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TECHNICAL INNOVATIONS

Procedure for rapid oocyte selection based on quantitative analysis of cumulus cell gene expression

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Abstract
Purpose To develop a procedure for the analysis of gene expression in cumulus cells during the interval between ovum pick up and insemination to select the best oocytes for fertilization.
Methods Five RNA extraction methods, three reverse transcription procedures followed by Real-time quantitative PCR and one single-step mRNA quantification kit were tested to measure the expression of five genes in cumulus cells.
Results Two RNA extraction kits gave the best combination of efficiency and purity. One reverse transcription procedure gave the best speed and efficiency. The single-step kit required more biological material than would be available from single cumulus oocyte complexes (COCs).
Conclusions Our test identified a combination of RNA extraction and reverse transcription procedures that enables the level measurement of 5 selected cumulus cell transcripts within 4 h. Using this combination it was possible to obtain a reliable quantification of gene expression in 44 out of 46 individual COCs collected from seven patients.

Keyword Marker genes · Oocyte selection · Prospective study · Real-time PCR

Capsule Development of a procedure for oocyte selection during the interval between ovum pick-up and insemination, based on gene expression levels in cumulus cells.

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Introduction

Non invasive embryo selection is used to optimize the number of embryos to be transferred into the uterus in order to achieve the best balance between reducing the risk of multiple gestation and maximizing the probability of pregnancy. Current selection methods are based mainly on morphological and developmental criteria performed during in vitro development [1]. However, it is generally acknowledged that the reliability of such methods for predicting embryo viability could be improved by combining the evaluation of different parameters at successive time points [1, 2].

In this respect the analysis of cumulus cells is a good candidate for providing such supplementary parameters. These cells, in fact, form a functional interconnection with the oocyte during follicular development and ovulation thanks to a gap junction network that links the somatic compartment to the gamete [3, 4]. Cumulus expansion, in particular, is normally associated with oocyte development, ovulation and fertilization [5–7] whereas oocytes, whose cumulus does not expand, have limited potential for implantation [8]. Growth and differentiation factor 9 (GDF9), a member of the transforming growth factor-β

(TGF-β) superfamily, was the first oocyte-specific gene shown to cause cumulus expansion [6]. GDF9 regulates several key granulosa cell enzymes involved in cumulus cells expansion and promotes the acquisition of oocyte developmental competence acting as a paracrine factor [4]. Recent data clearly indicate that the expression of GDF9 downstream target genes in the cumulus cells reflects GDF9 activity and predicts oocyte health as well as the grade of the resulting embryos [9–11].

In a previous work [9] we performed a retrospective study determining a positive relationship between the expression of two GDF9 target genes, namely HAS2 (Hyaluronic Acid Synthase 2) and Grem1 (Gremlin1), in cumulus cells and in vitro development of fertilized oocytes.

However when it is desirable that only a portion of the retrieved oocytes is fertilized it would be necessary to perform a prospective evaluation. This requires to measure the gene expression levels within the 3-4 h interval separating oocyte collection and insemination. Moreover only a portion of the cumulus can be removed for analysis so that the damage that would result from leaving the oocyte completely denuded during the whole period is kept to a minimum.

Aim of the present study was to develop a procedure for RNA extraction, reverse transcription and quantitative amplification that could be reliably performed within the required limits of time and cell number and would not compromise oocyte viability.

Material and methods

Patients and treatments

The local Institutional Review Board approved the study and all recruited patients signed an informed consent. Oocytes were obtained following controlled ovarian hyperstimulation using a long protocol with gonadotropin-releasing hormone agonist, triptoreline (Decapeptyl; Ipsen S.p.A., Milan, Italy), or a protocol using gonadotropin-releasing hormone antagonist, cetrorelix (Cetrotide; Merck Serono, Milan, Italy), depending on the clinical situation. In the first case, triptoreline (0.1 mg per day) was administered for at least 14 days for pituitary depression before gonadotropin stimulation. For the second type of stimulation protocol, cetrorelix (0.25 mg per day) was given by daily injection as soon as one or more follicles ≥14 mm were seen on transvaginal ultrasound during ovarian stimulation protocol. In both cases, based on patient's characteristics and history, recombinant follicle-stimulating hormone (r-hFSH; GONAL-f 75; Merck Serono, Milan, Italy) was initiated on cycle day 3 of the menstrual cycle onwards by once-daily injection, at a dose determined by the physician to achieve ovarian hyperstimulation; follicular development was monitored via ovarian ultrasonography and serum estradiol-17β assay. When at least three follicles with a mean diameter ≥18 mm were present, patients were instructed to administer 250 mcg of recombinant human chorionic gonadotropin (r-hCG, Ovitrelle; Merck Serono, Milan, Italy). Transvaginal follicular aspiration for oocyte retrieval was performed 36 h post-hCG.

