Assessment of vitrification outcome by xenotransplantation of ovarian cortex pieces in γ -irradiated mice: morphological and molecular analyses of apoptosis

Mina Jafarabadi · Maasoume Abdollahi ·
Mojdeh Salehnia

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Abstract

Purpose The aim of this study was the investigation of caspase-3/7 activity and apoptosis related gene expression after vitrification and xenotransplantation of human ovarian fragments.

Methods Ovarian specimens were obtained from normal female-to-male transsexual women during laparoscopic surgery and cut into small pieces and were considered as vitrified and non-vitrified groups. The morphological study, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, caspase-3/7 activity and apoptosis related gene expression analysis were done in both non-vitrified and vitrified groups in two steps (before transplantation of ovarian tissues and 30 days after transplantation).

Result(s) In spite of high rate of normal follicles in both non-transplanted tissues these rates were significantly decreased in vitrified and non-vitrified grafted tissues, moreover grafted-vitrified tissue showed significantly less normal follicles than

grafted-non-vitrified group ($P<0.05$). The expression of some pro and anti-apoptotic genes in vitrified-warmed tissues were not changed compared to non-vitrified ones but the expression of Fas and caspase8 was increased and the expression of BRIC5 was decreased in this group ($P<0.05$). In transplanted vitrified group the Bcl2, FasL and BRIC5 gene expression was high and caspase8 was low ($P<0.05$). The expression of all genes in both grafted groups was more than non-grafted tissues except for caspase8 ($P<0.05$). The TUNEL positive signals and caspase-3/7 activity were increased in both grafted groups compared to non-grafted groups and this enzyme activity in grafted-vitrified group was more than grafted-non-vitrified group ($P<0.05$).

Conclusion(s) This study provides the first evidence on the significant effect of vitrification on follicular apoptosis of grafted human ovarian tissue at mRNA level. The signs of follicular survival or degeneration detected by morphological assessment and caspase-3/7 activity were closely correlated to the changes in expression of apoptosis-related genes.

Capsule Our study demonstrated for the first time the signs of follicular survival or degeneration detected by morphological assessment and caspase-3/7 activity were closely correlated to the changes in expression of apoptosis-related genes.

Keywords Vitrification · Apoptosis gene expression · Transplantation · Human ovarian tissue

M. Jafarabadi
Reproductive Health Research Center, Tehran University of Medical Sciences, Tehran, Iran

M. Abdollahi
Department of Anatomical Sciences, Faculty of Medicine,
Qom University of Medical Science, Qom, Iran

M. Salehnia (✉)
Department of Anatomical Sciences, Medical Sciences Faculty,
Tarbiat Modares University, P. O. BOX: 14115-111, Tehran, Iran
e-mail: salehnia@modares.ac.ir

M. Salehnia
e-mail: mogdeh@dr.com

Introduction

The cryopreservation of human ovarian tissue has gradually become routine clinic practice using a slow-freeze program since 1996 and it is one method of preserving fertility in young women with cancer prior to chemo- or radiotherapy [1–5].

Vitrification represents a novel, alternative, simple and low-cost technique [6–8]. However, previous studies have provided controversial results regarding the structural integrity and developmental potential of follicles after vitrification and warming of human cortical tissue, depending on the methods used [9–17].

We previously showed that the morphology and ultrastructure of human ovarian follicles and stromal cells were well-preserved after vitrification of ovarian tissue using a solution containing ethylene glycol, Ficoll and sucrose (EFS40). We also found that the integrity of vitrified human tissue was preserved and the incidence of apoptosis was unchanged after 24 h in vitro culture [17]. However, we recently demonstrated that the expression levels of some apoptosis-related genes were changed immediately after warming of vitrified human ovarian tissue [18].

Apoptosis regulates cell death during ovarian follicular development and atresia [19]. Apoptosis may be initiated during the cryopreservation of ovarian tissue. Several investigations using immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) have shown that the incidence of apoptosis was unchanged after vitrification and warming [17, 9]. However, the longer-term effects of vitrification on the integrity, degeneration, and function of ovarian tissues may be assessed after transplantation. Several reports have been published in this regard [20–27]. Rahimi et al. [27, 25] observed a higher incidence of apoptosis in grafted vitrified ovarian tissue sample by TUNEL and immunohistochemistry assay. In contrast, Amorim et al. used the TUNEL assay and showed that pre-antral follicles were well-preserved after xenografting of vitrified human ovarian tissue [23].

