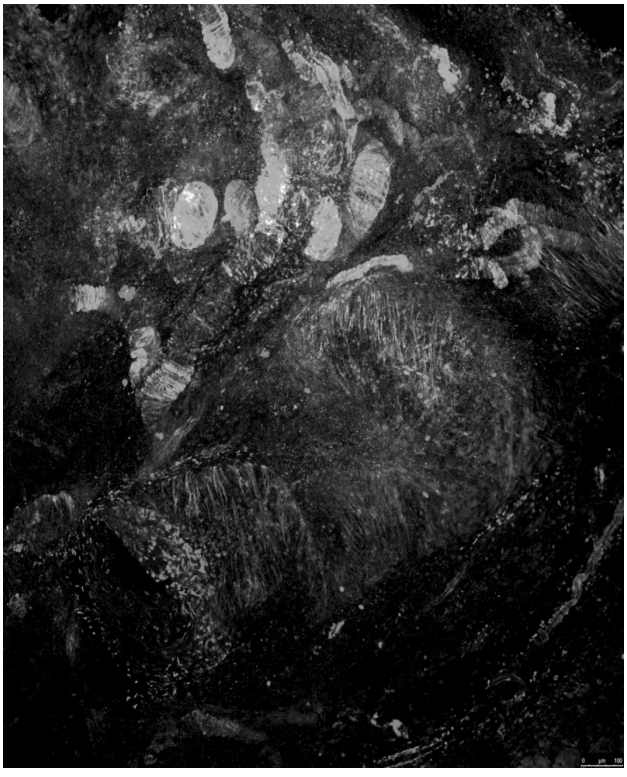

Figure 2. — Ovarian tissue examination through immunofluorescence microscope.

Table 3. — *Percentage viability rate of ovarian tissue.*

Number of experiment	Figure	Green (live)	Red (dead)	% Green (live)	% Red (dead)
Experiment 1	Figure 1	36058.394	55051.415	39.57685171	60.4231483
	Figure 2	120908.946	95616.708	55.84047145	44.1595286
Experiment 2	Figure 1	139105.440	280824.764	33.12584774	66.8741523
	Figure 2	51266.450	133990.623	27.67314045	72.3268596
Experiment 3	Figure 1	553497.230	307573.270	64.28012921	35.7198708
	Figure 2	296756.055	331929.066	47.20265282	52.7973472
Experiment 4	Figure 1	127664.360	115977.509	52.39836672	47.6016333
	Figure 2	448442.907	3529.412	99.21910881	0.78089119
Experiment 5	Figure 1	235779.813	16240.040	93.55604735	6.44395265
	Figure 2	340536.332	23961.938	93.42604891	6.57395109
Experiment 6	Figure 1	93900.687	205176.379	31.39681964	68.6031804
	Figure 2	72490.253	193194.060	27.2843557	72.7156443
Experiment 7	Figure 1	296206.525	34395.199	89.59618281	10.4038172
	Figure 2	682943.042	682943.042	50	50
	Figure 3	299615.274	69696.659	81.12796994	18.8720301
	Figure 4	67413.905	352807.274	16.04248152	83.9575185
	Figure 5	141797.904	169840.720	45.50074769	54.4992523
	Figure 6	108633.853	337787.695	24.33436591	75.6656341
Experiment 8	Figure 1	68606.580	66366.574	50.82979686	49.1702031
	Figure 2	192894.822	128582.299	60.00265941	39.9973406
	Figure 3	70323.406	12290.479	85.12298629	14.8770137
	Figure 4	105474.761	182528.385	36.62278085	63.3772192
	Figure 5	169686.827	36250.470	82.3973265	17.6026735
	Figure 6	70280.172	156150.610	31.03825875	68.9617413
Experiment 9	Figure 1	73894.532	84752.573	46.57792652	53.4220735
	Figure 2	35788.790	92573.356	27.88110912	72.1188909
	Figure 3	51195.356	308137.547	14.2473332	85.7526668
	Figure 4	33088.071	224940.061	12.82343547	87.1765645
Experiment 10	Figure 1	276503.277	237.626	99.91413412	0.08586588
	Figure 2	213174.383	135263.999	61.17993712	38.8200629
	Figure 3	308624.567	3140.138	98.99278592	1.00721408
Experiment 11	Figure 1	227804.358	203741.808	52.78794621	47.2120538
	Figure 2	182344.568	162246.503	52.91621964	47.0837804
Experiment 12	Figure 1	244812.190	615330.598	28.46180813	71.5381919
Experiment 13	Figure 1	179839.523	48461.492	78.77298443	21.2270156
	Figure 2	303283.606	171.027	99.94364001	0.05635999
	Figure 3	260257.872	489146.998	34.72860698	65.271393
Experiment 14	Figure 1	224886.781	145703.554	60.68339073	39.3166093
	Figure 2	380424.104	177859.769	68.1416968	31.8583032
	Figure 3	277302.467	359.103	99.87066881	0.12933119
Experiment 15	Figure 1	419714.613	286829.708	59.40386194	40.5961381
Experiment 16	Figure 1	149394.747	792814.748	15.85578874	84.1442113
	Figure 2	165891.630	172589.376	49.01061716	50.9893828
	Figure 3	378571.048	161811.498	70.05612058	29.9438794
	Figure 4	90379.349	357926.901	20.16018046	79.8398195
	Figure 5	179431.509	94715.755	65.45077503	34.549225
Retransplantation	Figure 1	206072.664	79247.405	72.22508558	27.7749144
	Figure 2	310916.955	244826.990	55.94608053	44.0539195
	Figure 3	344074.394	228235.294	60.12031619	39,8796838
	Figure 4	53240.741	53642.574	49.81202258	50.1879774
	Figure 5	121766.099	60253.582	66.89721591	33.1027841
	Figure 6	189188.554	39674.601	82.66448743	17.3355126
Average				*56.21387639*	*43.7861236*