Cumulus cells isolation

During ovum pick up, oocyte–cumulus complexes (COCs) were immediately separated from follicular fluid, washed in Modified HTF-HEPES Medium (Quinn's Advantage® HEPES Medium; Sage, Trumbull, CT), and singularly transferred to 40 μl drops of the same medium, where a portion of cumulus cells (not more than 50%) was mechanically removed using two syringes with 25 G needles. COCs were then kept in culture in Modified HTF Medium (Quinn's Advantage® Medium; Sage, Trumbull, CT) medium whereas removed cumulus cells were transferred into 1.5 ml tubes (Eppendorf Srl, Milan, Italy) with a minimal amount of medium.

Cumulus cells were exposed to 40 IU/ml hyaluronidase (SAGE BioPharma, Bedminster, NJ, USA) for 30 s before being washed with Phosphate Buffered Saline. Cumulus cells from different COCs were pooled together in order to have enough material to enable the direct comparisons of different procedures.

The number of cells for each pool was determined measuring the amount of DNA extracted from an aliquot of each pool with Wizard® Genomic DNA Purification Kit (Promega Italia, Milan, Italy) and quantified using the NanoDrop ND-1000 spectrophotometer (EuroClone S.p.A., Milan, Italy).

RNA extraction

RNA extraction was performed with the following five methods, each one repeated three times.

Method A: TRI reagent™ (Sigma-Aldrich, Milan, Italy) according to manufacturer's instructions, starting from 500 μl of reagent and re-suspending in 20 μl of water with DEPC (diethyl pyrocarbonate).

Method B: cell lysis procedure of the TaqMan® Gene Expression Cells-to-Ct™ Kit (Applied Biosystems Italia, Monza, MI, Italy).

Method C: GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Milan, Italy) using the protocol for Cell preparation but with 25 μl of Elution Solution instead of 50 μl.

Method D: RNeasy® Micro Kit (Qiagen S.p.A., Milan, Italy) according to manufacturer's instructions for total RNA isolation from animal cells.

Method E: SV Total RNA Isolation System (Promega Italia, Milan, Italy) using the protocol for cultured cells lysis and purifying the RNA by centrifugation (spin) using 25 μl of elution buffer instead of 100 μl.

Efficiency was evaluated measuring the following parameters:

a) time required for completing the extraction;
b) amount of extracted RNA, measured with a NanoDrop ND-1000 spectrophotometer (EuroClone S.p.A., Milan, Italy);
c) purity, assessing the 260/280 and 260/230 ratios, with the same spectrophotometer;
d) amplification efficiency, retro-transcribing with High-Capacity cDNA Rerverse Transcription Kits (Applied Biosystems Italia, Monza, MI, Italy) and amplifying the cDNA as described below.

Reverse Transcription

Reverse transcription (RT) was performed with the following three methods each one repeated three times.

Method H: High-Capacity cDNA Rerverse Transcription Kits (Applied Biosystems Monza, Italy) according to manufacturer's instructions.

Method S: RT procedure of the Enhanced Avian HS RT-PCR Kit (Sigma-Aldrich, Milan, Italy).

Method P: ImProm-II™Transcription System (Promega Italia, Milan, Italy) according to manufacturer's instructions, using 2.0 mM final concentration of $MgCl_2$.

Efficiency was evaluated measuring the following parameters:

a) time required for completing the extraction;
b) amplification efficiency following the Real-time PCR procedure described below.

RNA extracted with procedures C and D was used to perform the comparison.

Real-time PCR

Real-time analysis was performed using a Fast modality protocol with TaqMan Fast Universal PCR Master Mix no AmpErase UNG and Assay on Demand on a 7500 Fast Real-time PCR System (Applied Biosystems Italia, Monza, MI, Italy). Three target (GREM1, HAS2 and PTX3) and 2 endogenous (18S and HPRT1) genes were analysed.

