Differences in cumulus cells gene expression between modified natural and stimulated in vitro fertilization cycles

Tanja Burnik Papler · Eda Vrtačnik Bokal ·
Klementina Fon Tacer · Peter Juvan · Irma Virant Klun ·
Rok Devjak

Received: 6 August 2013 / Accepted: 29 October 2013 / Published online: 13 November 2013
© Springer Science+Business Media New York 2013

Abstract

Purpose The aim of our study was to determine whether there are any differences in the cumulus cell gene expression profile of mature oocytes derived from modified natural IVF and controlled ovarian hyperstimulation cycles and if these changes could help us understand why modified natural IVF has lower success rates.

Methods Cumulus cells surrounding mature oocytes that developed to morulae or blastocysts on day 5 after oocyte retrieval were submitted to microarray analysis. The obtained data were then validated using quantitative real-time PCR.

Results There were 66 differentially expressed genes between cumulus cells of modified natural IVF and controlled ovarian hyperstimulation cycles. Gene ontology analysis revealed the oxidation-reduction process, glutathione metabolic process, xenobiotic metabolic process and gene expression were significantly enriched biological processes in MNIVF cycles.

Capsule: The purpose of our study was to compare cumulus cell gene expression between unstimulated and stimulated IVF cycles to determine whether differences in gene expression could reveal why unstimulated IVF has lower success rates.

Electronic supplementary material The online version of this article (doi:10.1007/s10815-013-0135-6) contains supplementary material, which is available to authorized users.

T. B. Papler · E. V. Bokal · I. V. Klun · R. Devjak (✉)
Department of Human Reproduction, Division of Gynecology,
University Medical Centre Ljubljana, Ljubljana, Slovenia
e-mail: rok.devjak@gmail.com

K. F. Tacer
Department of Physiology, UT Southwestern Medical Center, Dallas,
TX, USA

P. Juvan
Centre for Functional Genomics and Bio-Chips, Institute of
Biochemistry, Faculty of Medicine, University of Ljubljana,
Ljubljana, Slovenia

Among differentially expressed genes we observed a large group of small nucleolar RNA's whose role in folliculogenesis has not yet been established.

Conclusion The increased expression of genes involved in the oxidation-reduction process probably points to hypoxic conditions in modified natural IVF cycles. This finding opens up new perspectives for the establishment of the potential role that oxidation-reduction processes have in determining success rates of modified natural IVF.

Keywords Cumulus cells · Gene expression · Stimulated IVF · Unstimulated IVF

Introduction

Since its first introduction in 1978 [1], IVF in a natural menstrual cycle has mostly been replaced with stimulated IVF due to its high cancellation and low pregnancy rate. The use of exogenous gonadotropins in combination with GnRH analogues for controlled ovarian hyperstimulation (COH) raises the number of oocytes retrieved and thus improves the results of IVF procedures [2], with cumulative pregnancy rate being approximately 63 % after 4 cycles [3, 4]. However, the use of COH is associated with serious clinical problems like multiple pregnancies [5] and ovarian hyperstimulation syndrome (OHSS) [6].

In recent years, there has been a tendency towards the use of milder IVF stimulation protocols and patient friendlier IVF [7]. Modified natural IVF (MNIVF) is considered to be patient friendlier and cheaper than COH cycles [8]. Furthermore, it does not have the adverse effects of the stimulated cycles and it probably offers the best follicular environment for oocyte maturation [9]. The problem of natural IVF is in the high cancellation of oocyte retrieval due to premature LH surge and consequently spontaneous ovulation [10, 11], and low

pregnancy rates [12]. However, it can be repeated on a monthly basis, which improves its overall success rate [8], with cumulative pregnancy rate being 46 % after 4 cycles [13].

The acquisition of quality oocyte and consequently quality embryo during folliculogenesis relies on bidirectional communication between oocyte and its surrounding cumulus cells (CC) [14, 15]. This means that CC gene expression pattern is an indirect marker of oocytes' state [16]. Cumulus cells represent a convenient biological material for gene expression studies since they are easily obtainable and discarded during the IVF procedure. Cumulus cells gene expression analysis can provide information about the factors influencing folliculogenesis [17], furthermore it has been proposed as a non-invasive method for oocyte and embryo quality assessment [18, 19].

The impact of COH on embryo morphology in humans has already been characterized, and no differences were found between embryos deriving from natural or stimulated cycles [20] moreover, excessive response to ovarian stimulation was shown to have no negative impact on embryo quality as assessed by morphology [21]. However, limited information are available on the impact of COH on the molecular level and to date, and the best of our knowledge, one study comparing CC gene expression between MNIVF and COH cycles has been published [9].

Besides that, human ovarian folliculogenesis is far from being understood and thus, a study of CC gene expression profile derived from MNIVF, is probably the best way to improve our understanding of the underlying mechanisms and pathways of this process, as it is known that the use of exogenous gonadotropins causes changes in CC gene expression profile [22]. Understanding folliculogenesis is fundamental for optimisation of ovarian stimulation protocols and in vitro maturation processes.

According to the aforementioned background, our hypothesis states that there are no gene expression differences between CC of MNIVF and COH deriving from oocytes that developed to the morula or blastocyst stage on day 5 of IVF. Also, we aimed to determine whether we could find the reason for lower success rates of MNIVF cycles by analysing CC gene expression.

