Human Ovarian Cryopreservation: Vitrification Versus Slow Freezing From Histology To Gene Expression.

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Research

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Abstract

Background:

Cryopreservation of ovarian tissue is one of the strategies offered to girls and women needing gonadotoxic treatment, as a means of preserving their fertility. There are two methods of ovarian tissue cryopreservation: slow freezing, the reference method, and vitrification, an alternative method. The aim of the present study was to evaluate which of the two is the best method for human ovarian tissue cryopreservation. Each ovary was divided into 3 groups: Fresh, Slow freezing and Vitrification. In each group a histological study to evaluate follicular density and quality; and an evaluation of 6 gene expression (*CYP11A, STAR, GDF9, ZP3, CDK2, CDKN1A)* were performed.

Results:

We observed no significant difference in follicular density within these 3 groups. Slow freezing altered the pool of primordial follicles compared to the Fresh tissue (31.8% *vs* 55.9%, p = 0.046, respectively). The expression of genes involved in steroidogenesis varied after cryopreservation compared to the fresh group; *CYP11A* was under-expressed in both freezing groups compared to the fresh group and significantly under-expressed in the slow freezing group (p = 0.01), *STAR* was over-expressed in the slow freezing group and significantly under-expressed in the vitrification group (p = 0.01). Regarding the expression of genes involved in cell cycle regulation, *CDKN1A* was significantly under-expressed in both freezing groups (slow freezing: p = 0.0008; vitrification: p = 0.03) compared to the fresh group.

Conclusion:

Vitrification had no effect on the histological quality of the follicles at any stage of development compared to Fresh tissue. There was no significant difference in gene expression between the two techniques.

Background

Ovarian tissue cryopreservation is a strategy to preserve fertility of young women suffering from cancer. Various treatments (chemotherapy, radiotherapy and surgery) can induce great damage to ovarian reserves (1) despite progress in oncology diagnosis and treatments. There are two methods for cryopreservation of ovarian tissue (OT); slow freezing and vitrification. Slow freezing is the reference method for cryopreservation of human OT, although it can reduce the ovarian follicular reserve and induce damage to stroma cells (2). Vitrification is a relatively new and alternative method; it has attracted attention due to apparent advantages over slow freezing, for the preservation of both the cells and stroma matrix as well as the survival of a vast pool of viable quiescent follicles that represents the ovarian reserve of the graft (3–9). Over the last decade, many studies have attempted to compare slow freezing and vitrification but results have been inconsistent. Some studies have reported the superiority of slow freezing (10–14), while others report no significant difference between the two methods (15–20). To our knowledge, these conflicting results appear to be due to conflicting methods employed to vitrify OT, as well as to differing evaluation criteria (follicular morphology, follicular vitality, apoptosis and gene expression). After literature review, we found a lack of information with regards to the impact of gene expression after cryopreservation. Our previous study highlighted that vitrification preserved follicular morphology better than slow freezing and led to genes being overexpressed, while slow freezing led to genes being under expressed (21).

The aim of the present study was to evaluate which of the two methods, slow freezing versus vitrification, is best for human ovarian tissue cryopreservation at histological and molecular levels.

Methods

Collection of ovaries

The use of human tissue for this study was approved by the Ethics Committee of *Hospices Civils de Lyon*. After obtaining informed consent, both oral and written, ovarian tissues were collected by hysterectomy and oophorectomy from 5 transsexual women (female to male) aged 23–40 years old, suffering from gender identity disorder at *Centre Hospitalier Lyon Sud.* Ovaries were immersed in Leibovitz L-15® (Eurobio, Couraboeuf, France) and transported to the laboratory at 10°C within 30 minutes. First, ovaries were cut in two hemi-ovaries using a scalpel in a Petri dish under sterile conditions. Then, the medulla was removed with a sterile chisel in order to obtain two hemi-cortexes. Finally, cortexes were cut into small pieces of 200mg ± 20mg, representing dimensions of approximately 5 mm (length) x 3 mm (width) 1 mm (thickness). The same ovary was cut into several pieces to be studied fresh (control tissue), after slow freezing and vitrification.

