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DNA repair signalling pathway genes are overexpressed in poor-quality pre-implantation human embryos with complex aneuploidy

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ABSTRACT

Objective: Chromosomal abnormalities and poor quality are correlated with DNA damage in the pre-implantation stage in humans. This study aimed to explore the altered expression of DNA damage signalling pathways – including apoptosis, cell cycle and DNA repair pathways – in poor-quality pre-implantation human embryos with complex aneuploidy.

Study design: Surplus Day 4 embryos from candidates undergoing pre-implantation genetic screening were pooled into two groups. Group 1 included good-quality embryos that had simple aneuploidy, a single chromosome [according to fluorescence in situ hybridization-based pre-implantation genetic diagnosis (PGD) on Day 3], a normal rate of cell division, and graded as A or B (excellent to good). Group 2 included embryos with more than one aneuploid chromosome on PGD on Day 3, an abnormal rate of cell division, and graded as C or D (fair to poor). Gene expression of DNA damage signalling pathways was analysed using a real-time polymerase chain reaction-based array, which included 84 genes after specific pre-amplification of cDNA by a primer mix, including all array genes.

Results: In Group 2, five of the 84 genes studied showed significant overexpression ($p < 0.05$): *MSH3*, *XRCC1*, *RAD50*, *LIG1* and *CDK7*. Alterations were in agreement with genetic relationships in pathway analyses on DAVID.

Conclusions: The five overexpressed genes are involved in DNA repair. Therefore, in comparison with cell cycle control and apoptotic pathways, DNA repair pathways are more activated in poor-quality pre-implantation human embryos with complex aneuploidy. This suggests that the dominant response to DNA damage in such embryos is DNA repair rather than cell division or apoptosis.

1. Introduction

The frequency of chromosomal mosaicism [1–3] and instabilities ranging from whole-chromosome aneuploidies to segmental aberrations [4,5] is known to be high in the pre-implantation stage in human embryos. There is an association between chromosomal instability and aneuploidies in human cells [6]. Chromosomal abnormalities, independent of their type, are related to the morphology and development of human embryos [1,2,7,8]. DNA damage is incident in poor-quality and aneuploid embryos [8–10], and fragmentation, an important morphological parameter, is strongly associated with chromosomal abnormalities. Fragmentation exceeding 35% is related to a chromosomal abnormality rate of 70–90%. Embryos with less than seven cells or more than nine cells on Day 3 are also strongly associated with chromosomal abnormalities [7].

Several cellular and molecular mechanisms are involved in the detection of, and response to, DNA damage for the maintenance of DNA integrity [11]. The success of assisted reproductive technology (ART) with pre-implantation genetic screening (PGS), particularly for women of advanced maternal age, is a point of discussion [12]. Gene expression studies in ART have been considered recently for the assessment of embryo quality [13]. These studies

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use micro-arrays to focus on different sources including oocytes [14,15], blastocysts [14] and cumulus cells [15]. Comparison of DNA repair gene expression in oocytes and blastocysts has shown differential expression of some of the genes at these two stages [15]. Comparison of gene expression in normal and aneuploid oocytes using micro-array also showed altered expression related to meiotic aneuploidies [14]. Wells et al. compared the expression of nine genes related to DNA damage between good- and poor-quality pre-implantation embryos, and concluded that a causal relationship may exist between changes in gene expression and the formation of abnormal embryo morphologies [10].

As the frequency of poor quality and chromosomal aneuploidies is high in the pre-implantation stage, and such embryos are susceptible to DNA damage, gene expression studies to determine which genes are correlated with the DNA damage response could further our knowledge on pre-implantation life. This study compared gene expression in poor-quality embryos with complex aneuploidy with gene expression in good-quality embryos with simple aneuploidy. A comparison study was undertaken using real-time polymerase chain reaction to investigate gene expression in 84 genes considered to be involved in apoptosis, DNA repair and cell cycle control.