Materials and methods

Patient selection and experimental design

The study was approved by the National Medical Ethics Committee of the Republic of Slovenia, and all patients signed informed consent. Seventeen patients ($n=17$) undergoing conventional IVF for tubal cause of infertility were included in the study. Patients included were under the age of 35, had the body mass index (BMI) between 17 and 26 kg/m² and normal

partners spermiogram according to the WHO criteria. All patients first underwent MNIVF followed by a COH cycle. Cumulus cells were collected after oocyte retrieval. The RNA from CC was isolated and gene expression analysis was performed by microarrays. The quantitative real-time PCR (qPCR) analysis was used to validate the selected genes. All CC used for analyses derived from MII oocytes, which developed into morulae or blastocyst on day 5 of the IVF cycle.

Microarray analysis was performed on 8 individual CC samples, derived from the first 5 patients included in the study. Altogether, 3 CC samples from MNIVF and 5 CC samples from COH cycles were included in the microarray analysis. The oocyte was not retrieved from 2 patients in the MNIVF cycle. For qPCR validation analysis the CC samples from following 12 patients were added. Altogether, the qPCR analysis was performed on 33 individual CC samples; 15 derived from MNIVF and 18 from COH cycles. Table 1 represents the patients and the number of their individual CC samples included in the study.

Stimulation protocols and oocyte retrieval

Transvaginal ultrasonographic examination was made on day 9 of the MNIVF cycle. The measurement of serum estradiol

Table 1 A list of patients ($n=17$) included in the study with the number of their corresponding CC samples ($n=38$) included in the study and the experiment for which the samples were used

Patient	MNIVF CC		COH CC	
	Microarray	qPCR	Microarray	qPCR
1				4
2		1		2
3		1		2
4	1*	1*	1	
5		1		
6	1*	1*	1	
7				3
8	1*	1*	1	
9		1		
10		1		
11		1		
12		1	1	
13		1		
14		1	1	3
15		1		2
16		1		1
17		1		1

MNIVF CC - cumulus cells derived from modified natural IVF cycle; COH CC - cumulus cells derived from stimulated cycles; qPCR - real time PCR. * The same sample of CC was used for the microarray and qPCR experiments

and testing of urine for LH surge presence was performed at the same time. A commercially available kit (Oestradiol-2; Sorin Biomedica, Saluggia, Italy) was used for the measurement of serum estradiol. The detection of LH surge was also made with a commercially available kit (RapiTest LH; Morwell Diagnostics GmbH, Egg, Switzerland). Five thousand IU of human chorionic gonadotropin (HCG) (Pregnyl; N.V. Organon, Oss, The Netherlands) was injected subcutaneously as soon as the dominant follicle reached ≥ 16 mm in diameter, serum estradiol exceeded 0.39 nmol/l and there was no LH surge detected in the urine sample. Ultrasound-guided transvaginal oocyte aspiration was performed 31–32 h after HCG administration [23].

Stimulation protocols and oocyte retrieval was performed according to long or short COH protocols. Briefly, busserelin acetate (Suprefact; Hoechst AG, Frankfurt/Main, Germany), was administered subcutaneously from day 22 of menstrual cycle in long protocols. Ggonadotropin follitropin alpha (Gonal F; Industria Farmaceutica Serono S.p.A., Bari, Italy) was administered subcutaneously when the criteria for ovarian desensitization were fulfilled. In short protocols gonadotropin follitropin alpha (Gonal F; Industria Farmaceutica Serono S.p.A., Bari, Italy) was administered subcutaneously from day 2 of the menstrual cycle. Cetorelix acetate (Cetrotide; Asta Medica AG, Frankfurt, Germany) was administered subcutaneously when the dominant follicle measured 13 mm in diameter. Ten thousand IU of HCG (Pregnyl; N.V. Organon) was administered in both protocols when at least three follicles measured ≥ 17 mm in diameter and serum estradiol exceeded 0.40 nmol/l per follicle. Ultrasound guided transvaginal oocyte aspiration was performed 34–36 h after HCG administration.

Cumulus cells collection and oocyte follow up

A part of CC surrounding a single oocyte was removed by using a sharp needle immediately after the retrieval of cumulus-oocyte complex (COC). Individual CC samples were washed in PBS, snap-frozen in liquid nitrogen and stored in vials at -80°C until RNA isolation.

The oocytes were cultured individually in the Universal IVF Medium (MediCult a/s, Jyllinge, Denmark) and fertilization was carried out 2 h after oocyte retrieval. The fertilization status was assessed after 24 h. Embryos were later cultured in a sequential media (BlastAssistSystem; MediCult, Denmark) and one or two embryos at the stage of morulae or blastocyst were transferred on day 5 after oocyte retrieval; supernumerary good quality blastocysts were cryopreserved.

Implantation was assessed 14 days after embryo transfer using serum β -HCG measurement. Transvaginal ultrasound was performed 4 weeks after embryo transfer to confirm intrauterine pregnancy and the number of gestational sacs with heartbeat activity.

RNA preparation

Total RNA was extracted using TRI reagent (Sigma - Aldrich, St. Louis, USA) according to manufacturer's instruction with small modification due to the small sample volume. Briefly, CC from individual follicle were homogenized in 500 μL TRI reagent supplemented with 125 μg of glycogen. Glycogen (Ambion, Woodward, USA) was used as a carrier to increase the RNA yield. One hundred μL of chloroform was added after incubation at room temperature and the sample was vortexed vigorously. Aqueous phase was transferred and RNA precipitated with 250 μL of isopropanol after 15 min centrifugation at $12,000 \times g$ and 4°C . RNA pellet was washed 3 times with 75 % ethanol after 25 min centrifugation at $12,000 \times g$ and 4°C . Total RNA was resuspended in 15 μL of RNase-free water and stored at -80°C . The integrity of RNA was assessed on Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA) to assure high quality of total RNA; the RIN number was more than 7 for each sample. Total RNA quantity and purity was measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA).