Slow freezing protocol and thawing procedure

OT was frozen according to the method described by our team that allowed live birth ewes (22)(23) which we have modified by 10% replacing fetal calf serum (FCS, Sigma-Aldrich, St Quentin Fallavier, France) with 10% serum substitute supplement (SSS, Irvine scientific, Santa Ana, USA). After

OT dissection, fragments were incubated in BM1 (Eurobio) and placed in 800 µL of freezing solution composed of 14.2% (2M) dimethyl sulfoxide (DMSO: Sigma-Aldrich), 10% SSS and BM1 within a sterile straw (CryoBioSystem, L'Aigle, France) for ten minutes at room temperature between 20 and 23°C, then subsequently freezing solution composed of 14.2% (2M) dimethyl sulfoxide (DMSO; Sigma-Aldrich), 10% SSS and BM1 within a sterile straw (CryoBioSystem, L'Aigle, France) for ten minutes at room temperature between 20 and 23°C then subsequently frozen with a semi-automatic self-seeding programmable freezer (Minicool 40 PC, Air Liquide Santé, France) held at 20°C. The cooling rate was – 2°C/min from 20°C to -35°C, at which point temperature nucleation was induced by semi-automatic seeding. The semi-automatic seeding was performed by the release of negative calories at -11°C. Then the temperature was lowered to -150°C at 25°C/min. When the temperature reached – 150°C, the straws were plunged in liquid nitrogen for storage. For thawing, straws were removed and immersed in a water bath at 37°C for one minute. OT were removed from the straw and placed in three successive thawing solutions composed of BM1 for five minutes at room temperature. Thawed OT were placed in 4% formaldehyde (VWR, Strasbourg, France) for investigation of morphology.

Vitrification protocol and warming procedure

OT were vitrified in a solution composed of DMSO, ethylene glycol (Sigma-Aldrich), SSS and sucrose (Dutscher, Brumath, France). The protocol was based on several vitrification protocols described in the literature (17, 37). Fragments were incubated in equilibration solution (BM1 containing 5.58% (1M) ethylene glycol, 3.55% (1M) DMSO 2.50% SSS and 0.125 M (sucrose) for five minutes at room temperature between 20 and 23°C. Then they were placed into a second bath (BM1 containing 11.16% (2M) ethylene glycol, 7.10% (1M) DMSO, 5.00 % SSS, and 0.25 M sucrose) for seven minutes at room temperature between 20 and 23°C, after which they were placed in the vitrification solution (BM1 containing 22.32% (4M) ethylene glycol, 14.20% (2M) DMSO, 10% SSS, and 0.5 M sucrose) at 4°C for ten minutes. OT were placed on a piece of semi-rigid 1 mm thick absorbent paper developed by our team, then cooled by direct contact with liquid nitrogen. For warming, OT were removed from straws and placed in a warming solution of 0.4 M sucrose for five minutes at room temperature, and then in sucrose-free BM1 for five minutes at room temperature. Warmed OT were placed into 4% formaldehyde for investigation of follicle morphology.

Histological evaluation

Five fresh, five frozen/thawed, and five vitrified/warmed OT were fixed in formaldehyde 4% for 24 hours at room temperature, paraffin embedded after dehydration, and cut into 4 µm serial sections. Ten sections were stained with hematoxylin (Millipore, Burlington, USA), eosin (Sigma-Aldrich) and safran (RAL Diagnostic, Martillac, France). The entire section was photographed to make a count and classification of the follicles using the Image J software. Then sections were checked by light microscopy to evaluate follicle morphology. The follicles were counted by two blind operators and were classified according to the description of reported by Gougeon and counted as altered or intact (Fig. 2). Follicles were classified as altered if there were at least one sign of oocyte or granulosa cell degeneration: the presence of pycnotic oocyte or follicular cell nuclei, detachment of the oocyte from surrounding granulosa cells, vacuolization of ooplasm, partially degenerated granulosa cells, or detachment of the basal membrane.

