Molecular profiling of human oocytes after vitrification strongly suggests that they are biologically comparable with freshly isolated gametes

To assess the effects of vitrification on the biomolecular profile of oocytes, we analyzed through real-time reverse transcriptase–polymerase chain reaction eight genes encoding critically important proteins for embryo development and compared this partial transcriptome with that of freshly collected gametes isolated from the same women. The comparison of the molecular profiles demonstrated that our vitrification protocol does not alter the biomolecular quality of oocytes: in fact, between the two groups we found the absence of statistically significant variations. Accordingly, this cryopreservation technique might be helpful in preserving women's fertility. (Fertil Steril® 2010;94:2804–7. ©2010 by American Society for Reproductive Medicine.)

Key Words: Human oocytes, gene expression, molecular markers, vitrification, real time RT-PCR

Oocyte cryopreservation is a helpful fertility preservation technique for women at risk of losing their ovarian functions after disease, surgery, or chemotherapy (1). Moreover, avoiding embryo cryopreservation would solve religious, ethical, and legal problems, connected to the laws that actually regulate medically assisted reproduction in various countries. There are two major techniques for cryopreservation: slow freezing and vitrification (2, 3). Many published studies have compared frozen-thawed human oocytes, after either slow freezing or vitrification, with fresh collected ones, and they have analyzed their biologic behavior (e.g., survival, fertilization, early embryonic development), as well as more specific structural cellular features (e.g., meiotic spindle assembly, chromosome alignment) (4–11). However, there are no published data on the molecular profile of oocytes after cryopreservation.

To assess the effects of vitrification on the biologic quality of oocytes, we compared the expression profile of messenger RNAs in single vitrificated-thawed oocytes with that of fresh collected oocytes without cryopreservation. In this article, we report the expression analysis of eight different genes: three perform housekeeping functions, because they encode proteins involved in the basic cellular functions and are expressed constantly in all human cells (12) (hypoxanthine phosphoribosyltransferase [HPRT], glyceraldehyde-3-phosphate dehydrogenase [GAPDH], and peptidylprolyl isomerase A [cyclophilin A, CYCLOPHILIN]); the other five genes encode proteins essential for oocyte development and specific functions (bone morphogenetic protein 15 [BMP15], growth differentiation factor 9 [GDF9], folliculogenesis-specific basic helix-loop-helix [FIGLA], POU class 5 homeobox 1 [POU5f1-OCT4], and TATA box binding protein [TBP]–associated factor 4B [TAF4B]). GDF9 and BMP15 (also known as GDF9b) are two closely related oocyte-specific growth factors, members of the transforming growth factor–β (TGFβ) superfamily, that are expressed in oocytes throughout most of folliculogenesis. Both GDF9 and BMP15 are involved in specific functions of granulosa and cumulus cells (i.e., proliferation, cumulus expansion, apoptosis) (13–15). FIGLA, OCT4, and TAF4B are transcription factors preferentially expressed in germ cells. FIGLA (factor in the germline), expressed exclusively in germ cells, is a critical transcription factor during the early steps of