Ovarian tissue apoptosis involves intrinsic and extrinsic pathways. The intrinsic pathway involves Bcl-2, Bax, p53, and BIRC5 genes, while Fas and FasL genes contribute to the extrinsic pathway. Some of these genes are anti-apoptotic (Bcl-2 and BIRC5), and some are pro-apoptotic (Fas, FasL, Bax and p53) [19]. Cysteine proteases such as caspase8 and caspase3 are activated in both pathways and act as a proteolytic cascade to remove dead cells.

To the best of our knowledge, there is little information on the effects of transplantation of vitrified human ovarian tissue on apoptosis at the mRNA level.

The present study evaluate the effects of vitrification of human ovarian tissue (using EFS40) on follicular morphology, caspase-3/7 activity, and the expression of apoptosis-related genes after xenotransplantation.

Material and methods

All reagents were obtained from Sigma-Aldrich except cited otherwise.

Ovarian tissue collection

Ovarian specimens were obtained from 15 normal female-to-male transsexual women aged between 21 and 35 years old during laparoscopic surgery (5×5×1 mm) by informed

consent, under a protocol approved by the Ethics Committee of the Faculty of Medical Science of Tarbiat Modares University (Ref. No. 5274856). These women had not used any exogenous hormones for 3 months prior to surgery and showed no endocrinological dysfunction. Human samples details were summarized in Table 1.

The tissue samples were transferred to the laboratory on ice within 1–2 h in pre-warmed and equilibrated Leibovitz L-15 medium supplemented with 10 mg/ml human serum albumin (HSA), 100 IU/ml penicillin and 100 µg/ml streptomycin. The ovarian cortices were cut into approximately 2×1×1 mm small pieces and the tissue fragments of each biopsy were randomly divided into non-vitrified (control) and vitrified groups.

Experimental design

In order to assess the effect of vitrification procedure on the follicular survival, growth and expression of some apoptosis related genes following xenotransplantation, ovarian fragments were divided into four experimental subgroups: (1) non-grafted non-vitrified, (2) non-grafted vitrified, (3) grafted non-vitrified and (4) grafted-vitrified. The histological study, TUNEL assay, caspase-3/7 activity assay and molecular analysis of apoptosis by real time RT-PCR were done before and after grafting.

Vitrification and warming procedure

The ovarian cortical fragments (90 fragments from 15 human samples) were cryopreserved as we described elsewhere [28] with some modifications. The vitrification solution was EFS40%, composed of 40 % ethylene glycol (EG; *v/v*), 30 % Ficoll 70 (*w/v*), 1 M sucrose and supplemented with 0.21 % human serum albumin (HSA). Tissues were equilibrated in three changes of vitrification solution for 5 min. Subsequently, the individual tissue fragments were loaded into cryovials with a minimal amount of vitrification solution (50 µl) and exposed to nitrogen vapor for 30 s then plunged into liquid nitrogen and stored for 2 weeks. Vitrified ovarian fragments were warmed at room temperature for 30 s then placed in a 37 °C water bath for 20 s. The tissues were then rinsed in 1, 0.5 and 0.25 M sucrose solutions, respectively for 5 min at room temperature. The warmed tissue fragments were equilibrated in McCoy's culture media supplemented with 10 mg/ml HSA, 100 IU/ml penicillin and 100 µg/ml streptomycin for 30 min before any assessments.

Preparation of γ-irradiated mice and transplantation of human ovarian tissue

The 6–8-weeks-old female National Medical Research Institute (NMRI) mice were cared for and used according to the university Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Committee