RNA extraction and complementary DNA synthesis for molecular assessment

Total RNA was extracted from fresh OT, frozen/thawed OT and vitrified/warmed OT using an RNeasy® Plus Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. We used the whole warmed tissue. Before the RNA samples were treated with DNase to remove any genomic DNA contamination prior to proceeding DNA synthesis. RNA was eluted twice in 30 µL of RNase free-water after extraction and consecutively collected in a final volume of 30 µL and was conserved at -80°C. The RNA concentration was determined by spectrophotometry; the ratio of the readings at 260 nm and 280 nm (A260/A280) provided an estimate of the purity of RNA. A total of 500 ng of the extracted RNA was used for cDNA synthesis using the High- Capacity RNA-to-cDNA® kit (Applied Biosystem, Waltham, Massachusetts, US). The cDNA synthesis was performed at 37°C for 60 minutes and stopped by heating to 95°C for five minutes. The obtained cDNA was stored at -20°C and prepared for real-time PCR analyses.

Real-time reverse transcription polymerase chain reaction

The primers for real-time RT-PCR were found by literature review (Table 1) or by our own design. Each primer was verified by using the University California, Santa Cruz (UCSC) Genome browser (https//genome.ucsc.edu) to check their specificity, target region, and size. Only verified primers were used for the PCR analyses. A total of six genes were analyzed: *CYP11A (Cytochrome P450 Family 11 Subfamily A Member 1), STAR (Steroidogenic Acute Regulatory Protein), GDF9 (Growth Differentiation Factor 9), ZP3 (Zona Pellucida Glycoprotein 3), CDK2 (Cyclin Dependant Kinase 2), and CDKN1A (Cyclin-Dependant Kinase Inhibitor 1A)*. We chose to evaluate *GDF9* and *ZP3* due to their dominant role in follicular development, in which they code for proteins produced by the oocyte. *CYP11A* and *STAR* encode proteins secreted by granulosa cells and are essential for the proper functioning of steroidogenesis. Finally, *CDK2* and *CDKN1A* are involved in cell-cycle regulation. *CDK2 is* involved in the control of the cell cycle; essential for meiosis, but non-essential for mitosis. *CDKN1A* encodes an inhibitory protein to regulate cell growth and cell response to DNA damage. One-step RT-PCR was performed using the StepOneplus real-time thermal cycler (Applied Biosystems) and using the Quantitect SYBR Green RT-PCR kit (Thermofisher, Waltham, USA). The reference gene was GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase). Prior to quantitative analysis, optimization procedures were performed by running real time RT-PCR with or without a template to verify the reaction condition, including the annealing temperatures of the primers and specific products. The real-time thermal conditions included a holding step at

50°C for two minutes and 95°C for two minutes, a cycling step at 95°C for 15 seconds, 60°C for 15 seconds, followed by a melt curve step 95°C for 15 seconds, 60°C for one minute and 95°C for 15 seconds. Each sample was analyzed in triplicate; water was used as negative control.

Table 1
Oligonucleotide primer sequences for PCR.

Accession number	Target gene	Primer sequence Forward (5'->3') : F Reverse (3'->5') : R
NM_002046	*GAPDH (*Wang *et al.* 2016*)*	R : GGATTTGGTCGTATTGGG F : GGAAGATGGTGATGGGATT
NM_005260	*GDF9 (*Wang *et al.* 2016*)*	F : CGTCCCAACAAATTCCTCCTT R : AGGCCAGCTCTGTCTCTCTCAT
NM_001110354	*ZP3 (*Wang *et al.* 2016*)*	F : GAGGCAGCCTCATGTCATG R : AGGCAAAGCCCACTGCTC
NM_000349	*STAR (Wang et al* 2016)	F : CCTGCTGTTCCCAACTGTG R : AGCCTCATCCCTGTTTTCTTG
NM_000781	*CYP11A (*Wang *et al.* 2016*)*	F : TGGAGTCGGTTTATGTCATCG R : GGCCACCCGGTCTTTCTT
NM_001798	*CDK2*	F : GGCCATCAAGCTAGCAGACT R : GAATCTCCAGGGAATAGGGC
NM_078467	*CDKN1A*	F : AGGTGGACCTGGAGACTCTCAG R : TCCTCTTGGAGAAGATCAGCCG

Statistical analysis

The analysis was performed with the R software (R Core Team 2014). In order to compare non-independent proportions a Generalized Linear Mixed Model (GLMM) was used. In this way, the ratios estimated by the GLMM were not a simple division of two values but the inverse of a logit function. However, the estimated ratios by the GLMM were near the ratios calculated by a simple division. Moreover, in order to take account of the overdispersion of the values, a quasibinomial distribution was used for the calculation and the test of the difference among the ratios. Thus, a precise analysis was performed, with ratios correctly calculated. To perform this study the so-called glmmPQL function of the MASS package was used. The Kruskal-Wallis non-parametric test was used to analyze the results of real-time RT-PCR data. A test was considered statistically significant when the p value was under 0.05.