totoxicity Kit, the ovarian tissue was examined and analyzed depending on the calcein AM and ethiomer-1 levels. Living cells are characterized by the presence of intracellular esterase activity, determined by the enzymatic conversion of non-fluorescent calcein AM to the intensely fluorescent calcein. While calcein AM maintains well within live cells, producing an intense uniform green fluorescence in live cells (ex / em ~495 nm / ~515 nm), ethD-1 enters in cells with damaged membranes, undergoing an amplification by fluorescence 40-fold, binding to nucleic acids and producing a bright red agent fluorescence in dead cells (ex / em ~ 495 nm / ~635 nm) (Figures 1, 2).

Confocal scanning microscopy with an argon-krypton laser was

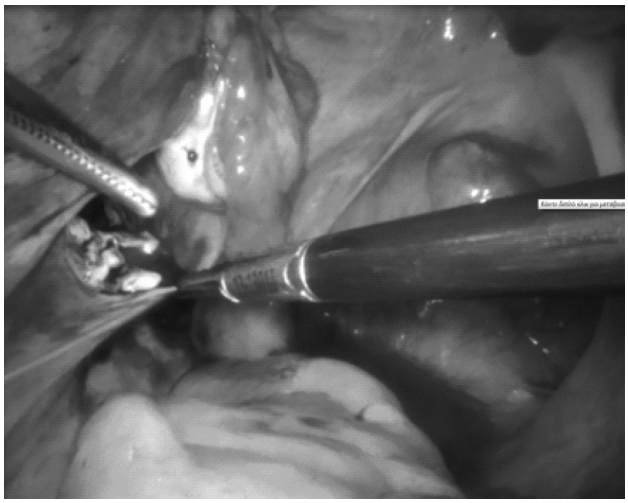

Figure 3. — Retransplantation of ovarian tissue in the fossa ovarica.

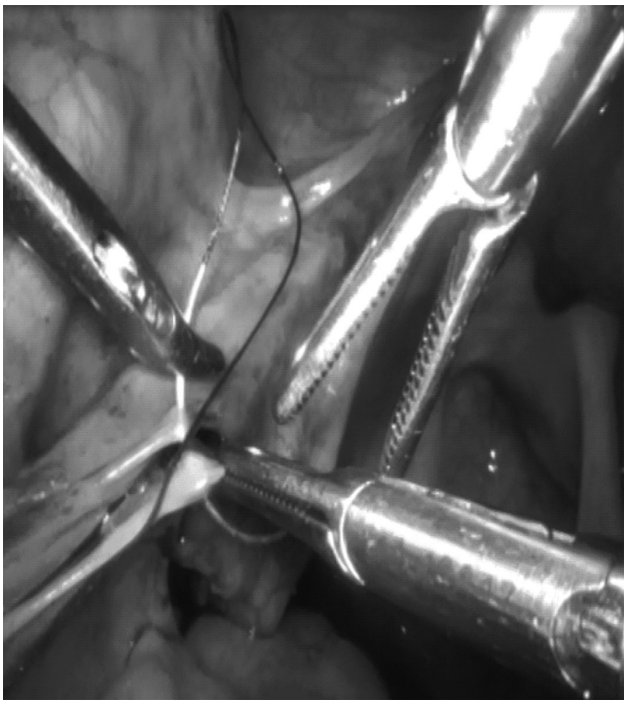

Figure 4. — Stitching and occlusion of the fossa ovarica.

used to record the fluorescent images. Confocal microscopy is an optical imaging technique for increasing optical resolution and contrast of a micrograph by means of adding a spatial pinhole placed at the confocal plane of the lens to eliminate out-of-focus light. It enables the reconstruction of three-dimensional structures from the obtained images. Through assiduous analysis of all images, it is able to measure the viability rate of all ovarian tissue.