Table 1 Detail of human samples characteristics

Samples	Age	BMI	Serum 17- β estradiol levels pg/mL	Serum progesterone levels ng/mL	No of tissue fragments	Assessments
No 1	21	23	110	1.01	30	All assessments before and after grafting for vitrified and non-vitrified groups
No 2	31	22	97.5	0.72	12	Morphological study and Caspase assay for vitrified and non-vitrified groups before grafting
No 3	26	26	89	0.98	10	Morphological study and TUNEL assay for vitrified and non-vitrified groups before grafting
No 4	21	29	96.3	0.87	16	Morphological study and Caspase assay for vitrified and non-vitrified groups after grafting
No 5	30	19	79	0.64	28	Morphological study and Caspase assay for vitrified and non-vitrified groups before grafting
No 6	26	22	71	0.90	9	Morphological study and TUNEL before grafting for vitrified and non-vitrified groups
No 7	28	27	83.6	0.69	15	Morphological study and TUNEL assay for vitrified group after grafting
No 8	24	26	102.2	0.91	32	All assessments before and after grafting for vitrified and non-vitrified groups
No 9	27	20	90.6	0.81	13	Morphological study and TUNEL after grafting for both vitrified and non-vitrified groups
No 10	26	30	75	0.49	10	Morphological study and Caspase assay for vitrified group after grafting
No 11	35	27	54.8	0.4	5	Low number of follicles Ignored
No 12	24	24	99.8	1.04	10	Morphological and molecular studies for vitrified and non-vitrified group before grafting
No 13	25	26	111	0.77	8	Morphological and Caspase assay for vitrified and non-vitrified group before grafting
No 14	28	22	85.9	0.81	14	Morphological and molecular studies for vitrified and non-vitrified group after grafting
No 15	22	29	103.6	0.98	12	Morphological and Caspase assay for vitrified and non-vitrified group after grafting

for Animal Research of the University. The mice ($n=15$) were given single dose of 7.5 Gy whole body γ -irradiation for 6 min (Theratron 780C, Canada) [29]. Tissue transplantation was performed 72 h after irradiation. Prior to transplantation the mice were anesthetized by an intra peritoneal injection of a mixture of ketamine 10 % (75 mg/kg body weight) and xylazine 2 % (15 mg/kg).

Their gluteus maximus muscles were bilaterally exposed and scratched via a dorso-horizontal incision using a strictly aseptic technique. Three pieces of vitrified or non-vitrified tissue fragments were inserted and stitched within each muscle (six fragments from one woman for each mouse) then the wound was closed (eight mice for vitrified and seven mice for non-vitrified samples).

Ovarian transplanted mice were maintained under sterile conditions with free access to food and water. Thirty days after transplantation the mice were sacrificed and the recovered ovarian grafts from each mouse were randomly fixed for light microscopic study or kept at -80°C for enzyme and molecular analysis.

Histological evaluation

Non-vitrified and vitrified-warmed human ovarian fragments before and after transplantation (eight fragments from different human samples for each sub group) were fixed in 10 % formalin and embedded in paraffin. Tissue sections were prepared serially at 5 μm thickness and every 10th section was stained with hematoxylin and eosin and assessed under a light microscope (near 15–20 sections per each fragment).

The follicles were considered as normal (containing an intact oocyte and granulosa cells) or as degenerated (containing pyknotic oocyte nuclei, shrunken ooplasm, and or disorganized granulosa cells). The follicles at different developmental stages were classified as described earlier [30].

TUNEL assay

The human ovarian tissue fragments before grafting (three fragments for each group) and after grafting (three fragments for each group) in both vitrified and non-vitrified groups were

fixed in 4 % formaldehyde and paraffin embedded, serially cut into 5 µm sections and three sections per each fragments were selected randomly for TUNEL assay. The sections were stained according to the instructions of the kit (In Situ Cell Death Detection Kit, Roche, Germany). The slides were rinsed in PBS three times and examined under a fluorescence microscope (Zeiss, Axiophot, Germany).

A second set of tissue sections was incubated with 50 µl reaction buffer without terminal deoxynucleotidyl transferase (TdT) treatment, as a negative control. As an external positive control, apoptosis was induced in thymus tissue by intraperitoneal injection of 10 mg/kg of dexamethasone into 2-week-old mice [31]. After 16 h, the thymus was removed and fixed in 4 % formaldehyde and embedded in paraffin wax. The thymic sections were processed in the same way as the ovarian samples.

RNA extraction and cDNA synthesis

Total RNA was extracted from the human ovarian tissue samples (three fragments from different human samples in each subgroup) using an RNeasy MiniKit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Using oligo dT, RNA was reverse-transcribed by RevertAid M-MuLV reverse transcriptase and amplified by specified primers (Table 2). Primers were synthesized based on human mRNA coding sequences. GAPDH gene was used as an internal control.