2. Materials and methods

2.1. Sample preparation

This study was approved by the Ethics Committee of Royan Institute, Iran, and was performed on surplus embryos from PGS candidates who gave their informed consent. The embryo biopsy for PGS was performed 72 h after fertilization. The inclusion criteria for this study were: mother's age $\leq$37 years, normal karyotype for both parents, stimulation by the long protocol described previously [16], and fertilization by intra-cytoplasmic sperm injection. As advanced maternal age could change the gene expression profile [17], including genes involved in the cell cycle, embryos of mothers aged >37 years were excluded. Regarding the possible effect of the ovarian stimulation protocol on the chromosomal abnormality rate [18], only patients stimulated using the long protocol were included in this study. PGS was performed with two panels of three-colour fluorescence in situ hybridization (FISH) probes: locus-specific identifier (LSI) 21, chromosome enumeration probe (CEP) 18 and LSI 13 for the first round; and CEPX, CEP Y and CEP15 (Vysis, USA) for the second round, as described previously [19].

Surplus Day 4 embryos were used in this study. The protocol for embryo freezing was performed in accordance with similar studies [14,15]. In order to obtain reliable amounts of mRNA, 15–20 selected embryos for each biological replicate were pooled. Of note, the amount of mRNA decreased from zygote to blastocyst [20].

2.2. Embryo classification

Morphological characteristics and pre-implantation genetic diagnosis (PGD) results were used to classify the embryos. Good-quality embryos with simple aneuploidy were allocated to Group 1, and poor-quality embryos with complex aneuploidy were allocated to Group 2.

The inclusion criteria for Group 1 were: graded A or B (excellent to good), normal rate of cell division, seven or eight cells on Day 3 post-fertilization, and FISH results indicating aneuploidy in a single chromosome. Embryos were graded based on their fragmentation pattern and morphological characteristics, which included blastomere expansion, compaction, equal size and absence of vacuoles. Due to limited access to normal good-quality embryos for research use and ethical reasons, the study was not based on normal embryos. Inclusion criteria for Group 2 were: graded as C or D (fair to poor), abnormal rate of cell division, less than seven or more than nine cells on Day 3 post-fertilization, and aneuploidy in two or more chromosomes.

2.3. Gene expression study

Three independent biological replicates were studied in each group. For each biological replicate, 15–20 embryos were pooled and RNA was isolated using the Absolutely Nanoprep Kit (Stratagene, USA). After the RNA concentration was measured using Nanodrop 2000 (Thermo, USA), 2 ng of RNA from each biological replicate was used for the pre-amplification cDNA synthesis step by the RT^2 Preamp cDNA Synthesis Kit (SABiosciences, USA). These kits use specific primer mixes including the 84 genes of the signalling pathway and the five housekeeping genes. A260/A280 > 1.8 is considered to indicate good quality when analysing RNA using Nanodrop.

A 96-well PCR array (Human DNA Damage Signaling Pathway RT^2 Profiler^{TM}, SABiosciences, USA) was used to compare genes related to DNA damage. The PCR array contained pre-dispensed primer sets including the 84 genes related to the DNA damage signalling pathway. These genes belong to three functional groups: apoptosis, cell cycle control and DNA repair. The quantitative PCR reaction was performed using the Applied Biosystems 7500 system (Applied Biosystems, USA). Two technical replicates were performed for each biological replicate. Reactions with a threshold cycle >35 were considered to be undetectable, and those with unspecific amplification were excluded from the study.

2.4. Bioinformatic approaches and statistical analysis

RT^2 profiler PCR array data analysis web-based software (Version 3.5) was used for data analysis. As proposed by the manufacturer, five housekeeping genes were considered in order to select the best housekeeping gene for data normalization. Differences in expression between groups were calculated using the $2^{-\Delta\Delta Ct}$ method. p-Values were calculated based on Student's t-test, and $p < 0.05$ was considered to indicate significance. $p < 0.1$ and $p > 0.05$ were taken to indicate a marginal difference in expression.

The functional analysis of genes with significant ($p < 0.05$) and marginal ($0.1 < p < 0.05$) differences in expression was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID Bioinformatics Resources 6.7; http://david.abcc.ncifcrf.gov), linked to the Kyoto Encyclopedia of Genes and Genomes (KEGG) for pathway identification.

A minimum of two counts and a Benjamini value < 0.05 were considered to indicate the significant relevance of pathways.

3. Results

One hundred and three out of 437 surplus embryos were included in this study: 46 in Group 1 and 57 in Group 2. Tables 1 and 2 show the distribution of embryos in both groups based on their grade, FISH results and cell numbers.

Table 1
Distribution of embryos in Group 1 by grade.[a]

Replicate number		I	II	III
Grade	A	2	3	3
	AB	6	7	5
	B	8	5	7
Patients (n)		13	12	13
Embryos (n)		16	15	15

[a] Data from three biological replicates.