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Original article

Developmental competence and apoptotic gene expression patterns of mature and immature human oocytes retrieved from controlled ovarian stimulation cycles

Farzaneh Fesahat[a,b], Mohammad Hasan Sheikhha[a,b], Seyed Mehdi Kalantar[a,b], Nasim Tabibnejad[b], Razieh Dehghani Firouzabadi[b], Hojjatollah saeedi[c], Mohammad Ali Khalili[b,*]

[a] Department of Genetic , Shahid Sadoughi University of Medical Sciences, Yazd,Iran
[b] Research and Clinical Center for Infertility, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[c] Department of Biology, Faculty of Science, University of Isfahan, Isfahan, Iran

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ABSTRACT

The purpose was to assess the developmental competence of the in vitro or in vivo matured human oocytes as well as the apoptotic genes expression of cumulus cells (CCs) regarding nuclear maturity status of associated oocytes retrieved from stimulated ICSI cycles. A total of 590 oocytes and the associated CCs were retrieved and divided into groups of test and control according to the nuclear maturity status in order to the developmental evaluation as well as expression patterns of apoptosis-related genes using real time PCR. The fertilization and embryo formation rates were 60.3% and 87.5% vs.69.1% and 92.8% in test and control groups, respectively. Good quality embryos on day 3 were 62.2% in test and 69.1% in control groups. There were significant differences in the rates of normal fertilized as well as unfertilized oocytes between the groups. Also, mRNA levels of some apoptotic genes were significantly higher in the CCs obtained from immature oocytes among patients with premature ovarian factors (POF) rather than other infertility etiologies ($p < 0.001$). The data demonstrated the developmental competence of in vitro matured oocytes −even to good quality cleavage embryos- is not completely consistent with molecular integrity and well-mannered gene expression patterns resulting to ICSI success. It seems that using immature oocytes could be helpful for patients at risk of ovarian hyperstimulation syndrome (OHSS) as the same as patients with diminished ovarian reserve.

1. Introduction

In vitro maturation (IVM) could be a good choice for patients who are hormone sensitive and have few oocytes in stimulated cycles [1]. Furthermore, IVM would broaden the potential of infertile population independent of age, reproductive maturity, or pregnancy status [2]. It has been shown that almost 15% of the retrieved oocytes in assisted reproductive technology (ART) cycles are immature [2,3]. Despite the advantages, IVM remains underutilized due to the decreased developmental competence of IVM oocytes in comparison to the in vivo mature oocytes obtained for in vitro fertilization (IVF) [4]. Actually, the precise molecular mechanisms involved in the cytoplasmic and nuclear maturations have not been fully understood. Recently, a study on poly cystic ovarian syndrome (PCOS) patients demonstrated that the clinical pregnancy rate was significantly lower in IVM group (51.3%) in comparison to the IVF treatment group (63.5%) [1].

In another point of view, the implantation potential of in vitro generated human embryos still remains low. One reason is that the morphological selection of the embryos,as the usual embryo selection technique, is not always a qualitative method for ART success [5,6]. Oocyte maturation is in turn promoted by communication with surrounding cumulus cells (CCs) and measurement of the genes involved in this mechanism provides non-invasive biomarkers of oocyte developmental competence [7,8].Different factors such as changes in metabolic necessities, cell cycle progression and apoptosis processes can alter regulation of gene expression [9].

Apoptosis is an underlying process in oocyte degeneration and embryo fragmentation [10]. It has been demonstrated that several pro- and anti-apoptotic genes are involved in the survival of follicles as well as their roles in atresia and follicular apoptosis [11–13]. Assou and associates using oligonucleotide microarrays compared wide gene expression of pooled immature and mature oocytes with associated CCs from patients who underwent IVF. They reported that *BCL2-like 10* and *BIRC5* are involved in apoptosis may be more up-regulated genes either

* Corresponding author.
E-mail address: khalili59@hotmail.com (M.A. Khalili).

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in mature or immature oocytes compare to CCs [8].

Previous studies have reported that immature oocytes at the time of oocyte retrieval are discarded due to their reduced potential for creation of good quality embryo under routine culture conditions [14]. Thus, these oocytes are useful for studies aimed at elucidating the mechanisms of oocytes IVM and might finally contribute to the pool of embryos available for embryo transfer [15]. One study reported that CCs isolated from different oocyte stages; present different expression profile. [16]. However, to our knowledge, there was no report on apoptotic gene pattern at different stages of human oocytes. Therefore, our purpose was to assess the developmental competence of the in vitro or in vivo matured oocytes as well as the apoptotic genes expression of CCs regarding nuclear maturity status of associated oocytes retrieved from stimulated ICSI cycles.

2. Material and methods

2.1. Participants

The patients referred to Yazd Reproductive Sciences Institute entered into this prospective randomized study. This investigation was conducted from March 2015 to February 2017. The patients were randomly divided into two groups by computer generated random numbers: control group (N = 60) were collected from in vivo matured oocytes, and the test group (N = 60) consisted of normal in-vivo immature oocytes (at least one or more) at germinal vesicle (GV) stage. The exclusion criteria was patients with previous IVF failure, non-ejaculated spermatozoa, cycles using donor gametes, advanced maternal age (37 years or older) as well as those with any history of genetic disorders or chromosomal aneuploidy. In addition, patients with severe endocrine disorders, and severe male factor infertility were excluded. The inclusion criteria for both groups were: oocytes with normal morphology, normal ejaculates according to WHO criteria [17] and women $^{<}$37 years old without the history of recurrent pregnancy loss and genetic disorders. The Ethics Committee of Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran approved this study and we have confirmed that all experiments involving human participants were performed in accordance with the 1964 Helsinki declaration and relevant guidelines. Written informed consents were obtained from all patients who participated in our research study.