Results

Follicular density

There was no significant difference observed in the initial follicular density within the three groups (fresh, slow freezing and vitrification). The percentage of primordial follicle was higher in the slow freezing and vitrification group compared to the fresh group (Table 2).

Table 2
Impact of cryopreservation protocol compared to fresh OT on follicular density: mean ± SD (follicles/mm^2)

	Follicles				
Techniques	Primordial	Intermediate	Primary	Secondary	Total
Fresh	15,5 ± 12.5	1,9 ± 3,1	4,3 ± 3,8	2,1 ± 1,2	21,6 ± 8,4
Slow freezing	35,8 ± 32,0	0,3 ± 0,7	5,1 ± 4,1	1,8 ± 2,5	42,0 ± 28,2
p(1)					0,063
Vitrification	30,6 ± 25,3	0,0 ± 0,0	3,5 ± 4,6	0,8 ± 1,7	34,1 ± 22,5
p(2)					0,375

p(1) p value for slow freezing *versus* fresh

p(2) p value for vitrification *versus* fresh.

Follicle morphology

We classified follicle quality using two conditions: intact or damaged at different stages of development: primordial, primary, intermediate and secondary follicle.

Slow freezing significantly altered the pool of primordial follicles compared to the fresh tissue (31.8% 55.9% p = 0.046 respectively) while this effect was not statistically significant after vitrification (48.8%, p = 0.508). The percentage of intact primordial follicle was higher but not significantly after vitrification compared to slow freezing (p = 0.129).

The analysis of intermediate follicles was not possible because of the low number of follicles in the slow-freezing group (0.3 follicles/mm2 ± 0.7). Moreover we did not observe intermediate follicles in the vitrification group.

There was no negative impact on the percentage of primary follicle after slow freezing and vitrification compared to the fresh group (slow freezing 27.6% p = 0.085; vitrification 89,05% p = 0.690). The percentage of intact primary follicle was higher in the vitrification group than slow freezing but not significant (p = 0.064).

The secondary follicle results were difficult to study because of the low number of follicles. We observed secondary follicles were more damaged after slow freezing compared to vitrification. (Table 3)

Table 3

Proportion of intact follicles according to cryopreservation technique: mean ± SD (follicles/mm^2)

	Primordial follicle			Primary follicle			Secondary follicle		
	Fresh	Slow freezing	Vitrification	Fresh	Slow freezing	Vitrification	Fresh	Slow freezing	Vitrification
Follicular density (mean ± SD)	15.45 ± 10.19	35.79 ± 32.01	30.59 ± 25.34	4.28 ± 5.74	5.10 ± 4.12	3.49 ± 4.63	2.14 ± 2.29	1.76 ± 2.50	0.75 ± 1.68
Density of intact follicles (mean ± SD)	7.13 ± 2.79	9.01 ± 5.86	13.96 ± 11.02	3.09 ± 3.81	1.50 ± 1.65	2.67 ± 2.96	1.66 ± 1.36	0.00 ± 0.00	0.75 ± 1.68
Proportion of intact follicles, %	55.90	31.72	48.81	82.02	27.6	89.05	96.51	0.00	100.00
[CI 95%]	[25.59;70.52]	[14.47;43.79]	[29.82;64.64]	[32.76 ;97.71]	[6.59 ; 67.31]	[38.35;99.07]	[77.88; 99.54]	[0.0 ; 0.0]	[100;100]
p	-	0.0464$^{(1)}$	0.5083$^{(2)}$	-	0.0847$^{(1)}$	0.6900$^{(2)}$	-	0.0002$^{(1)}$	0.0177$^{(2)}$

