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Comparison of Xenografting in SCID Mice and LIVE/DEAD Assay as a Predictor of the Developmental Potential of Cryopreserved Ovarian Tissue

T. MALTARIS[1], H. KAYA[2], I. HOFFMANN[1], A. MUELLER[1], M.W. BECKMANN[1] and R. DITTRICH[1]

[1]*Department of Obstetrics and Gynaecology, University Hospital Erlangen, D-91054 Erlangen, Germany;*
[2]*Department of Obstetrics and Gynecology, School of Medicine, Suleyman Demirel University, TR-32260 Isparta, Turkey*

Abstract. *This study compared the predictive value of the LIVE/DEAD fluorescence viability assay to xenotransplantation in SCID mice, regarding the developmental potential of cryopreseved human ovarian tissue for fertility preservation purposes. The thawed ovarian tissue of ten patients was partly examined by LIVE/DEAD viability staining or histologically examined after transplantation and gonadotropin stimulation in 30 SCID mice. The LIVE/DEAD assay showed 87.1±3.5% (mean±SD, n=10) viable follicles (intact oocyte and more than 50% of granulosa cells alive). Histological examination showed follicles in all developmental stages in the transplanted grafts. The total number of follicles found was much lower than with the LIVE/DEAD assay (8.9±3.1 versus 54.4±20.0, p<0.001). If the LIVE/DEAD assay yields > ~85% viable follicles, it can be assumed that the follicles in the cryopreserved tissue have maintained their developmental potential. This assay is, therefore, a suitable diagnostic method before an intended retransplantation.*

Combinations of chemotherapy and radiotherapy in young women with cancer have substantially improved life expectancy in these patients, but these treatments often cause irreversible ovarian failure. It is estimated that approximately 70% of adolescent patients with lymphoma and leukaemia survive (1), whereas a third of these young women are rendered sterile (2). These young women have to face years of hormone replacement therapy as well as the prospect of definite infertility which causes an additional psychological stress (3, 4). Frozen semen was effective for preservation of fertility in one-third of male patients who returned to use their semen after chemotherapy (5). However, there are currently no routine methods to preserve female fertility following cancer treatment (6) or treatment for severe autoimmune conditions such as rheumatoid arthritis (7, 8). A promising method of preserving fertility is cryopreservation of ovarian tissue (9, 10). Ovarian biopsy can be expediently performed without delaying cancer treatment. Follicular viability after cryopreservation and thawing has been demonstrated in several studies (11). The most likely follicles to survive cryopreservation are the primordial follicles (12, 13), probably because of their lower metabolic rate (14).

Although only two pregnancies have been achieved to date (15, 16), cryopreservation of ovarian tissue is now being offered by many groups due to its future therapeutic potential.

The aim of this study was to compare two methods of assessing follicular viability after the freezing/thawing procedure, as it is known, for example, that there are variations in survival rates of cryopreserved sperm or embryos from different individuals (17).

An open freezing system was used for ovarian tissue cryopreservation (CTE-920, CTE, Hoechstadt, Germany) and a slow freezing protocol to cryopreserve human ovarian tissue. The survival of the tissue after thawing was examined by fluorescent LIVE/DEAD viability assay and follicular development after transplantation into SCID mice.

Materials and Methods

Animals. Thirty female SCID mice (C.B-17/IcrHanHsd SCID, 6 weeks of age) were obtained from Harlan-Winkelmann (Borchen, Germany). The animals were housed in a high efficiency particulate air-filtered positive pressure room. The cages (Techniplast, Milano, Italy) were filter-topped and the animals had free access to food (Altromin 1314, Altromin, Lage, Germany) and water under 12-hour light / 12-hour darkness conditions. Groups of 5-9 mice were housed in one cage. Upon arriving from the

Correspondence to: Dr. rer. nat. R. Dittrich, Dept. of Obstetrics and Gynecology, University Hospital Erlangen, Universitaetsstrasse 21–23, D-91054 Erlangen, Germany. Tel: +49 (0) 9131 853 3553, Fax: +49 (0) 9131 853 3552, e-mail: ralf.dittrich@frauen.imed.uni-erlangen.de