Real time RT-PCR

After cDNA synthesis real time RT-PCR was performed by Applied Biosystem real time thermal cycler according to QuantiTect SYBR Green RT-PCR kit (Applied Biosystems, UK) and melt curve analysis was used to confirm the amplified product. For each sample, the reference gene (GAPDH) and the target genes were amplified in the same run. Real time thermal condition including holding step: 95 °C, 5', cycling step: 95 °C 15', 58 °C 30', 72 °C 30' was continued by a melt curve step: 95 °C 15', 60 °C 1', 95 °C 15'. The relative quantification of target genes was determined using the Pfaffel method. All real time RT-PCR experiments were repeated three times.

Caspase-3/7 assay

The caspase-3/7 activity of human ovarian tissues in vitrified and non-vitrified groups before and after grafting (ten fragments from five human samples were considered for vitrified group and ten fragments for non-vitrified group; five fragments before grafting and five fragments after grafting in each group) and dexamethasone treated mouse thymus as positive control ($n \leq 5$ for each groups) were done using the Caspase-Glo® 3/7 Assays kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Ovarian fragments were homogenized in 200 µl hypotonic extraction buffer containing 25 mM HEPES (pH =7.5), 5 mM MgCl₂ and 1 mM EDTA.

Table 2 Oligonucleotide primers

Accession numbers	Gene	Primer sequence	PCR product size (bp)
NC_000012.11	GAPDH	Forward: 5'CTGGGCTACACTGAGCACC 3' Reverse: 5'AAGTGGTCGTTGAGGGCAATG3'	101
NC_000010.10	Fas	Forward: 5'TGAAGGACATGGCTTAGAAGTG 3' Reverse: 5'GGTGCAAGGGTCACAGTGTT3'	118
NC_000001.10	FasL	Forward: 5'GCAGCCCTTCAATTACCCAT 3' Reverse: 5'CAGAGGTTGGACAGGGAAGAA3'	101
NC_000018.9	Bcl2	Forward: 5'TTGCTTTACGTGGCCTGTTC3' Reverse: 5'GAAGACCCTGAAGGACAGCCAT3'	94
NC_000019.9	Bax	Forward: 5'CCCGAGAGGTCTTTTCCGAG3' Reverse: 5'CCAGCCCATGATGGTTCTGAT3'	155
NC_000017.10	p53	Forward: 5'GAGGTTGGCTCTGACTGTACC3' Reverse: 5'TCCGTCCAGTAGATTACCAC3'	133
NC_000017.10	BIRC5	Forward: 5'AGGACCACCGCATCTCTACAT3' Reverse: 5'AAGTCTGGCTCGTTCTCAGTG 3'	118
NC_000002.11	caspase8	Forward: 5'ATTGCTGTATGCCCGAGC 3' Reverse: 5'CCTGAGTGAGTCTGATCCACAC3'	105
NC_000004.11	caspase3	Forward: 5'AGAGGGGATCGTTGTAGAAGTC 3' Reverse: 5'ACAGTCCAGTTCTGTACCACG3'	81

Then the extracts were clarified by centrifugation at 13,000 rpm for 15 min at 4 °C and the supernatants were used for the assays. Total protein concentrations were determined by Bradford method (Bio-Rad). Diluted (10 µg/ml) extract was mixed with Caspase-Glo® Reagent and incubated at 37 °C for 1 h. Then, it was put in a sirius-single tube luminometer (Berthold Detection Systems GmbH, Germany) and read on luminometer RLU (Relative Light Unit). Then the activity of caspase-3/7 per mg/ml of protein was determined.

Statistical analysis

Statistical analyses were done using SPSS 19.0 software. Quantitative variables were expressed as means±SE. The results of all experimental studies between non-vitrified and vitrified transplanted groups were compared by independent sample *T*-test and the data in the same groups were compared by pair *T*-test before and after transplantation of ovarian fragments. *P*-values less than 0.05 were considered as statistically significant.

Results

Light microscopy observation

Before grafting of human ovarian tissue, the morphology of the vitrified tissue was well preserved and it was similar to the non-vitrified group. The percent of morphological normal follicles in the non-vitrified and vitrified group were 93.75 %±5.12 and 91.07 %±4.80 respectively. There were a few normal primordial follicles within the tissue sections (Fig. 1). The proportion of primordial, primary and growing follicles in the non-vitrified and vitrified group were summarized in Table 3. There was not significant difference between the proportion of normal follicles in vitrified and non-vitrified groups before grafting.