Results are expressed as cycle threshold (Ct), the cycle number at which the fluorescence generated within a reaction well exceeds the defined threshold. The amount of a specific transcript is inversely related to the Ct value. We consider Ct values up to 35 cycles as a limit of physiologically meaningful gene expression .

One-step quantitative RT-PCR

We also evaluated the Quantitative RT-PCR ReadyMix™ (Kit O, Sigma-Aldrich, Milan, Italy) which allows to perform reverse transcription and cDNA amplification in one-step using RNA extracted with TRI reagent. We compared the amplification efficiency obtained using the 200 ng of RNA for single reaction as recommended by the manufacturer instruction.

Statistical analysis

Threshold cycles of investigated genes were compared with Wilcoxon Signed Rank test using software Statistics Package for Social Sciences (SPSS 15.0, Chicago, IL). Data are expressed as mean±SD. A value of $P<0.05$ was considered to be statistically significant.

Complete analysis

In order to verify if the whole procedure could actually be performed within the 3-4 h interval separating oocyte collection from fertilization, we analyzed the expression levels of 46 cumulus collected from 7 patients, using method C for RNA extraction in combination with method P for reverse transcription. In this case cumulus cells obtained from single COCs were kept separated and processed individually.

The quality of the resulting embryos was scored according to developmental stage and morphological quality, as specified in the grading system of Veeck [8]. This classification is based primarily on equal-sized blastomeres, pattern of fragmentation, and cytoplasmic appearance. Grade 1 represents perfect morphology, and grade 5 represents the worst. We considered as "good quality embryos" those of grade 1 and 2.

Results

The comparison among the different extraction and reverse transcription methods was performed on cell aliquots adjusted to the same size as determined by DNA quantification.

RNA extraction

RNA extraction with method A yielded the highest RNA concentration and of the highest purity but required 60 min to be completed. Methods C, D and E were completed in 30 min and the extracted RNAs had similar values of purity, but method D gave a higher yield; method B took only 15 min and gave the second higher yield but the extracted RNA had the lowest purity values. After Real-time amplification, samples extracted with methods A, C, D gave the best results (Table 1) although difference was not statistically significant.

Reverse Transcription

Reverse-transcription with method H required 2.5 h to be completed whereas methods S and P required only 1.5 h. Threshold Cycles obtained with method S were not reliable because upper then 35 (Table 2).

Real-time PCR

We performed Real-time PCR using Fast modality with 7500 Fast Real-time PCR System (Applied Biosystems). This system gave reliable results and was completed in 40 min.

One-step quantitative RT-PCR

We also tested kit O which enables Reverse Transcription and Real-time procedures to be performed in one-step, that is completed in 1 h and 45 min and, considering 60 min for RNA extraction with TRI reagent, entire analysis is done in 2 h and 45 min. Ct values of 26.09±0.13 for the endogenous gene (HPRT) and of 28.08±0.02 for the less expressed target gene (GREM1) were obtained with this method. They were comparable to those obtained with the best combinations described above. However 3 μg of total RNA are required for analysing five genes.

Complete analysis

Based on the results described above we performed the whole procedure on individual cumulus fragments of COC collected from 7 patients using method C for RNA extraction in combination with method P for reverse transcription. We were able to analyse the cumulus cells gene expression within the interval between ovum pick-up and fertilization for all patients. We obtained a valid measurement from 44 out of 46 cumulus fragments (about 96%).

Embryological parameters from oocytes included and not included in the study, such as fertilization and good quality embryos rates per inseminated oocyte in the same period, were comparable (Table 3).

Discussion

We demonstrated that it is possible to quantify the level of expression of genes in individual *cumuli oophori* within the interval between pick-up and fertilization which, usually, does not exceed 4 h. This will enable us to perform a prospective evaluation of each oocyte. This analysis could support the traditional morphological evaluation in those cases where it may be desirable to fertilize only a portion of the retrieved oocytes. It is important to note that the procedure had no detrimental effects on oocyte competence and development after fertilization.

The commercial kits used in our work were originally developed for high-throughput analysis which usually rely on amounts of starting RNA higher than those available to us. Therefore we tested them to their lower limits since we had to work with less than half of the cells of each cumulus.

Due to the fact that we had to face the double problem of restricted time and limited amount of material we had to search for the best compromise that would enable us to get a good result in the shortest time.