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Table I. *Pre-freeze follicular count (per mm^2 of ovarian cortex), survival of follicles assessed with LIVE/DEAD viability assay after cryopreservation and total number of follicles found per graft histologically (mean±SD, n=3).*

Patient diagnosis	Age	Follicular count mean number/mm^2 (percentage of primordial)	Survival count (%) (cryopreserved ovarian tissue) with the LIVE/DEAD assay				Total number of follicles found per graft			
			Complete follicle	Oocyte + >50% granulosa	Oocyte + <50% granulosa	Dead	Primordial	Primary	Preantral	Antral
Hodgkin	28	17 (94.2%)	15 (39%)	19 (50%)	4 (11%)	0	1.0±0.8	2.3±0.4	1.6±1.2	5±0.8
Hodgkin	26	23 (95.5%)	18 (44%)	19 (46%)	4 (10%)	0	0.6±0.9	1.6±0.4	2.6±0.4	7±0.8
Hodgkin	24	21 (95.7%)	17 (42%)	15 (38%)	8 (20%)	0	0.3±0.4	1.3±0.4	2.0±0.8	4.0±0.8
CML	16	26 (98%)	28 (41%)	32 (47%)	7 (11%)	1 (1%)	1.0±0.8	0.6±0.4	2.0±0.8	4.0±1.4
Hodgkin	34	9 (88%)	10 (45%)	9 (41%)	2 (9%)	1 (5%)	1.3±0.4	2.3±1.2	2.3±0.4	7.6±2
CML	15	28 (95%)	32 (44%)	32 (44%)	8 (12%)	0	1.0±0.8	0.3±0.4	1.3±0.4	2.0±0.8
CML	19	25 (96%)	23 (55%)	15 (36%)	3 (7%)	1 (2%)	0.3±0.4	0.0	2.6±0.9	3.3±0.4
Breast cancer	31	12 (90%)	16 (38%)	21 (50%)	5 (12%)	0	0.6±0.4	0.3±0.4	1.3±0.4	2.6±0.9
Neuroendoblastoma	22	28 (94%)	30 (52%)	20 (35%)	6 (11%)	1 (2%)	0.3±0.4	0.3±0.4	4.0±0.8	6.0±1.6
ALL	19	33 (95%)	33 (48%)	28 (41%)	7 (10%)	1 (1%)	0.6±0.4	0.3±0.4	3.3±0.4	7.6±2.5

breeding company, the mice were allowed to acclimatize for one week. All procedures, tests and injections were performed under a laminar flow hood in a positive pressure room. Approval for the study was obtained from the local ethical committee on animal experiments. The animals were maintained in accordance with the Animal Care and Use Committee regulations.

Patients. Ten patients, between 15 and 34 (median 23) years of age, were included in this study following informed consent and approval of the local university ethical committee. All patients suffered from malignant diseases (Table I) and wanted to preserve ovarian tissue for a future pregnancy. A maximum of 5% of frozen tissue from each patient was used for our experiments. Prior to cryopreservation, a histological examination of the ovarian cortex was performed in order to secure a sufficient amount of primordial follicles. All patients had age-related normal follicular distribution.

Cryopreservation protocol. The ovarian cortex was gained through an operative laparoscopy by dissecting an area of about 20 x 10 x 3 mm ovarian tissue anti-mesenterically. The biopsies were cut into small pieces (about 1 x 1 x 1 mm) and equilibrated in ascending equimolar concentrations of DMSO/propandiol up to a concentration of 1.5 M in steps of 0.25 M. The tissue pieces remained in each concentration at 37˚C for 7 minutes and at the last concentration of 1.5 M for 30 minutes. The tissue was then placed in special cryovials (CTE, Erlangen, Germany) and loaded into an open freezing system which provides self seeding (CTE). The freezing protocol was as follows: a) cool at –5˚C/min to –3.8˚C; b) cool at –1˚C/min to –5.3˚C; c) cool at –0.2˚C/min to –6˚C; d) unchanged for 20 min; e) cool at –0.3˚C/min to –30˚C; f) cool at –0.1˚C/min to –35˚C; g) cool at –0.3˚C/min to –80˚C; h) cool at –10˚C/min to –110˚C; i) immersion into liquid nitrogen. After storage in liquid nitrogen for at least 1 month, the probes were thawed at room temperature. Removal of the cryoprotectant was done in the reverse order of the freezing equilibration procedure. The tissue blocks were then cultured in an antibiotic-supplemented Medicult IVF-Medium (Gück, Berlin, Germany).