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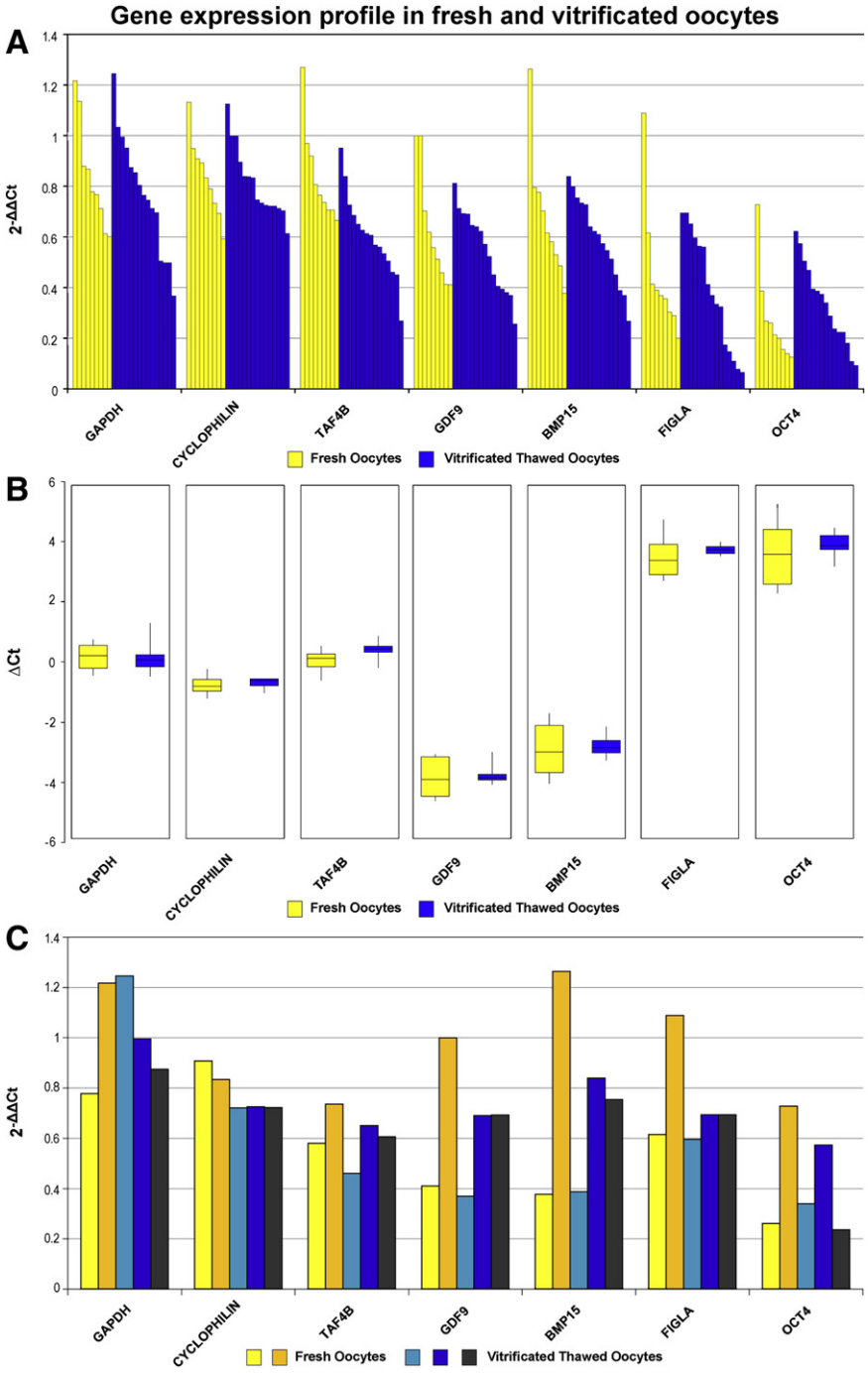
FIGURE 1

Gene expression profile in fresh and vitrificated oocytes. (**A**) The histogram shows the expression profiles of analyzed genes in 9 fresh (*yellow*) and 15 vitrificated oocytes (*blue*) estimated with use of the $2^{-\Delta\Delta Ct}$ method. We used hypoxanthine phosphoribosyltransferase as reference gene and the same fresh oocyte as calibrator. (**B**) Box and whisker plot of ΔCt means of the analyzed genes in fresh and thawed oocytes after vitrification. It provides a simple description of a distribution of values by depicting the 25th and 75th percentile values as the bottom and top of a box, respectively. The Y axis represents the ΔCt values. The median expression values of fresh and vitrificated thawed oocytes are marked by *horizontal lines* in the boxes. (**C**) Expression profile of analyzed genes in five oocytes collected from a woman during the same ovarian stimulation protocol. Normalization has been performed by $2^{-\Delta\Delta Ct}$ method with use of hypoxanthine phosphoribosyltransferase as reference gene and the same fresh oocyte as calibrator. Statistical analysis of data demonstrates that the expression profiles of the analyzed genes are not significantly different ($P<.01$).



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folliculogenesis. It regulates transcription of zona pellucida (Zp) genes through an E-box motif (CANNTG); in FIGLA-knockout mice, Zp1, Zp 2, Zp3 expression is absent (16, 17). OCT4 is expressed at high levels in mature oocytes. It plays an important role in embryonic development, especially during early embryogenesis, and it is necessary for embryonic stem cell pluripotency (18, 19). TAF4B, a general transcription factor expressed at high levels in mature oocytes, seems to be an important regulatory factor in mammalian female gametogenesis (20, 21). It has been demonstrated that TAF4B-null female mice are infertile, with defects in early follicle formation, oocyte maturation, and zygotic cleavage. A recent article has shown that TAF4B-deficient female mice exhibit premature reproductive senescence (22). On the basis of the data reported earlier in this article, the transcripts chosen for our analysis encode proteins that are essential for mature oocytes, because of their role in gamete viability and in follicular and embryo development: accordingly, they represent excellent molecular markers of oocyte quality (23).

Human oocytes were collected from an IVF center (Servizio di PMA–Azienda Ospedaliera Cannizzaro, Catania, Italy), after informed consent for the use of supernumerary ones. A total of 25 metaphase II oocytes were collected from five different women, whose primary infertility was due to a male factor: this excluded pathologies that could influence oocyte quality (e.g., endometriosis, ovarian insufficiency, polycystic ovaries). For each patient, we collected two fresh and three vitrificated oocytes, choosing those with optimal morphology, for a total of 10 fresh and 15 vitrificated oocytes. Recovery, culture, and cell preparation were performed with use of a previously published protocol (21).