Table 1 Work time, value of RNA concentration, purity and cycle threshold (Ct) resulting from Real-Time PCR. HPRT data are reported as endogenous Ct, GREM1 data are reported as target Ct since it was the gene expressed at the lower level

Extraction method	Work time	Total RNA (ng)	Abs 260/280	Abs 206/230	Total RNA (ng/μl)	Endogenous Ct	Target Ct
A	60 min	4,153±1,025	1.24±0.03	0.37±0.02	207.65±51.25	23.39±2.05	28.44±3.29
B	15 min	1283±641	0.40±0.08	0.06±0.06	25.66±12.82	35.86±2.89	36.04±3.27
C	30 min	124±48	0.92±0.05	0.11±0.03	4.97±1.92	22.43±2.17	28.11±3.17
D	30 min	103±38	1.28±0.03	0.08±0.02	7.36±2.72	20.94±2.12	26.44±3.16
E	30 min	61±21	0.96±0.04	0.07±0.04	2.42±0.84	27.73±2.81	34.59±3.77

Data are reported as mean±DS

Table 2 Work time and threshold cycle (Ct) resulting from real-time PCR of 5 genes

RNA extraction method	RT method	Work time	Ct HPRT	Ct 18S	Ct GREM1	Ct HAS2	Ct PTX3
C	H	2 h 30 min	26.419±2.159	13.254±2.952	32.030±3.361	27.522±3.652	23.582±3.869
	S	1 h 30 min	29.819±2.407	15. 841±3.014	35.272±3.128	30.049±3.322	26.541±3.749
	P	1 h 30 min	26.787±2.146	12.857±2.873	31.354±3.206	27.603±3.215	23.862±3.625
D	H	2 h 30 min	24.815±1.979	12.756±2.227	31.194±2.775	25.921±3.401	21.743±3.657
	S	1 h 30 min	28.339±2.535	14.859±2.364	34.647±3.129	28.983±2.985	25.428±3.945
	P	1 h 30 min	25.561±2.671	12.587±2.510	30.016±2.967	25.933±3.142	22.468±3.838

Data are reported as mean±SD

The extraction method A, the "classic" and the cheapest method to extract the RNA, even if gave good results in terms of efficiency, had a working time too long for our needs. On the other hand method B was faster, but, despite the fact that RNA output was high, when used for RT-PCR amplification, it gave an higher threshold level compared to other methods. This suggests a poor RNA quality leading to an inefficient retro-transcription. We hypothesise that this result may be due to the fact that this method did not include a proper extraction step, but the sample was lysed and immediately retrotranscribed. Higher levels of impurities were therefore likely to be present, leading to a decreased efficiency of the subsequent reactions.

Methods C, D and E were all column-based but with several important differences: method E presented an elution volume of 100 µl, too high for the amount of our starting material, leading to a not measurable RNA concentration in the sample. The problem was partially avoided decreasing the elution volume, but its efficiency remained lower than that of the other two methods. Method C showed a good efficiency using an elution volume of 25 µl (half of the 50 µl recommended by the manufacturer). Method D is designed for RNA extraction from small amount of starting material and indeed it gave the best results in our analysis. However it was also much more expensive compared to the other ones.

Retrotranscription kits have been tested after that the extraction was performed with method C and D. The method H has been discarded because it required an incubation time too long. Kits S and P had a working time shorter than H, but kit S showed Threshold Cycles not reliable in Real-time PCR

The amplification with the RT-PCR described in our experiments, allowed us to complete the procedure in half of the time required for a "standard" Real-time PCR.

We used various kits from distinct Companies which have different compatibility degrees that could influence efficiency and results of entire the procedure.

We also tested a kit for one-step quantitative RT-PCR. It requires 3000 ng of total RNA for analysing 5 genes but is not possible to obtain this quantity for all CC due to their great variability.

This study shows the possibility to use kits C and D, for the same efficiency of RNA extraction, but with kit C preferred for its lower cost, in view of a possible high-throughput screening in the future.

Following extraction, the retrotranscription is performed with the kit P and Real-time-PCR "Fast".

This protocol has been used to evaluate 7 patients, with a total of 46 cumulus fragments, and the loss of data limited to 4% of the cases.