Transplantation procedure. Surgery was performed under narcosis with ketamin (80 mg/kg body weight, Ketavet, Pharmacia & Upjohn, Erlangen, Germany) and xylazin (10 mg/kg body weight, Rompun, Bayer, Frankfurt, Germany), irrespective of the stage of the oestrus cycle. During surgery, the mice were kept on a warming plate, the incision site was disinfected with pure alcohol and covered with a one-way sterile towel. Both ovaries were removed by a small body wall incision which was sutured with absorbable thread. Xenografting of the ovarian cortex was performed within 2 hours after thawing to minimize ischaemic damage. Ovarian tissue pieces were placed in a pocket of the neck muscle.

Gonadotropin stimulation. Mice received daily *i.p.* injections of human menopausal gonadotropin (HMG, Menogon, Ferring, Kiel; 2 IU FSH / 2 IU LH per animal/every second day) or saline, starting from day 14 after transplantation for 15 weeks. This dose was adjusted from an earlier study by Oktay *et al.* (24).

LIVE/DEAD assay. One ovarian tissue piece from each patient was examined for estimation of its vitality. In order to estimate the number of follicles that had survived the freezing/thawing procedure *in vitro* we used a fluorescence staining. The LIVE/DEAD viability /cytotoxicity assay kit (L-3224, Molecular Probes, Leiden, The Netherlands) provides a two-colour fluorescence cell viability assay that is based on the simultaneous

determination of live and dead cells. The kit was used with a Zeiss fluorescence microscope (IM 35, Zeiss, Oberkochen, Germany) in order to examine the viability of the frozen-thawed tissue.

Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually non-fluorescent cell-permeating calcein acetoxymethyl (AM) to the intensely-fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (ex/em ~495 nm/~515 nm). Ethidium homodimer-1 (EthD-1) enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495 nm-635 nm).

To perform the viability assay, the following protocol was used: a) the thawed tissue was dissected to as small as possible pieces with a scalpel; b) 3 mg collagenase (Collagenase Type IV, Sigma-Aldrich, Steinheim, Germany) diluted in 3 ml Dulbecco's phosphate-buffered saline (D-PBS) was added to the tissue; c) the samples were left to incubate for approximately 2 hours at 37˚C, while the homogenate was stirred periodically every 20 minutes; d) the LIVE/DEAD reagent stock solutions were removed from the freezer and allowed to warm to room temperature; e) the reagents were combined by adding 10 μl of the 2 mM EthD-1 stock solution and 5 μl of the supplied 4 mM calcein AM stock solution to 10 ml of sterile, tissue culture-grade D-PBS; f) the resulting approximately 2 μm calcein AM and 4 μM EthD-1 working solution was added directly to the samples and left to incubate for 30 minutes in a dark place at room temperature; g) the labelled cells were viewed under the fluorescence microscope.

Microscopic evaluation of the number of follicles. Seventeen-week grafts were recovered under narcosis and fixed in formalin. After routine paraffin embedding, the samples were entirely serially sectioned (~3 μm), every 5 was stained with haematoxylin and eosin and examined microscopically as a reference section (Figure 1). The number of primordial, primary, preantral and antral follicles (intact and displaying ooplasma) were examined.

The calculation was done according to the method described earlier (18). The follicles were classified as follows: primordial follicles with one layer of flattened granulosa cells surrounding the oocyte; primary follicles having one layer of cuboid granulosa cells; preantral follicles having two or more layers of granulosa cells but no antrum; and antral follicles with an antral cavity.