Transcriptome analysis

Transcriptome analysis was performed using the GeneChip Human Gene 1.0 ST Arrays (Affymetrix, Santa Clara, USA). Array hybridizations were performed according to manufacturer's recommendations. Two hundred ng of RNA was amplified and converted to cDNA using the WT Expression Kit (Ambion, Austin, USA). The resulting cDNA was fragmented and labeled using the GeneChip WT Terminal Labeling and Controls Kit (Affymetrix, Santa Clara, USA) and hybridized to the arrays for 16 h.

The arrays were washed using GeneChip Fluidics Station 450 according to manufacturer's recommendations, and scanned on the Affymetrix GeneChip Scanner 3000 7G. The images were analyzed by Affymetrix GeneChip Command Console software and quality of data checked by Affymetrix GeneChip Expression Console software.

Data were processed and analyzed using different R/Bioconductor packages [24]. Data were normalized using the RMA algorithm from the XPS package. Annotation of genes and data representation was managed using ANNAFFY and AFFYCORETOOLS packages. The raw and normalized gene expression data together with experimental information were deposited to Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=oxwbcowpmbmxnmb&acc=GSE43684>) in compliance with MIAME standards, under series accession number GSE43684.

A mixed model analysis of variance using LIMMA package was used to assess the differential expression of genes between the two IVF procedures using patients as a mixing variable and controlling the false discovery rate (FDR) [25] at

significance level $\alpha=0.05$. Gene set enrichment analyses were performed using Parametric Gene Set Enrichment Analysis (PGSEA) package in combination with LIMMA package using an equal statistical modelling as for individual genes and controlling FDR at significance level $\alpha=0.05$. Gene sets related to different KEGG pathways were tested for their enrichment and sorted by their enrichment scores. Gene Ontology (GO) analysis of differentially expressed genes was performed using GeneCodis [26].

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was used to validate 7 selected differentially expressed genes using TaqMan® Gene Expression pre-designed assays (Applied Biosystems, Foster City, USA).

Genomic DNA contamination was eliminated by DNase treatment using DNase I (Roche, Basel, Switzerland). cDNA for qPCR assays was prepared from 150 ng DNase RNA using SuperScript RT III (Invitrogen, Carlsbad, USA) in 20 μ l final volume. Following cDNA synthesis, RNase-free water was added to increase the sample volume to 40 μ l. All qPCR reactions were performed in triplicates in 384-well formats in a 10 μ l final volume. The measurements were performed using LightCycler® 480 System (Roche Applied Science, Penzberg, Germany).

Selection of candidate genes for qPCR validation was based on the microarray results and using different parameters. We focused on the genes with absolute FC ≥ 1.3 and p value <0.05 , with a special emphasis on those genes that are known to be involved in the process of folliculogenesis and those that have already been described as potential biomarkers of oocyte competence (lymphatic vessel endothelial hyaluronan receptor 1 (*LYVE1*); cytochrome P450, family 3, subfamily A, polypeptide 4 (*CYP3A4*); hydroxysteroid-17-beta dehydrogenase 11 (*HSD17B11*); insulin-like growth factor 2 (*IGF2*); glutathione peroxidase 3 (*GPX3*); regulator of G-protein signaling 2 (*RGS2*); histone cluster 1, H4c (*HIST1H4C*)). Normalized mRNA levels were obtained by dividing the averaged, efficiency corrected values for mRNA expression by normalization factor, calculated from peptidylpropyl isomerase B (*PPIB*) and ribosomal protein, large, P0 (*RPLP0*) values, that were used as an internal control, and were expressed in arbitrary units [27]. The resulting values were log2 transformed for graphical representation.

Results

The study population and IVF cycle characteristics (age, body mass index (BMI), pregnancy rate and delivery rate) are presented in Table 2. Age and BMI were comparable between the two groups. As expected, significant differences were observed in pregnancy and delivery rate.

Table 2 Study population and IVF cycle characteristics

Variable	MNIVF	COH	p-value
Age (years)	31.13 $\pm$ 3.68	31.59 $\pm$ 3.03	0.74
BMI (kg/m ²)	20.52 $\pm$ 2.30	20.60 $\pm$ 2.52	0.93
Pregnancy rate per cycle (proportion)	0.07 $\pm$ 0.26	0.50 $\pm$ 0.52	0.01
Delivery rate per cycle (proportion)	0.07 $\pm$ 0.26	0.42 $\pm$ 0.51	0.03

Age, body mass index (BMI), pregnancy and delivery rates per cycle. Significance of differences between the two treatment groups was assessed using Student's t-test; two-tailed p-values are shown. MNIVF - modified natural IVF cycle; COH - stimulated cycles

Microarray analysis

We analyzed the differences in gene expression profile of individual CC samples derived from both MNIVF and COH cycles. There were 66 differentially expressed genes between MNIVF and COH cycles and only one differentially expressed gene when CC of morulae and blastocysts were compared (Fig. 1). Among 66 differentially expressed genes, 64 showed increased and 2 decreased expression in the MNIVF cycles when compared with COH cycles (Table 3). Functional characterization of differentially expressed genes using PGSEA of KEGG pathways yielded enrichments of olfactory transduction, RNA degradation, RNA transport, neuroactive ligand-receptor interaction, ribosome biogenesis in eukaryotes and ribosome (Table S1). The list of differentially expressed genes was subjected to GO analysis to determine which biological processes were significantly overrepresented (p value <0.05). Processes such as glutathione metabolic process, xenobiotic metabolic process, oxidation-reduction process and gene expression were represented in the list (Table S2).