In this study we analysed 3 genes downstream to GDF9 which have been previously shown to be related to oocyte competence [9–11]. However results on their value as predictive markers were controversial. More recently, the use of a custom-made complementary DNA microarray containing granulosa/cumulus expressed sequence tags (ESTs) has lead to the identification of alternative marker genes (i.e. 3-beta-hydroxysteroid dehydrogenase 1, Ferredoxin 1, Serine (or cysteine) proteinase inhibitor clade E member, Cytochrome P450 aromatase and Cell division cycle 42, [12] for oocyte quality as well as to the identification of two predictors for ongoing pregnancy (phosphoglycerate kinase 1 and regulator of G-protein

Table 3 Embriological parameters during the entire study period obtained from oocytes subjected to removal of cumulus cells versus oocytes not subjected to removal of cumulus cells

	% oocytes fertilized	% cleavaged oocytes	% good quality embryo
Oocytes subjected to cumulus cells removal	79,6% (187/235)	98% (176/187)	62% (109/176)
Oocytes not subjected to cumulus cells removal	81,3% (703/865)	98% (689/703)	61% (421/689)

signalling 2, [13]. The value of the method described in the present manuscript to the clinical embryology laboratory will depend on the reliability of the marker genes tested and mining transcriptomic data looks a promising way to identify the most reliable candidates. Once these are established it will be possible to complement the current morphological methods and improve the selection of healthy oocytes resulting in good pregnancy rates. This may be useful in all those situations where the fertilization of only part of the oocytes may be desirable for legal or ethical consideration. The information about genes expressed in competent follicles will also be of help for the refinement of hormonal treatments in problematic patients.

In conclusion we demonstrated that it is possible to evaluate the expression level of genes that are predictive for the development of an embryo with good quality within the interval between pick-up and insemination.

This method can be applied without changing the protocol of a routine ICSI procedure and provides the embryologist with a molecular evaluation that can support other parameters used to choose which oocyte to inseminate.

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References

1. Scott L. The biological basis of non-invasive strategies for selection of human oocytes and embryos. Hum Reprod Update. 2003;9:237–49.
2. Wang Q, Sun QY. Evaluation of oocyte quality: morphological, cellular and molecular predictors. Reprod Fertil Dev. 2007;19(1):1–12.
3. Gilchrist RB, Ritter LJ, Armstrong DT. Oocyte-somatic cell interactions during follicle development in mammals. Anim Reprod Sci. 2004;82-83:431–46.
4. Pangas SA, Matzuk MM. The art and artifact of GDF9 activity: cumulus expansion and the cumulus expansion-enabling factor. Biol Reprod. 2005;73:582–5.
5. Chang H, Brown CW, Matzuk MM. Genetic analysis of the mammalian transforming growth factor-beta superfamily. Endocr Rev. 2002;23:787–823.
6. Elvin JA, Clark AT, Wang P, Wolfman NM, Matzuk MM. Paracrine actions of growth differentiation factor-9 in the mammalian ovary. Mol Endocrinol. 1999;13:1035–48.
7. Vanderhyden BC, Macdonald EA, Nagyova E, Dhawan A. Evaluation of members of the TGFbeta superfamily as candidates for the oocyte factors that control mouse cumulus expansion and steroidogenesis. Reprod Suppl. 2003;61:55–70.
8. Veeck LL. An atlas of human gametes and conceptuses: an illustrated reference for assisted reproductive technology. Parthenon: New York; 1999.
9. Cillo F, Brevini TA, Antonini S, Paffoni A, Ragni G, Gandolfi F. Association between human oocyte developmental competence and expression levels of some cumulus genes. Reproduction. 2007;134:645–50.
10. McKenzie LJ, Pangas SA, Carson SA, Kovanci E, Cisneros P, Buster JE, et al. Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. Hum Reprod. 2004;19:2869–74.
11. Zhang X, Jafari N, Barnes RB, Confino E, Milad M, Kazer RR. Studies of gene expression in human cumulus cells indicate pentraxin 3 as a possible marker for oocyte quality. Fertil Steril. 2005;83 Suppl 1:1169–79.
12. Hamel M, Dufort I, Robert C, Gravel C, Leveille MC, Leader A, et al. Identification of differentially expressed markers in human follicular cells associated with competent oocytes. Hum Reprod. 2008;23(5):1118–27.
13. Hamel M, Dufort I, Robert C, Leveille MC, Leader A, Sirard MA. Genomic assessment of follicular marker genes as pregnancy predictors for human IVF. Mol Hum Reprod. 2009;16(2):87–96.