Statistical evaluation. SPSS was used for data evaluation. Nominal data were expressed as mean±SD and compared using the *t*-test. A p value of 0.05 was considered statistically significant.

Results

Pre-frozen follicular histological count and follicular viability after thawing assessed with the LIVE/DEAD assay. All patients showed age-related normal follicular counts (Table I). The primordial follicle was the most predominant type of follicle. The results of the fluorescence staining of one ovarian tissue piece (approx. 1 x 1 x 1 mm) of each patient are given in Table I. After cryopreservation with the slow freezing protocol, a total of 44.9±5.6% intact follicles, 42.2±6.6% follicles with intact oocyte and >50% granulosa cells and 11.8±3.7% with intact oocyte and <50% granulosa cells were found. Dead follicles were very rare (1.1±1.5). Figure 1 demonstrates a LIVE/DEAD assay sample.

Follicular survival and development. In the frozen/thawed tissue transplanted into the SCID mice, follicles in all developmental stages were found. Primordial: 0.7±0.7; primary: 0.9±1.0; preantral: 2.3±1.1; and antral: 4.9±2.4 (mean±SD, n=30), Figure 1. The total number of follicles found in the transplanted grafts was much lower than with the LIVE/DEAD assay (8.9±3.1 *versus* 54.4±20.0, $p<0.001$) (Table I).

Discussion

It is estimated that, by 2010, one in every 250 women of reproductive age will be a cancer survivor (19). However this lifesaving treatment can provoke early menopause and subsequent infertility, due to the destruction of a significant proportion of ovarian follicles by chemo- and radiotherapy. Methods to preserve fertility in these young patients are chemoprotection with GnRH-analogues (20) and apoptosis inhibitors (21) before the planned chemotherapy, the ovarian transposition before the radiation and the cryoconservation of oocytes or ovarian tissue.

To date, two pregnancies have been obtained after autotransplantation of cryopreserved ovarian tissue (15, 16). Many groups are cryopreserving ovarian tissue for future clinical use (22-26).

The LIVE/DEAD assay as well as xenotransplantation in SCID mice are established methods for the evaluation of survival of cryopreserved human ovarian tissue (23, 27). However, there has been no direct comparision of these methods in an equal-sized tissue piece of the same patient. The aim of the study was to compare these two different assessment methods of the development potential of cryopreserved human ovarian tissue in the same patient collective.

The pre-freezing histological follicular count showed normal age-related follicular distribution in the ovaries. After thawing, a LIVE/DEAD assay was performed. Follicles were considered as viable only if they had an intact oocyte and more than 50% of the granulosa cells, because the maturation of oocytes from small follicles will succeed only when sufficient granulosa cells are intact (28).The results of the staining with the dye of the LIVE/DEAD fluorescent assay confirmed that a high percentage of oocytes, as well as granulosa cells, survive the cryopreservation and thawing procedure. This is in accordance with earlier findings of our group in animal experiments and in other studies (18, 27-29).