For the vitrification and warming procedure, we used the Irvine Vitrification Freeze Kit (Vit Kit-Freeze; Vit Kit-Thaw , Santa Ana, CA) including two vitrification and three warming solutions. The two vitrification solutions have to be used in sequence and comprise the equilibration solution, a HEPES-buffered solution of Medium-199 containing gentamicin sulfate (35 μg/mL), 7.5% (vol/vol) each of dimethyl sulfoxide and ethylene glycol, and 20% (vol/vol) dextran serum supplement; and the vitrification solution, a HEPES-buffered solution of Medium-199 containing gentamicin sulfate (35 μg/mL), 15% (vol/vol) each of dimethyl sulfoxide and ethylene glycol, 20% (vol/vol) dextran serum supplement, and 0.5 mol/L sucrose. The three warming solutions include the thawing solution, a HEPES-buffered solution of Medium-199 containing gentamicin sulfate (35 μg/mL), 1.0 mol/L sucrose, and 20% (vol/vol) dextran serum supplement; the dilution solution, a HEPES-buffered solution of Medium-199 containing gentamicin sulfate (35 μg/mL), 0.5 mol/L sucrose, and 20% (vol/vol) dextran serum supplement; and the washing solution, a HEPES-buffered solution of Medium-199 containing gentamicin sulfate (35 μg/mL) and 20% (vol/vol) dextran serum supplement. Vitrification procedure was performed at 23°C to 25°C (room temperature). After three oocytes were transferred with a minimal volume of medium from the culture dish (37°C–5% CO_2) into the 20-μL drop of culture medium (Gamete; Vitrolife, Göteborg, Sweden) for 1 minute, the drop was merged first to 20-μL equilibration solution drop with the tip of the transfer pipette; spontaneous mixing of the two solutions was allowed to occur for 2 minutes; subsequently, a second 20-μL drop of equilibration solution was merged to the previously merged drops and left for 2 minutes; then, the oocytes were transferred with minimal volume of solution to the bottom of a third 20-μL equilibration solution drop for 3 minutes.

After a quick incubation in three subsequent 20-μL drops of vitrification solution for 5 seconds, 5 seconds, and 10 seconds each, the oocytes were aspirated in a small volume of vitrification solution and immediately loaded onto a high-security vitrification kit (HSV; CryoBioSystem, L'Aigle, France) on the distal part of the capillary gutter. The capillary was inserted immediately into the HSV straw and sealed with use of a thermal sealer (CryoBioSystem). In <1 minute from the start of exposure to the vitrification solution, the sample was vitrified by plunging the HSV in the liquid nitrogen. Warming was performed at room temperature.

The device was cut, keeping the area containing the oocytes immersed in the liquid nitrogen. The capillary was extracted and immediately plunged into the first thawing solution, in which the oocytes were incubated for 1 minute and then transferred into a second 20-μL thawing solution drop for another minute. Then the oocytes were incubated in two subsequent 20-μL drops of dilution solution for 2 minutes each and in three subsequent 20-μL drops of washing solution for 2 minutes each. Single oocytes were rinsed several times in ribonuclease-free H_2O (molecular biology quality) to remove any trace of proteins from the culture medium; then they were placed in polymerase chain reaction tubes in 2 μL ribonuclease-free H_2O (molecular biology quality) and frozen in liquid nitrogen to avoid RNA degradation.

For RNA extraction and complementary DNA synthesis, we used a previously published protocol (21). Real-time reverse transcriptase–polymerase chain reaction primers were designed with use of Primer Express version 3.0 (Applied Biosystems, Foster City, CA). Real-time reverse transcriptase–polymerase chain reaction was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems).

As a first step for data analysis, we performed the relative quantification of our results through the $2^{-\Delta\Delta Ct}$ method by using HPRT as reference gene (24, 25) and a single fresh oocyte as calibrator. Our data clearly show that the expression profile of the genes, chosen as biomarkers, did not change between fresh and vitrificated oocytes ($0.3 < 2^{-\Delta\Delta Ct} < 1.3$) (Fig. 1A). To validate our results statistically, we compared the Ct values, normalized to HPRT (ΔCt), in fresh and vitrificated oocytes. Even though some were collected from the same woman, we consider each oocyte as an independent sample: this is due to the specific differentiation history even of gametes from a single individual. In fact, each gamete may undergo specific genomic mutations or specific epigenetic DNA modifications and show an evident level of molecular diversity, leading to differences in oocyte competence, fertilization grade, and pregnancy rate. Accordingly, we used the independent Student's *t*-test and demonstrated that there are no significant variations between fresh and vitrificated-thawed oocytes ($P<.01$) (Fig. 1B). This was further confirmed by comparing five different oocytes (two fresh and three vitrificated), collected from a woman during the same ovarian stimulation protocol (Fig. 1C).

This article proves for the first time that the vitrification protocol keeps unaltered the oocyte molecular profile and does not cause messenger RNA degradation, as normally found in poor-quality oocytes. Our molecular data, together with published results on oocyte survival, oocyte fertilization, and pregnancy rates, confirm that vitrification might be very helpful for preserving women's fertility (4–11).

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