We wanted to determine whether the age of included patients, regardless of therapy, affected the expression of oxidation-reduction process gene set. The Pearson correlation

Fig. 1 Number of differentially expressed genes between MNIVF and COH cycles (a) and between morulae and blastocysts (b). MNIVF - modified natural IVF cycle; COH - stimulated cycles; Mo- morulae; Bl- blastocyst

Table 3 Genes differentially expressed between COH CC and MNIVF CC, their p value and fold change

Symbol	Description	p-value	Fold change
SNORD45B	small nucleolar RNA, C/D box 45B	0.045	−2.19
CYP3A4	cytochrome P450, family 3, subfamily A, polypeptide 4	0.037	−1.95
PRND	prion protein 2 (dublet)	0.011	−1.94
LSM3	LSM3 homolog, U6 small nuclear RNA associated (<i>S. cerevisiae</i>)	0.04	−1.85
SNORD27	small nucleolar RNA, C/D box 27	0.024	−1.85
SNORD5	small nucleolar RNA, C/D box 5	0.029	−1.85
GBP4	guanylate binding protein 4	0.024	−1.82
SNORD44	small nucleolar RNA, C/D box 44	0.034	−1.74
SNORD28	small nucleolar RNA, C/D box 28	0.044	−1.73
CPE	carboxypeptidase E	0.01	−1.71
HIST1H4C	histone cluster 1, H4c	0.003	−1.67
SNORD78	small nucleolar RNA, C/D box 78	0.048	−1.66
RPS29	ribosomal protein S29	0.021	−1.65
SNORD47	small nucleolar RNA, C/D box 47	0.045	−1.65
LYVE1	lymphatic vessel endothelial hyaluronan receptor 1	0.024	−1.63
SNORD75	small nucleolar RNA, C/D box 75	0.04	−1.62
HINT2	histidine triad nucleotide binding protein 2	0.032	−1.6
ATP1B3	ATPase, Na ⁺ /K ⁺ -transporting, beta 3 polypeptide	0.029	−1.59
MED17	mediator complex subunit 17	0.025	−1.58
MGST3	microsomal glutathione S-transferase 3	0.047	−1.58
RPS10	ribosomal protein S10	0.024	−1.57
HIGD1A	HIG1 hypoxia inducible domain family, member 1A	0.036	−1.56
SNORD61	small nucleolar RNA, C/D box 61	0.032	−1.54
ZNF737	zinc finger protein 737	0.025	−1.54
NDUFB5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5, 16 kDa	0.049	−1.53
SNORA5A	small nucleolar RNA, H/ACA box 5A	0.034	−1.52
DECR1	2,4-dienoyl CoA reductase 1, mitochondrial	0.047	−1.49
SSBP1	single-stranded DNA binding protein 1	0.014	−1.49
GSTA1	glutathione S-transferase alpha 1	0.034	−1.48
ZNF431	zinc finger protein 431	0.028	−1.48
SCARNA9	small Cajal body-specific RNA 9	0.034	−1.44
GSTA2	glutathione S-transferase alpha 2	0.044	−1.43
SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1	0.032	−1.43
GPX3	glutathione peroxidase 3 (plasma)	0.045	−1.42
GSTA3	glutathione S-transferase alpha 3	0.042	−1.42
SNORD54	small nucleolar RNA, C/D box 54	0.025	−1.42
GCG	glucagon	0.034	−1.4
PAK1IP1	PAK1 interacting protein 1	0.026	−1.4
TXN	thioredoxin	0.029	−1.4
SNORD42A	small nucleolar RNA, C/D box 42A	0.037	−1.39
ATP1B1	ATPase, Na ⁺ /K ⁺ -transporting, beta 1 polypeptide	0.01	−1.38
SNORD38B	small nucleolar RNA, C/D box 38B	0.037	−1.38
DTWD1	DTW domain containing 1	0.035	−1.37
SNORD50B	small nucleolar RNA, C/D box 50B	0.04	−1.37
VBP1	von Hippel-Lindau binding protein 1	0.025	−1.37
C13orf27	chromosome 13 open reading frame 27	0.024	−1.36
PRPS2	phosphoribosyl pyrophosphate synthetase 2	0.025	−1.34
C11orf73	chromosome 11 open reading frame 73	0.025	−1.33
NMI	N-myc (and STAT) interactor	0.015	−1.33

Table 3 (continued)