The morphology of follicles and stroma after 30 days grafting were shown in Fig. 2a–f. In both grafted samples, the growing rate of follicles was significantly increased than non-grafting tissue as the percent of primordial follicles was significantly lower and the rate of primary and growing follicles was higher than non-grafted groups ($P<0.05$; Table 3). But the proportion of normal follicles in the grafted-vitrified group (Table 3) was significantly lower than the grafted-non-vitrified group ($P<0.05$).

Well structured call-exner bodies were seen in preantral follicles of vitrified grafted ovarian section. These structures were roset shape of granulosa cells which arranged around the central cavity which filled with fluid. Several blood vessels with blood cells were prominent in theca layer of this preantral follicle (Fig. 2d).

In two vitrified grafted tissue samples, especially at their superficial parts, the neovascularization were seen. These vessels mainly composed of small capillaries (Fig. 2e).

In two grafted tissues from both vitrified and non-vitrified ovarian cortical fragments, the fibrotic area was prominent (Fig. 2f).

TUNEL assay

No TUNEL-positive cells were found before grafting in the oocytes or follicular cells in both vitrified and non-vitrified groups but a few TUNEL signals were observed in stromal cells (Fig. 3a and b). However there were several TUNEL positive cells in the follicles and stromal cells of recovered grafted tissues in both vitrified (2.1 ± 0.264 per 1,000 µm²) and non-vitrified (1.65 ± 0.360 per 1,000 µm²) groups (Fig. 3c and d).

Mouse thymic tissue, used as a positive control (Fig. 3e), showed a high number of TUNEL signals per 1,000 µm² (14.22 ± 1.056). There was no significant difference in TUNEL positive signals between grafted-vitrified and grafted-non-vitrified groups (Fig. 3f, $P<0.05$).

Expression of apoptosis-related genes in non-vitrified and vitrified human ovarian tissue

The mRNA levels of several apoptosis-related genes including pro-apoptotic (Bax, p53, Fas, FasL, caspase3 and 8) and anti-apoptotic (Bcl-2, BIRC5) genes to the housekeeping gene (GAPDH) were evaluated and compared in both non-vitrified and vitrified human ovarian tissue before and after grafting (Fig. 4a–h).

As the results demonstrated, among the studied genes in non-grafted vitrified-warmed group, the expression of Bcl-2, Bax, FasL, P53 and caspase3 were not changed compared to non-vitrified ones. But the expression ratio of Fas and caspase8 mRNA was higher and the expression level of BIRC5 was lower in vitrified group than non-vitrified control group ($P<0.05$).

Analysis of gene expression in ovarian tissue after 30 days grafting showed that the mRNA of Bcl-2, FasL and BIRC5 in the vitrified samples was higher and caspase8 was lower than the non-vitrified control groups ($P<0.05$).

As the results demonstrated, the ratio expression of all studied gene in both grafted groups was higher than non-grafted control groups, except the caspase8 gene expression ratio was lower in vitrified-grafted samples than non-grafted vitrified group ($P<0.05$).

Caspase-3/7 assays

The caspase-3/7 activity as determined RLU/mg protein was not significantly different in non-grafted vitrified

Fig. 1 Light microscopic observation of non-grafted human ovarian cortical tissue after hematoxylin and eosin staining. The morphology of several primordial and secondary follicles were seen in non-vitrified group (**a** and **b**) and in vitrified group (**c** and **d**) with low magnification. The normal morphology of primordial and primary follicles was seen in non-vitrified (**e**) and in vitrified group (**f**) with high magnification

Table 3 The percentage of normal follicles at different developmental stages in all groups of study

Groups	Total no of follicles	No. of primordial F. (Mean%±SE)	No. of primary F. (Mean%±SE)	No. of growing F. (Mean%±SE)	No. of normal F. (Mean%±SE)	No. of degenerated F. (Mean%±SE)
Non-grafted non-vitrified	48	39 (81.25±3.91)*	7 (14.58±0.33)*	2 (4.17±0.17)*	45 (93.75±5.12)*	3 (6.25±1.42)*
Non-grafted vitrified	56	49 (82.14±4.06)**	8 (14.29±0.48)**	2 (3.57±0.11)**	51 (91.07±4.80)**	5 (8.93±0.11)**
Grafted non-vitrified	36	19 (52.8±2.30)* ^a	11 (30.56±1.20) ^a	6 (16.7±1.68) ^a	24 (66.7±2.23)* ^a	12 (33.3±1.85)* ^a
Grafted vitrified	42	26 (61.9±2.45) ^a	11 (26.19±1.53)* ^a	5 (11.9±1.59) ^a	23 (54.76±2.04)* ^a	19 (45.2±1.76)* ^a