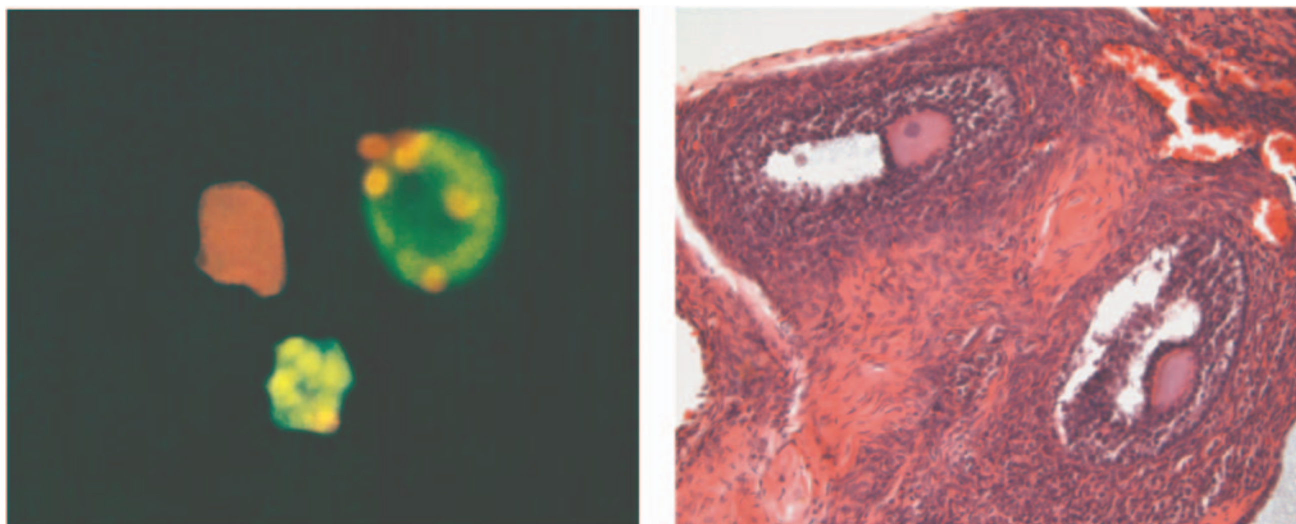

Figure 1. *LIVE/DEAD assay with one dead and two viable follicles (intact oocyte and > 50% granulosa cells alive) (left, x ~300) and histological stain of ovarian tissue 17 weeks after transplantation (right, HE, x 100).*

Table II. *Advantages (+) and disadvantages (–) of the LIVE/DEAD assay and xenotransplantation of cryopreserved ovarian tissue.*

LIVE/DEAD assay		Xenotransplantation	
+	–	+	–
Relatively cheap and easy to perform	Probably overestimates the survival rate	Shows the development of primordial follicles to the antral stage	Animal experiments required
Results in the same day	Fluorescence microscope required	Can be used for experimental proposes (*i.e.* optimizing the transplantation procedure)	SCID mice handling difficult
No animal experiments			Costly, hMG-stimulation over 15 weeks, time-consuming
Minimal tissue requirement			More tissue-consuming Histological examination necessary Loss of follicles due to ischaemia after transplantation

After thawing of cryopreserved ovarian tissue, a small piece (1 x 1 mm) was transplanted in SCID mice. The mice were then stimulated with gonadotropins for 15 weeks, because although the endogenous gonadotropins in ovarectomised mice are sufficient to support follicular growth in grafts, many researchers use human gonadotropins to maximise follicular development (24, 30). In the histological examination of the grafts, follicles at all developmental stages were found, which shows that the human ovarian tissue survived the cryopreservation and transplantation procedure. More antral follicles were found than primordial, which can be explained by the long stimulation period.

The comparison of the absolute number of follicles in the LIVE/DEAD assay and in the transplanted grafts, in equal-sized tissue pieces from the same patient, showed a significant loss of follicles through the transplantation procedure. This can be explained by the known effect of follicle loss because of ischaemia after transplantation (31, 32).

Our experiments showed that if more than ~85% intact follicles are found by the LIVE/DEAD assay in the

frozen/thawed ovarian tissue, it can be assumed that the primordial follicles have retained their developmental potential and that this tissue can be used for retransplantation.

The LIVE/DEAD assay has the following advantages, in comparison to xenotransplantation in SCID mice, regarding the evaluation of the cryopreserved tissue before a retransplantation: it is easier, significantly more economical and requires only a minimum of ovarian tissue in contrast to the xenotransplantation, which also involves animal experiments. For the evaluation of the results, only a fluorescence microscope is needed and no histological examination. The only real advantages of xenotransplantation are that it actually shows the development of primordial follicles to the antral stage and can also be used for experimental purposes such as optimizing the transplantation procedure (Table II).

Conclusion

Ovarian tissue, which yields more than 85% viable follicles with the LIVE/DEAD assay after cryopreservation has retained its developmental potential. We conclude that the LIVE/DEAD assay is a reliable and reproducible predictive method to study the survival of ovarian tissue after the cryopreservation procedure, before an intended retransplantation for fertility purposes.

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