Symbol	Description	p-value	Fold change
SOD2	superoxide dismutase 2, mitochondrial	0.04	−1.33
TAF1D	TATA box binding protein (TBP)-associated factor, RNA polymerase I, D, 41 kDa	0.034	−1.33
RGS2	regulator of G-protein signaling 2, 24 kDa	0.037	−1.32
SNORA8	small nucleolar RNA, H/ACA box 8	0.04	−1.32
SNORD76	small nucleolar RNA, C/D box 76	0.039	−1.32
COX7B	cytochrome c oxidase subunit VIIb	0.047	−1.31
HIST1H3B	histone cluster 1, H3b	0.036	−1.31
HIST1H4L	histone cluster 1, H4l	0.038	−1.31
MMADHC	methylmalonic aciduria (cobalamin deficiency) cblD type, with homocystinuria	0.028	−1.31
SNORA16A	small nucleolar RNA, H/ACA box 16A	0.04	−1.31
SNORD6	small nucleolar RNA, C/D box 6	0.024	−1.31
C6orf115	chromosome 6 open reading frame 115	0.037	−1.3
IL7R	interleukin 7 receptor	0.036	−1.3
RPS7	ribosomal protein S7	0.007	−1.3
HSD17B11	hydroxysteroid (17-beta) dehydrogenase 11	0.04	−1.3
SCG2	secretogranin II	0.037	1.31
IGF2	insulin-like growth factor 2 (somatomedin A)	0.007	1.49

coefficient between oxidation-reduction process gene set (*GPX3*, *SOD2*, *GSTA1*, *GSTA2*, *GSTA3*) and the patients age was low and non-significant (Table S3).

Microarray validation by qPCR

Expression levels of 7 genes were checked using qPCR for microarray data validation. The checked genes were *IGF2*, *LYVE1*, *RGS2*, *GPX3*, *HIST1H4C*, *HSD17B11* and *CYP3A4*. Six ($n=6$) of the selected genes showed increased expression and *IGF2* showed decreased expression in MNIVF cycles according to the microarray data. The differential expression between MNIVF and COH CC could be confirmed by qPCR for 6 out of the 7 selected genes (86 %) (Fig. 2).

Two out of 7 candidate genes selected for the qPCR analysis were significantly differentially expressed following the qPCR analysis between MNIVF and COH cycles: *IGF2* ($p=0.02$), which showed decreased expression in MNIVF, and *GPX3* ($p=0.002$), which showed increased expression in MNIVF. *RGS2*, which showed increased expression in MNIVF, showed a tendency to be differentially expressed ($p=0.07$) (Table 4).

Discussion

The results of this study have shown that the gene expression profile of CC derived from MNIVF differs from those derived from COH cycles even between MII oocytes that develop to morulae or blastocyst stage on day 5 after oocyte retrieval.

The most pronounced changes are seen with genes involved in the oxidation-reduction process.

In our previous study [17] we have discovered that there are no differences in CC gene expression between unfertilized oocytes and those that developed to the blastocyst stage. Thus, we did not expect differences in CC gene expression between morulae and blastocysts. We have confirmed this, as comparison of CC gene expression between morulae and blastocysts resulted in a single differentially expressed gene and no enrichment of KEGG pathways. The differentially expressed gene is vault RNA 1–2 (*VTRNA1-2*), whose potential role in human ovarian folliculogenesis has not yet been established.

Among 66 differentially expressed genes, 64 show increased expression and 2 show decreased expression in CC derived from MNIVF as compared to COH cycles. In GO analysis of differentially expressed genes glutathione metabolic process and oxidation-reduction process are overrepresented. In the group of genes related to these two processes there are *GPX3*, glutathione S-transferase alpha 1, 2 and 3 (*GSTA 1, 2, 3*) and superoxide dismutase 2 (*SOD2*). The correlation between patients' age and log2 expression values for genes related to oxidation-reduction process, regardless of therapy, is not statistically significant. A negative correlation between age and gene expression of genes involved in defense against reactive oxygen species (ROS) in follicles is well known and has been reported in several studies [28–31]. It seems that in our study we have controlled this effect by including patients of similar age and younger than 35 years, which is proved by a non-significant correlation (Table S3-Regardless of therapy). When therapy is

Fig. 2 Relative gene expression (log2 fold change) of *CYP3A4*, *IGF2*, *HIST1H4C*, *GPX3*, *LYVE1*, *HSD17B11* and *RGS2* in CC derived from COH cycles as compared to CC derived from MNIVF cycles. The black

bars represent the gene expression as measured with microarrays (MA). The white bars represent the gene expression as measured with qPCR

taken into account the most age sensitive according to our data are *GSTA* genes (Table S3-COH vs MNIVF).

The role of ROS in human reproduction has been well described [32]. It seems that ovarian ROS production is triggered by LH and is essential for ovulation, as the use of antioxidants significantly reduces ovulation rate, prevents LH-induced cumulus expansion, lowers progesterone secretion and down-regulates genes essential for ovulation [33]. However, pathological levels of ROS in follicular fluid diminish oocyte and embryo quality [34–36] and have a negative impact on IVF outcome [37]. Increased percentage of ROS producing granulosa cells (GC) has a negative impact on the number of oocytes retrieved and diminishes implantation rate in IVF procedures [38]. *GPX3*

catalyzes the reduction of free hydrogen peroxide, organic hydroperoxide and lipid peroxides and protects cells against oxidative damage [39]. *GPX3* has also been proposed to be a marker of poor embryo quality as it was down-regulated in CC of early-cleaving embryos and up-regulated in CC of non-early cleaving embryos [40]. Glutathione (GSH) function in oocytes is mainly related to antioxidative properties [41] furthermore, it protects the oocyte's meiotic spindle against oxidative damage [42]. Glutathione S-transferases (*GSTA*) provide protection against cellular oxidative stress through neutralization of a wide range of hydrophobic and electrophilic endogenous compounds or xenobiotics by catalyzing their conjugation to reduced GSH [43]. The expression of *GSTA 1* and *GSTA2* is modulated by gonadotropins in the developing