SD: standard deviation; CI: Confidence interval

p$^{(1)}$ p value for slow freezing *versus* fresh

p$^{(2)}$ p value for vitrification *versus* fresh

Gene expression

Concerning the genes expressed by the granulosa cells, the mean fold change ± SEM fold change of *CYP11A* was 0.53 ± 0.18 in frozen OT and 0.46 ± 0.50 in vitrified OT; the change after slow freezing was significantly lower (p = 0.01) and there was no significant difference between vitrified OT and fresh tissue (p = 0.12). The mean ± SEM fold change of *STAR* mRNA was 2.25 ± 3.41 in frozen OT and this was 0.38 ± 0.22 in vitrified OT; there was no significant difference between frozen OT and fresh tissue (p = 0.47) and the change after vitrification was significantly lower (p = 0.01). Concerning the genes expressed by the oocytes, the mean fold change ± SEM of *GDF9* was 1.81 ± 1.90 in frozen OT and this was 1.47 ± 1.43 in vitrified OT; there was no significant difference between cryopreserved OT and fresh tissue (p = 0.39 and p = 0.50). The mean ± SEM fold change of ZP3 mRNA was 1.47 ± 0.73 in frozen OT and this was 1.82 ± 2.55 in vitrified OT; there was no significant difference between cryopreserved OT and fresh tissue (p = 0.28 and p = 0.51). Concerning cell cycle gene, the mean fold change ± SEM of *CDK2* was 1.38 ± 1.53 in frozen OT and this was 1.41 ± 2.01; there was no significant difference between cryopreserved OT and fresh tissue (p = 0.60 and p = 0.67). The mean ± SEM fold change of *CDKN1A* mRNA was 0.45 ± 0.13 frozen OT and this was 0.58 ± 0.29 in vitrified OT; the change after slow freezing and vitrification were significantly lower (p = 0.0008 and p = 0.03), as per Fig. 1.

Discussion

Vitrification is a simple alternative method for cryopreservation of ovarian tissue. In our study we worked on human ovaries from transsexual women. This model has also been used in the literature (22–24). The neutrality of androgenic treatments on follicular and tissue quality has been reported (25, 26). Few studies have studied women without prior androgenic treatment (27–29). We have shown a greater alteration of the primordial follicles after slow freezing compared to fresh tissue. Our study showed slow freezing would seem to be affected the normal morphology of primary and secondary follicle. Vitrification had no effect on the histological quality of the follicles on the human ovary at any stage of development compared to the fresh tissue which is in agreement with previous studies (3, 4, 22, 23, 28–30). This data is reassuring, but follicular histology is not sufficient to define the effectiveness of the technique. Indeed, some studies demonstrated that OT vitrification affect the normal morphology of oocytes follicles (29). It would be interesting to evaluate follicular vitality and apoptosis after thawing and after *in vitro* culture. Abdollali *et al.* 2013 reported the absence of apoptosis at the histological and molecular level after vitrification compared to the fresh tissue (28). In our study, stromal tissue was not evaluated and may be the key to longer graft survival. Moreover, it plays an important role in follicular development (31). We also found a decrease in granulosa cell gene expression after slow freezing and vitrification. In Wang's study the results are similar: they observed a decrease in CYP11A and STAR expression after vitrification and slow freezing (29). In histology, the anomalies observed were not only granulosa damage. It is difficult to link these expression abnormalities to histological abnormalities. The impact of these changes on gene expression is difficult to predict. The expression of CYP11A allows the production of pregnenolone by enzymatic cleavage of the cholesterol side chain. Similarly, STAR expression plays a role in the production of pregnenolone by controlling the entry of cholesterol into the mitochondria. Pregnenolone assay could be considered to evaluate the impact of these cryopreservation techniques.

We also observed a decrease in the expression of the CDKN1A gene, a gene that inhibits cell cycle initiation. This gene was under expressed by both cryopreservation techniques. To our knowledge, no studies have evaluated the expression of cell cycle genes after vitrification and slow freezing on human ovarian tissue. In the bovine model, CDKN1A expression was decreased after cryopreservation techniques (32). Under-expression can trigger the cell cycle. An initiation of primordial follicles into growth follicles after tissue transplantation is reported in the literature, which is secondary to an interruption of the PI3K/Akt/mTOR when dissecting ovarian tissue (33–35). This pathway plays a role in the cell cycle (35, 36). The CDKN1A gene is not reported as a target for this signaling pathway. Its change in expression may also be an explanation for this entry into reserve follicle growth. Functional analyses are required to complete these results. Oocyte gene expression was not altered after cryopreservation techniques. This result was similar in the ovine model. The absence of effect of slow freezing and vitrification on oocyte genes is rather reassuring. Wang *et al*. observed a decrease in ZP3 expression after cryopreservation techniques (29). Our study only focused on two genes, which is not enough to know the real impact on the oocyte.