Four of 17 samples were fresh, for instance experiment No. 4 and No. 10, and as a result this appears with a greater proportion of green signal which reflects the percentage of live cellular tissue; hence indirectly it is proved that the fresh tissue affects more the quality of results than the cryopreserved one.

Results

After a thorough analysis of all images using immunofluorescence microscopy, the measurement of ovarian tissue viability was performed. Image J can display, edit, analyze, process, save and print 8-, 16-, and 32-bit images and supports most major formats. Image J can calculate the area and the pixel values, which may be useful for graphic designers. Furthermore it allows the practitioner to measure distances and angles, create density histograms, and also line profile plots. It also supports other standard image processing functions, such as contrast manipulation, sharpening, smoothing, edge detection, and median filtering.

Using the program Image J, it is feasible to measure the ovarian tissue viable area, depending on the green and the red signals. The green and red areas reflect the viability rate inside the ovarian follicles through the emission of calcein AM and ethiomer-1.

Using mathematical analysis, the viability percentage rate can be achieved. The average was quantified to be 56.21% and 43.78% of green and red signals, respectively. The later measurements represent the mean average of the photographical analysis of each patient. This indicates the numeral difference between the red and the green signal (Table 3).

The ultimate goal remains the retransplantation of viable ovarian tissue in the fossa ovarica after completion of chemo-, radio- or hormonal therapy and through assisted reproductive techniques, a conception of a healthy pregnancy. The present authors managed in their last experiment to retransplate the viable ovarian tissue orthotopically in the fossa ovarica (Figures 3, 4). After the completion of chemo-, radio-, and hormonal therapy due to breast cancer and a thorough hormonal and ultrasonic examination, the patient underwent IVF protocols in order to fulfill the main scope, the conception of a healthy pregnancy. This successful attempt was the first mentioned in accordance with the Hellenic bibliography.

Discussion

The presence of neoplasmatic diseases in women of reproductive age is increasing. Recent data estimate that more than 679,540 women in the USA, many of premenopausal age, are diagnosed with invasive cancer annually [8].

The option of chemotherapeutic, radiotherapeutic or hormonal approach leads mathematically to premature ovarian failure and decrease of ovarian reserves [9]. The following follicular destruction results in the loss of endocrine and reproductive functions, depending on the dose of the chemotherapeutic agent and the age of the patient.

The main scope remains fertility preservation in women of reproductive age. As potential options, there are many under developmental stages, such as cryopreservation of ovarian oocytes or embryos and many at the experimental level, such as cryopreservation of ovarian tissue [10].

Due to variations in the cancer type, dose and type of chemotherapy and age of the patient each case should be individualized and requires a different strategy in fertility preservation [11]. Cryopreservation of ovarian tissue cannot be undertaken in women who have already received high-dose chemotherapy or presented clinical signs of ovarian failure. It consists in a new promising option available for premenopausal women and for those who cannot delay the departure of chemotherapy [12].

There are adequate advantages regarding the preservation of ovarian tissue. The tissue can be harvested immediately laparoscopically without ovarian stimulation. There is no need of male partner/gamete donor. It is feasible resumption of reproductive and endocrine function, prohibiting a hormonal replacement therapy, and preventing the use of immunosuppressant medications in case of autotransplanation, respectively [13]. After the histological analysis of the tissue, the measurement of the quality and viability using immunofluorescence microscope and the completion of the chemo-or hormonal therapy, the next step will be without doubt the transplantation of the tissue in the fossa ovarica [14]. The ultimate option consists of orthotopic autotransplantation (fossa ovarica). Eterotopic autotransplantation (peritoneal cavity) or allotransplantation (same species) and xenografting (different species) provide alternative therapeutic options [15]. The graft must be vascular in comparison with ovarian cortical tissue. Through the vascularisation, the problem of ischemic injury can be minimized.

All the aforementioned future promising techniques, focusing on the fertility preservation in premenopausal women, infected by neoplasmatic diseases, raise a number of potential and legal issues related to both patient and offspring welfare [16]. The issue remains controversial. There are many obstacles regarding the pregnancy establishment of an older woman without fulfilling the health and responsibility criteria. Additionally, the morality of harvesting and thawing of ovarian tissue without a prior evaluation of the procedure's success rate is being questioned [17]. All these factors must be kept under consideration, for the fertility preservation specialists to prevent a misunderstanding and misuse of all these new technologic options.

The present study demonstrated the efficacy, quality, and viability of ovarian tissue thawing and the capability of autotransplantation in the fossa ovarica, while restoring the fertility preservation rate and increasing the possibilities of a healthy gestational consumption.

Conclusion

In young cancer patients, with possible premature ovarian failure and decrease of ovarian reserves after the completion of chemo-, radio-, or hormonal therapy, cryopreservation of ovarian tissue represents an ideal alternative option preserving the fertility rate and sets the conditions for future gestation. Many further studies must be conducted in order to achieve this ultimate scope.

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