Table 4 Quantification of mRNA levels by quantitative PCR for the selected genes

Gene	Mean COH	COH±SEM	Mean MNIVF	MNIVF±SEM	p-Value
IGF2	1.31290	0.37695	0.38550	0.07402	0.030
GPX3	2.18690	0.26037	6.46020	1.32718	0.006
RGS2	57.93010	8.34221	90.03170	16.53492	0.070
LYVE1	4.29250	2.06859	2.27510	0.70617	NS
HSD17B11	1.47790	0.29912	1.73640	0.40957	NS
HIST1H4C	27.50010	2.07581	31.84290	4.65529	NS
CYP3A4	0.94070	0.36385	1.55920	0.89303	NS

COH controlled ovarian hyper-stimulation; MNIVF modified natural IVF cycle. NS non significant

bovine ovarian follicles [44]. Since the highest level of *GSTA* expression was in steroidogenically active cells it has been proposed, that the role of *GSTA* may be linked to oxygen stress induced by ROS generated during steroidogenesis. Superoxide dismutases (*SOD*) are a group of enzymes that reduce superoxide anion to hydrogen peroxide and molecular oxygen [45]. In our study, *GPX3*, *GSTA1*, *GSTA2*, *GSTA3* and *SOD2* showed increased expression in MNIVF cycles and thus, suggesting higher exposure of the developing follicle to ROS than in COH cycles. This finding is in agreement with that of de los Santos et al. [9] and Jančar et al. [46], where follicular fluid LH concentration was significantly higher in MNIVF cycles as compared to COH cycles. Higher LH concentration suggests that in MNIVF cycles there is a spontaneous LH surge, despite the early administration of HCG in the late follicular phase. In unstimulated IVF, the optimum timing for HCG injection is difficult to determine, as we do not measure LH concentrations continuously over the 24 h. It is known that the start of the LH surge is more important than the LH peak itself. This has already been discussed in a paper by Lenton [11] where cycle-failure rate was 24 % in MNIVF due to the timing of oocyte retrieval which was either too early or too late. Furthermore, fertilization rate and clinical pregnancy rate were found to be dependent on the optimum timing of oocyte retrieval after HCG injection. As LH surge triggers acute inflammation and ROS production in the ovary [47], it is reasonable to expect that higher levels of LH lead to higher ROS production in MNIVF. The exposure of only one follicle to excessive inflammation and thus higher ROS levels could have detrimental effects on oocyte quality and success rates of MNIVF.

The most numerous group of differentially expressed genes in this study consisted of small nucleolar RNAs (*snoRNAs*). There were 18 different types of these genes that showed increased expression in the MNIVF group (Table 3), which represented almost a third (27 %) of all of the differentially expressed genes. Small nucleolar RNAs are a group of non-coding RNAs involved in posttranscriptional modification of ribosomal and small nuclear RNAs. This modification affects the diversity and structural stability of RNA molecule. A number of tissue-specific *snoRNAs* have been identified that are believed to be involved in the regulation of gene expression [48]. Ro et al. [49] identified novel *snoRNAs* that were preferentially expressed in the ovary, suggesting that they might have a role limited to ovarian functions. Further studies are needed in order to reveal their potential physiological role in folliculogenesis. If *snoRNAs* really are important regulators of gene expression during folliculogenesis, their mutations could lead to defects of this process.

Despite numerous studies comparing MNIVF and COH cycles considering embryo morphology [20], serum and follicular fluid endocrine profile [44], embryo implantation rate [50], DNA ploidy of granulosa cells [51], and success rates

[52], information about the differences between MNIVF and COH cycles on molecular level are still scarce. To the best of our knowledge there is only one study analyzing differences in CC gene expression between MNIVF and COH cycles [9]. Their results have shown that the use of COH induces changes in CC gene expression, particularly in genes involved in immune processes, meiosis and ovulation pathways; nevertheless these differences do not seem to have an impact on oocyte competence since no differences were observed in the fertilization ability and early embryo morphology. However, these pathways were not among the overrepresented ones in our study. Among the genes that showed higher expression in MNIVF cycles in our study were zinc finger proteins 737 (*ZNF737*) and *ZNF431*. This finding is in agreement with that of de los Santos, where *ZNF33B* was up-regulated in MNIVF cycles. Zinc finger protein has been shown to be involved in the regulation of meiosis in mammalian oocytes [53]. These findings warrant further studies in order to determine the role of zinc finger protein family in human ovarian folliculogenesis.

Conclusion

Our study shows that the differences in CC gene expression between MNIVF and COH cycles exist, mainly for genes related to oxidation-reduction processes and *snoRNAs*. Since ROS production is more pronounced in MNIVF as compared to COH cycles, it could have a negative effect on the success rates of MNIVF. The reason may lie in the inadequate control of MNIVF cycles and bad timing of oocyte retrieval which leads to the acquisition of poor quality oocytes. Moreover, *snoRNAs*, were largely represented in the group of differentially expressed genes between MNIVF and COH cycles and they should be addressed in future studies in order to improve understanding of their role in the development of ovarian follicles and competent oocytes.

Limitations

The study's limitation is in the small number of samples used for microarray analysis. In addition, insufficient mRNA yield from some of the samples did not allow us to perform qPCR validation on all of the samples used for microarray analysis. The potential importance of our findings warrant further investigation.