Conclusion

This initial work highlights the absence of deleterious effects of vitrification compared to slow freezing on human ovarian tissue just after warming. The long-term effect with functional studies remains to be defined and will be the subject of future work. Two authors studied the effect of vitrification after *in vitro* tissue culture with histological and molecular analysis. They observed an over-expression of FSH and GDF9 genes, which reflect the quantity of primary and secondary follicles (22, 23) and under-expression of FIGLA and KIT-L which reflect the quantity of primary follicles (24). These results show a resumption of folliculogenesis after vitrification of the ovarian tissue. Vitrification of human ovarian tissue is equivalent to slow freezing.

Abbreviations

CYP11A
Cytochrome P450 Family 11 Subfamily A Member 1
STAR
Steroidogenic Acute Regulatory Protein
GDF9
Growth Differentiation Factor 9
ZP3
Zona Pellucida Glycoprotein 3
CDK2
Cyclin Dependant Kinase 2
CDKN1A
Cyclin-Dependant Kinase Inhibitor 1A
OT
ovarian tissue
SEM
standard error of measurement
mRNA
messenger RiboNucleic Acid
PI3K/Akt/mTOR
phosphatidylinositol 3-kinase signaling pathway
FCS
Fetal Calf Serum
SSS
serum substitute supplement
DMSO
dimethyl sulfoxide
DNase
desoxyribonuclease
RT-PCR
reverse transcription polymerase chain reaction

Declarations

Ethics approval and consent to participate: The study was conducted after approval from the local ethics committee (*Hospices Civils de Lyon – reference number: 19-138*). All the procedures adhered to relevant ethical regulations.

Consent for publication: Not applicable

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: Participation in design (P.J., C.F., E.L., C.S., N.MJ.), data analysis (P.J., E.L., M.B., J.L.), execution and manuscript drafting (P.J., E.L.) and critical discussion (E.L., E.F., B.S., J.L.).