There was no statistically difference between non-grafted non-vitrified and non-grafted vitrified group in all columns

*Significant differences between grafted non-vitrified and non-grafted non-vitrified group in the same column ($P<0.05$)

**Significant differences between grafted vitrified and non-grafted vitrified group in the same column ($P<0.05$)

^aSignificant differences between grafted vitrified and grafted non-vitrified group in the same column ($P<0.05$)

Fig. 2 Representative photomicrograph of recovered grafted human ovarian tissue to γ -irradiated mice using hematoxylin and eosin staining. The morphology of growing follicle were seen in non-vitrified (a) and primary follicle in vitrified group (b; arrow). High magnification of growing follicle were seen in non-vitrified group (c) and morphology of preantral follicles with several call-exner bodies (white arrow) was seen in vitrified group (d). In vitrified grafted tissue the new formed capillaries and vessels (e; white arrow head) and some fibrotic area (red arrow head) were seen (f)

(2294 ± 169.2) and non-grafted non-vitrified (2231 ± 89.27) tissues. This enzyme activity was increased in both grafted groups compared to non-grafted tissue and it was significantly higher in grafted-vitrified group (2294 ± 169.2) than non-vitrified grafted group (2231 ± 89.27 ; $P < 0.05$). It was 16370 ± 658.5 RLU/mg protein in thymus tissue as positive control group.

Discussion

Xenotransplantation into animal models represents one method of evaluating the quality of vitrified human ovarian tissues before clinical usage. In the present study, we examined the morphology and follicular development of vitrified ovarian fragments 1 month after xenotransplantation. The safety of the vitrification solution EFS40 for preserving the integrity of mouse and human ovarian tissues in short time study has been demonstrated by our group and other studies [28, 30, 32, 17], which

confirmed its lack of toxicity in terms of the morphology and fine structure of follicles and stromal cells, immediately after warming or after short-term in vitro culture [28, 30, 32, 17].

Our data in the present study regarding to the caspase-3/7 activity and TUNEL assays in both non-grafted tissue samples also more confirmed that there was no increasing in the incidence of apoptosis in human ovarian tissue immediately after vitrification and warming.

Moreover, our data for long term study showed the normality rate of follicles was decreased in grafted-vitrified group than grafted non-vitrified group and these results in the present study provide the first evidence to suggest that this vitrification procedure had deleterious effects on follicular survival and morphology in human ovarian tissue after one month xenotransplantation. Current successes to obtain healthy follicles from xenotransplanted frozen-thawed human ovarian tissue were promising [33–36] whereas there was not any comparison between cryopreserved samples with fresh tissues in these reports.

Fig. 3 Florescent microscopy images of human ovarian sections subjected to TUNEL analysis to determine apoptosis. No TUNEL positive signal was seen in follicles (*white arrow*) in non-grafted-non-vitrified (**a**) and grafted-vitrified tissue (**b**). Several TUNEL positive cells within follicles and stroma were observed in grafted-non-vitrified (**c**) and grafted-vitrified ovarian tissue (**d**). Induced mouse thymic tissue as positive control showed TUNEL positive reaction as green staining (**e**). The comparison of TUNEL positive signals per $1,000 \mu\text{m}^2$ in vitrified and non-vitrified grafted tissues were shown (**f**). There was no significant difference between these groups regarding to the TUNEL signals

In spite of high rate of growing follicles in both transplanted groups the number of normal follicles was decreased than non-transplanted groups. The possible suggestion regarding to the low number of normal follicles in the recovered tissue may have been due to the small size of the grafted ovarian tissue and to post-transplant ischemia. Several investigations found that transplantation had a negative effect on follicular integrity, independent of the cryopreservation method used [22, 37, 38]. The fibrotic areas in grafted tissue have been shown to result in follicular loss following ischemia similar to our observation [39, 23].