Acknowledgements We thank all of the personnel at the Department of Human Reproduction, Division of Gynaecology, University Medical Center Ljubljana, for their contribution to this study. We would especially like to thank the IVF laboratory staff Mses Lili Bačar Kermavner, Jerneja Kmecl, Brigita Valentinčič Gruden and Jožica Mivšek for their work with the biological material.

References

1. Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. *Lancet*. 1978;312(8085):366.
2. Pelinck MJ, Hoek A, Simons AH, Heineman MJ. Efficacy of natural cycle IVF: a review of the literature. *Hum Reprod Update*. 2002;8:129–39.
3. Stanford JB, Mikolajczyk RT, Lynch CD, Simonsen SE. Cumulative pregnancy probabilities among couples with subfertility: effects of varying treatments. *Fertil Steril*. 2010;93:2175–81.
4. Lintsen AM, Eijkemans MJ, Hunault CC, Bouwmans CA, Hakkaart L, Habbema JD, et al. Predicting ongoing pregnancy chances after IVF and ICSI: a national prospective study. *Hum Reprod*. 2007;22:2455–62.
5. Fauser BC, Devroey P, Macklon NS. Multiple birth resulting from ovarian stimulation for subfertility treatment. *Lancet*. 2005;365:1807–16.
6. Rizk B, Smits J. Ovarian hyperstimulation syndrome after superovulation using GnRH agonists for IVF and related procedures. *Hum Reprod*. 1992;7:320–7.
7. Nargund G, Frydman R. Towards a more physiological approach to IVF. *Reprod Biomed Online*. 2007;14:550–2.
8. Janssens RM, Lambalk CB, Vermeiden JP, Schats R, Schoemaker J. In-vitro fertilization in a spontaneous cycle: easy, cheap and realistic. *Hum Reprod*. 2000;15:314–8.
9. de los Santos MJ, García-Láez V, Beltrán-Torregrosa D, Horcajadas JA, Martínez-Conejero JA, Esteban FJ, et al. Hormonal and molecular characterization of follicular fluid, cumulus cells and oocytes from pre-ovulatory follicles in stimulated and unstimulated cycles. *Hum Reprod*. 2012;27:1596–605.
10. Claman P, Domingo M, Garner P, Leader A, Spence JE. Natural cycle in vitro fertilization-embryo transfer at the University of Ottawa: an inefficient therapy for tubal infertility. *Fertil Steril*. 1993;60:298–302.
11. Lenton EA. Natural cycle IVF with and without terminal HCG: learning from failed cycles. *Reprod Biomed Online*. 2007;15:149–55.
12. Paulson RJ, Sauer MV, Francis MM, Macaso TM, Lobo RA. In vitro fertilization in unstimulated cycles: the University of Southern California experience. *Fertil Steril*. 1992;57:290–3.
13. Nargund G, Waterstone J, Bland J, Philips Z, Parsons J, Campbell S. Cumulative conception and live birth rates in natural (unstimulated) IVF cycles. *Hum Reprod*. 2001;16:259–62.
14. Li Q, McKenzie LJ, Matzuk MM. Revisiting oocyte-somatic cell interactions: in search of novel intrafollicular predictors and regulators of oocyte developmental competence. *Mol Hum Reprod*. 2008;14:673–8.
15. Buccione R, Schroeder AC, Eppig JJ. Interactions between somatic cells and germ cells throughout mammalian oogenesis. *Biol Reprod*. 1990;43:543–7.
16. Matzuk MM, Burns KH, Viveiros MM, Eppig JJ. Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science*. 2002;296:2178–80.
17. Devjak R, Fon Tacer K, Juvan P, Virant Klun I, Rozman D, Vrtačnik Bokali E. Cumulus cells gene expression profiling in terms of oocyte maturity in controlled ovarian hyperstimulation using GnRH agonist or GnRH antagonist. *PLoS ONE*. 2012;7:e47106.
18. Assou S, Haouzi D, Mahmoud K, Aouacheria A, Guillemin Y, Pantesco V, et al. A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study. *Mol Hum Reprod*. 2008;14:711–9.
19. 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.
20. Ziebe S, Bangsbo S, Schmidt KL, Loft A, Lindhard A, Nyboe AA. Embryo quality in natural versus stimulated IVF cycles. *Hum Reprod*. 2004;19:1457–60.
21. Ng EH, Lau EY, Yeung WS, Ho PC. Oocyte and embryo quality in patients with excessive ovarian response during *in vitro* fertilization treatment. *J Assist Reprod Genet*. 2003;20:186–91.
22. Grondahl ML, Borup R, Lee YB, Myrholm V, Meinertz H, Sørensen S. Differences in gene expression of granulosa cells from women undergoing controlled ovarian hyperstimulation with either recombinant follicle-stimulating hormone or highly purified human menopausal gonadotropin. *Fertil Steril*. 2009;91:1820–30.
23. Tomazevic T, Gersak K, Meden-Vrtovc H, Drobnic S, Veble A, Valentinčič B, et al. Clinical parameters to predict the success of *in vitro* fertilization-embryo transfer in the natural cycle. *Assist Reprod*. 1999;9:149–56.
24. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol*. 2004;5:R80.
25. Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J R Stat Soc B Met*. 1995;57:289–300.
26. Nogales-Cadenas R, Carmona-Saez P, Vazquez M, Vicente C, Yang X, Tirado F, et al. GeneCodis: interpreting gene lists through enrichment analysis and integration of diverse biological information. *Nucleic Acids Res*. 2009;37:W317–22.
27. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paep A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;3: RESEARCH0034.
28. Lim J, Luderer U. Oxidative damage increases and antioxidant gene expression decreases with aging in the mouse ovary. *Biol Reprod*. 2011;84:775–82.
29. Tatone C, Carbone MC, Falone S, Aimola P, Giardinelli A, Caserta D, et al. Age-dependent changes in the expression of superoxide dismutases and catalase are associated with ultrastructural modifications in human granulosa cells. *Mol Hum Reprod*. 2006;12:655–60.
30. Hamatani T, Falco G, Carter MG, Akutsu H, Stagg CA, Sharov AA, et al. Age-associated alteration of gene expression patterns in mouse oocytes. *Hum Mol Genet*. 2004;13:2263–78.
31. Matos L, Stevenson D, Gomes F, Silva-Carvalho JL, Almeida H. Superoxide dismutase expression in human cumulus oophorus cells. *Mol Hum Reprod*. 2009;15:411–9.
32. Agarwal A, Gupta S, Sharma R. Oxidative stress and its implications in female infertility - a clinician's perspective. *Reprod Biomed Online*. 2005;11:641–50.
33. Shkolnik K, Tadmor A, Ben-Dor S, Nevo N, Galiani D, Dekel N. Reactive oxygen species are indispensable in ovulation. *Proc Natl Acad Sci U S A*. 2010;108:1462–7.
34. Tarin JJ. Potential effects of age-associated oxidative stress on mammalian oocytes/embryos. *Mol Hum Reprod*. 1996;2:717–24.
35. Seino T, Saito H, Kaneko T, Takahashi T, Kawachiya S, Kurachi H. Eight-hydroxy-2'-deoxyguanosine in granulosa cells is correlated with the quality of oocytes and embryos in an in vitro fertilization-embryo transfer program. *Fertil Steril*. 2002;77:1184–90.
36. Du Plessis SS, Makker K, Desai NR, Agarwal A. Impact of oxidative stress on IVF. *Expert Rev Obstet Gynecol*. 2008;3:539–54.
37. Attaran M, Pasqualotto E, Falcone T, Goldberg JM, Miller KF, Agarwal A, et al. The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. *Int J Fertil Womens Med*. 2000;45:314–20.
38. Jancar N, Kopitar AN, Ihan A, Virant Klun I, Bokali EV. Effect of apoptosis and reactive oxygen species production in human granulosa cells on oocyte fertilization and blastocyst development. *J Assist Reprod Genet*. 2007;24:91–7.
39. Yu BP. Cellular defenses against damage from reactive oxygen species. *Physiol Rev*. 1994;74:139–62.
40. van Montfort AP, Geraedts JP, Dumoulin JC, Stassen AP, Evers JL, Ayoubi TA. Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis. *Mol Hum Reprod*. 2008;14:157–68.