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Figures

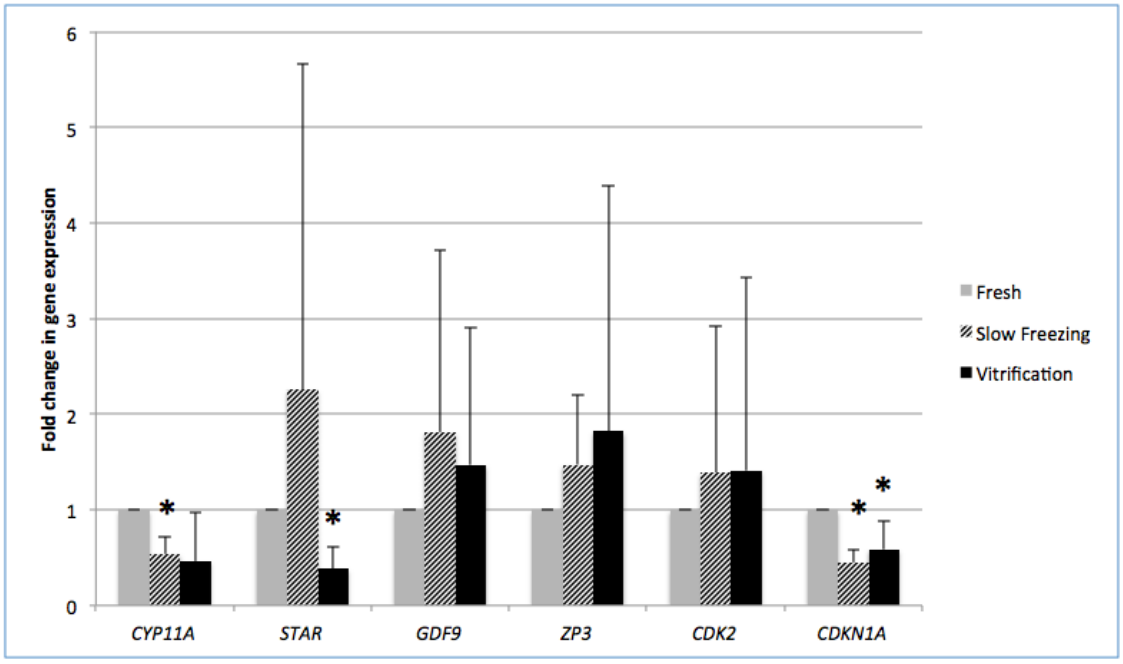

Figure 1

Change in gene expression as measured by mRNA extraction from ovarian tissue and PCR analysis. The value of the expression of each gene in fresh tissue was set at 1; results are presented as means ± SEM; *p<0.05 with respect to fresh OT. CYP11A, Cytochrome P450 Family 11 Subfamily A member 1; STAR, Steroidogenic Acute Regulatory Protein; GDF9, Growth Differentiation Factor 9; ZP3 Zona Pellucida Glycoprotein 3, CDK2, Cyclin Dependant Kinase 2, CDKN1A, Cyclin Dependant Kinase Inhibitor 1A.

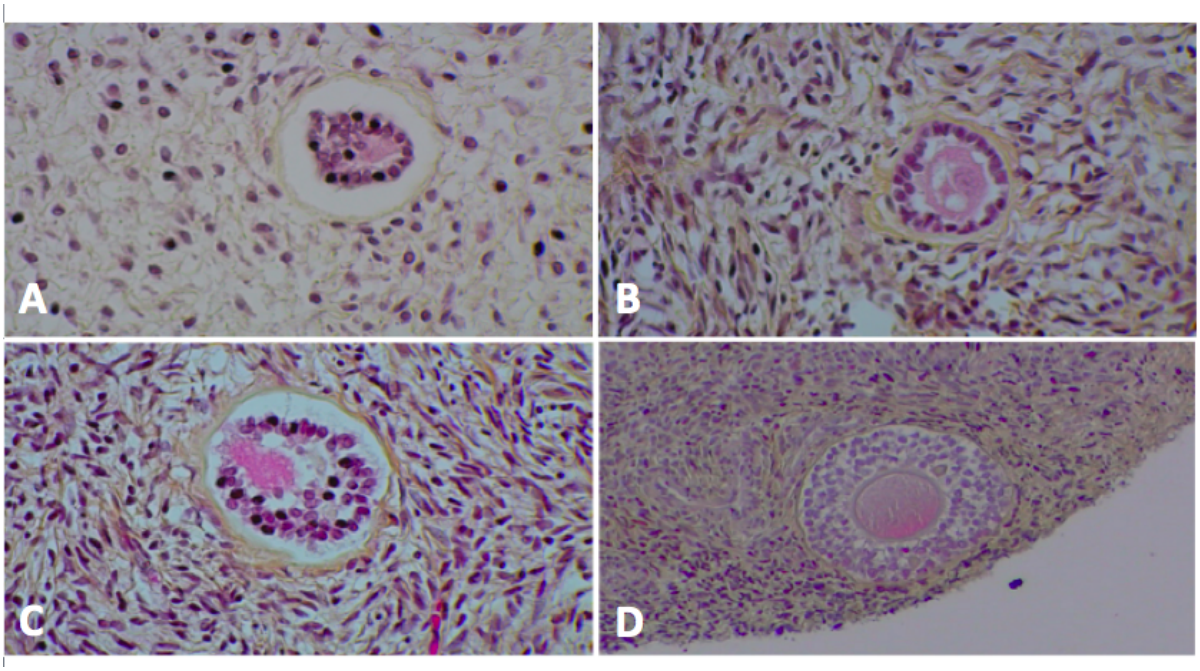

Figure 2

Light microscopic images of human ovarian cortical tissue after hematoxylin, eosin and safran (HES) staining. A and B: altered primary follicles (magnification 400); C: altered secondary follicle (magnification 400); D: intact secondary follicle (magnification 100).