We also observed several newly-formed capillaries (angiogenesis) and small vessels in the cortical part of two pieces of recovered tissue, suggesting that the vitrification and/or transplantation techniques can support angiogenesis in some transplanted tissues fragments. Neovascularization is critical for the survival of grafted tissue, and the newly-formed vessels may be derived from the host animal or from

the grafted tissue [40]. Hypoxia can induce the secretion and activation of angiogenic or endothelial growth factors during the first days of transplantation, and further studies are needed to investigate this suggestion. Laschke et al. [38] and Rahimi et al. [22] found similar results regarding increased surface microvascular networks after transplantation of both slow-frozen and vitrified ovarian tissue.

For RNA extraction according to the heterogeneity of the tissue fragments we have chosen more than one fragment for

Fig. 4 The relative expression ratio of apoptosis related genes to GAPDH were seen in non-vitrified and vitrified human ovarian tissue before ($n=3$ tissue fragments from three human samples for each group) and after grafting ($n=3$ tissue fragments from three human samples for each group) using real time RT-PCR. The expression ratio of Fas (**a**), FasL (**b**), Bax (**c**), p53 (**d**), caspase3 (**e**), caspase8 (**f**), Bcl2 (**g**), BIRC5 (**h**) to GAPDH. *a* significant difference between vitrified and non-vitrified groups ($P < 0.05$); *b* significant difference between grafted tissues with their non-grafted groups in the same group ($P < 0.05$)

each human sample however there was an internal control (housekeeping gene) which normalized the data as usual. However our conclusion related to gene expression data is related to ovarian tissue including to stromal cells, follicles and other types of the cells.

There were no significant differences in the expression levels of pro- and anti-apoptotic genes, including Bcl-2, Bax, FasL, p53 and caspase3, or in the Bcl-2/Bax ratio, between vitrified-warmed and non-vitrified samples. p53 induces apoptosis by increasing transcriptional activity of the Bax gene [41] while concomitantly suppressing expression of the Bcl-2 gene [42]. Fas and FasL lead to apoptosis via the extrinsic pathway [19], while Bax is involved in the intrinsic pathway of apoptosis [43]. The balance between these pro- and anti-apoptotic genes is critical for regulating cell survival and apoptosis.

Increased expression levels of Fas and caspase8, and reduced expression of BIRC5 in vitrified-warmed samples demonstrated an impact of the vitrification procedure on the expression of these genes during the short-term, though it was suggested that vitrification did not result in apoptosis during the short period of time after cryopreservation. Because apoptosis is a dynamic process, increases in Fas and caspase8 expression levels may induce DNA fragmentation during in vivo follicular development following xenotransplantation of vitrified-warmed ovarian tissue.

The expression levels of the anti-apoptotic genes BIRC5 and Bcl2 were increased in grafted-vitrified samples, but Fas expression was similar in both groups. BIRC5 protein acts as an apoptosis inhibitor by regulating the cell cycle [44, 45], and its mRNA levels increase during follicular development [46]. Bcl-2 is a mitochondrial membrane-anchored protein that has been detected in the ovary in several species, and is known as an inhibitor of follicular atresia and apoptosis [47, 48]. Its overexpression has been demonstrated in improved folliculogenesis, and its expression level was shown to decrease in follicular atresia [49]. It is possible that some of the alterations in apoptosis-related gene expression after warming of vitrified samples may be reversible, or may be insufficient to induce apoptosis in grafted-ovarian tissue. Additional studies at the protein level are required to investigate these suggestions.

The expression levels of all the studied genes were higher in the grafted compared with the non-grafted samples. It is likely that ischemia during grafting may induce both extrinsic and intrinsic apoptosis pathway. In addition, the Bcl2/Bax ratio was lower in grafted compared with non-grafted tissue, while the increased level of caspase-3/7 and TUNEL positive signals also confirmed that apoptosis was more activated in the grafted than in the non-grafted groups, especially in vitrified grafts.

Any alteration in the expression levels of the studied genes and caspase-3/7 activity likely represents changes in ovarian stroma, not oocytes or follicles. Moreover, our TUNEL assay observation showed more positive apoptosis signals in follicles and stroma of both grafted-vitrified and non-vitrified samples.

This study provides the first evidence on the significant effect of vitrification on follicular apoptosis of grafted human ovarian tissue at mRNA level. The signs of follicular survival or degeneration detected by morphological assessment and caspase-3/7 activity were closely correlated to the changes in expression of apoptosis-related genes.

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