41. Lubberda Z. The role of glutathione in mammalian gametes. *Reprod Biol*. 2005;5:5–17.
42. Zuelke KA, Jones DP, Perreault SD. Glutathione oxidation is associated with altered microtubule function and disrupted fertilization in mature hamster oocytes. *Biol Reprod*. 1997;57:1413–20.
43. Mannervik B, Danielson UH. Glutathione transferases. Structure and catalytic activity. *Crit Rev Biochem Mol Biol*. 1988;23:283–337.
44. Rabahi F, Brûlé S, Sirois J, Beckers JF, Silversides DW, Lussier JG. High expression of bovine alpha glutathione S-transferase (GSTA1, GSTA2) subunits is mainly associated with steroidogenically active cells and regulated by gonadotropins in bovine ovarian follicles. *Endocrinology*. 1999;140:3507–17.
45. Zelko IN, Mariani TJ, Folz RJ. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radic Biol Med*. 2002;33:337–49.
46. Jančar N, Virant-Klun I, Vrtačnik BE. Serum and follicular endocrine profile is different in modified natural cycles than in cycles stimulated with gonadotropin and gonadotropin-releasing hormone antagonist. *Fertil Steril*. 2009;92:2069–71.
47. Richards JS, Russell DL, Ochsner S, Espey LL. Ovulation: New dimensions and new regulators of the inflammatory-like response. *Annu Rev Physiol*. 2002;64:69–92.
48. Bratkovič T, Rogelj B. Biology and applications of small nucleolar RNAs. *Cell Mol Life Sci*. 2011;68:3843–51.
49. Ro S, Song R, Park C, Zheng H, Sanders KM, Yan W. Cloning and expression profiling of small RNAs expressed in the mouse ovary. *RNA*. 2007;13:2366–80.
50. Ata B, Yakin K, Balaban B, Urman B. Embryo implantation rates in natural and stimulated assisted reproduction treatment cycles in poor responders. *Reprod Biomed Online*. 2008;17:207–12.
51. Gersak K, Lavrencak J, Us-Krasovec M. DNA ploidy of human granulosa cells from natural and stimulated *in vitro* fertilization cycles. *Fertil Steril*. 2000;74:158–61.
52. MacDougall MJ, Tan SL, Hall V, Balen A, Mason BA, Jacobs HS. Comparison of natural with clomiphene citrate-stimulated cycles in *in vitro* fertilization: a prospective, randomized trial. *Fertil Steril*. 1994;61:1052–7.
53. Kim AM, Vogt S, O'Halloran TV, Woodruff TK. Zinc availability regulates exit from meiosis in maturing mammalian oocytes. *Nat Chem Biol*. 2010;6:674–81.