2.2. Sample collection

The gonadotropin-releasing hormone (GnRH) antagonist stimulation protocol were used for all participants [18]. Transvaginal oocyte aspiration was performed 36 h after the oocyte triggering. Cumulus oocyte complex (COCs) were removed from the collection fluid using a sterile pipette, washed two times with G-Mops-V1 (Vitrolife), and placed in G-IVF Plus (Vitrolife Co., Switzerland) in a 6% CO2 incubator (37° C) for 3 h [19]. After enzymatic and mechanical removal of CCs with 80 IU/ml hyaluronidase (Sigma Co, USA), denuded oocytes were assessed by the presence of the first polar body (PBI) under an inverted microscope (Nikon Co, Japan), normally considered to be a marker of oocyte nuclear maturity (completing meiosis I) [20]. Then, they were washed twice with G-Mops-V1 (Vitrolife) and placed in either Sage IVM medium supplemented with 75 IU/L of human menopausal gonadotropin (for immature oocytes) or G-IVF Plus (Vitrolife Co., Switzerland) for (MII oocytes) at 37 ˚C in 5% O_2, 6% CO_2 and 90% air with high humidity under paraffin oil (MediCult). Also, associated CCs of each patient were pooled based on maturity status of denuded oocytes and collected in separated numbered sterile 1.5 mL microtubes (Eppendorf) as test or control group. Then the cells were washed in PBS twice, centrifuge in 5000 g for 1 min. Finally, the pellet was stored at −80 ˚C using appropriate volume of RNA later RNA Stabilization Reagent (Qiagen, Europe) for further analysis.

2.3. Rescue IVM procedure

The selected immature oocytes at GV stage were cultured in 25 μL drops of blastocyst medium supplemented with 75 IU/L of human menopausal gonadotropin (IVF-M, LG Life Sciences, Jeonbuk-do, Korea), cultured for 24 h at 37 ˚C in 5% O2, 6% CO2 and 90% air with high humidity under paraffin oil (MediCult) and without medium renewal. In order to prepare for ICSI insemination, the oocytes were assessed for maturity after 24 h by presence of the PB1 under an inverted microscope (Nikon Co, Japan) [21].

2.4. Fertilization and embryo culture

The fertilization and embryo formation rates were determined and compared between groups. Fertilization rate was defined as 2PNs divided by MII oocytes and embryo formation rate was defined as formed embryos divided by 2PNs. After ICSI, oocytes were cultured in 20 μL drops of cleavage medium (Cook Medical) covered with paraffin oil. Fertilization was checked 16–18 h after ICSI. Then, fertilized oocytes were cultured in cleavage medium for 72 h and embryo development were assessed according to grading [22].

2.5. Extraction of total RNA and first strand cDNA synthesis

To reach this purpose, we used QuantiTect®, RNeasy Micro kit (Qiagen, Europe) for purification of total RNA that is applicable for small samples according to the manufacturer's guide. The RNA concentration was determined by NanoDrop spectrophotometer and adjusted to a concentration of 1000 ng/μl. Then cDNA was synthesized using RevertAid First Strand cDNA synthesis kit (Thermo Fisher scientific Inc.) in the same day according to the manufacturer's guide. The reverse transcription was performed in 20 μL reactions for 60 min at 42 ˚C, followed by 70 ˚C for 5 min to inactivate the reverse transcriptase. The reverse transcription reaction product was directly used in quantitative PCR (qPCR) in a separate step to amplify the targets.

2.6. Real time polymerase chain reaction

Using specified primers (Table 1), relative expressions of some apoptotic genes (*Bcl2*, *Bax*, *Caspase 8, Caspase 3, p53,* and *BIRC5*) were evaluated by quantitative real time PCR (qRT-PCR). *GAPDH* gene was used as an internal control. The PCR run was carried out according to QuantiTect SYBR Green RT-PCR kit (Applied Biosystems, UK, Lot no:1201416) on an ABI 7500 RT-PCR system (Applied Biosystems) using the following program: stage 1: 95 ˚C for 10 min, stage 2: 95 ˚C for 10s, 58 ˚C for 20 s, 72 ˚C for 30 s for a total of 40 cycles, and it was continued by a melt curve step at 95 ˚C for 15 s, 58 ˚C for 1 min, and 95 ˚C for 15 s. All samples were run in duplicate to reduce the sampling error and the mean value of each duplicate was used for all further calculations. Reverse transcriptase minus samples and no template controls were run together with main samples. Verification of Amplicon specificity and size were performed by 2% agarose gel and product length as well as by a melting curve analysis. The output data were transferred to Microsoft Excel for analysis. The relative expression ratios were calculated by a mathematical model, which included an efficiency correction for real time PCR efficiency of the individual transcripts [23] as follows: Ratio = (E target) ΔCt target (control-sample)/ (E ref) ΔCt ref (control-sample). The relative expression ratio of a target gene was determined from the real time PCR efficiency (E) and the threshold cycle difference for an unknown sample versus a control (Ct control-sample). For each gene, cDNA dilution curves were generated and used to calculate the individual real time PCR efficiencies [E = 10(-1/slope)]. The geometric mean of the two internal reference genes was used to correct the raw values for the genes of interest.