Reduction of connexin 43 in human cumulus cells yields good embryo competence during ICSI

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Abstract

Purpose The purpose of this study was to predict developmental competence of human oocytes during ICSI via analysis of connexin 43 (Cx43) in cumulus cells surrounding mature oocytes.

Materials and methods Human cumulus cells were manually separated from the oocyte–cumulus complex under a microscope. Cx43 mRNA was expressed by real-time quantitative polymerase chain reaction (RT-PCR) measurement in cumulus cells.

Results There was no significant relationship between expression of Cx43 and fertilisation or cleavage rate. However, Cx43 expression was lower in the good morphology group (blastomeres >7 cells with fragmentation <10% on day 3) when compared to the other groups ($p=0.035$).

Conclusions These results suggest that full reduction of Cx43 expression on cumulus cells at the time of oocyte collection during ICSI is essential for developmental competence of human oocytes.

Keywords Connexin 43 · Gap junction · Cumulus cells · IVF · Embryo development

Introduction

The recent literature focuses its attention on the role of factors involved in the regulation of nuclear and cytoplasmic maturation as well as cumulus expansion, mainly those factors contained in the homologous preovulatory follicular fluid [1–3]. The evidence in the literature has shown the channels between granulosa cells and the oocyte not only permit the transfer of metabolites for growth and development, but also play an important role in the maintenance of meiotic arrest of the oocyte [4]. The cumulus cells are functionally interconnected by gap junctions. The fundamental unit of gap junction is the connexon, which is a hexamer of proteins called connexins. The process of cumulus expansion is accompanied by modifications of gap junctions, which contain transmembrane channels formed by hexamers of proteins belonging to connexin family [3]. By facilitating the transfer of ions and small molecules from cell to cell, the gap junctions are thought to modulate numerous physiological processes, including folliculogenesis and oogenesis [5]. Meiotic resumption is induced by the disruption of gap junctions within cumulus cells, which blocks the conduction of meiosis inhibitory signals from the outer cumulus cells to oocyte [6], and is associated with the reduction of gap junctional protein connexin 43 (Cx43) in the outer layers of cumulus cells [7]. Initiation of meiotic resumption is associated with the reduction of Cx43 level in the cumulus–oocyte-complex (COC).

The recognition of oocyte maturation and regulation of cumulus cells, and the harvesting and culturing of numerous good-quality oocytes has clinical significance for improving fertility treatment. Objective of this study is to clarify whether developmental competence of human

Capsule Reduction of connexin-43 expression on cumulus cells at the time of oocyte collection during ICSI is essential for developmental competence of human oocytes.

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oocytes in ICSI can be predicted from analysis of Cx43 in cumulus cells surrounding mature oocytes. Relationship between the expression of Cx43 mRNA in human cumulus cells at the time of oocyte collection via real-time quantitative polymerase chain reaction (RT-PCR) and day 3 morphological embryo quality during ICSI cycles was examined.

Materials and methods

Subjects

The subject group comprised of 29 women undergoing ICSI. Their ages ranged from 29 to 44 years (mean, 36.2 years), and the indications for ICSI included tubal absence or occlusion, ovulation disorders, endometriosis, oligozoospermia, and idiopathic infertility. All patients gave written informed consent to participate in this study.

IVF procedure

The ovaries were stimulated with clomiphene citrate (Clomid[®]; Shionogi, Osaka, Japan) and human menopausal gonadotropin (hMG; HMG Injection TEIZO[®], Teikoku-zouki, Tokyo, Japan) or pure FSH (Fertinorm P[®]; Serono, Tokyo, Japan) after pituitary desensitization with gonadotropin-releasing hormone agonist (GnRH-a; Suprecur; Aventis Pharma, Tokyo, Japan), according to the long protocol. Ovarian follicle diameter was assessed by transvaginal sonography, and gonadotropins were administered daily until the second largest follicle reached a diameter of 18 mm. When the follicle grew beyond that diameter, human chorionic gonadotropin (hCG; Gonatropin[®]; Teikoku-zouki, Tokyo, Japan) 10,000 IU was administered between 30 and 35 h later, oocytes were retrieved under ultrasonographic guidance. Cumulus cells were manually separated from COC under a microscope after oocyte retrieval.

Oocyte and embryo culture

Oocytes were cultured in dishes with 500 μ l P-1TM medium (preimplantation-1 medium; Irvine Scientific, CA, USA) under mineral oil with 10% human serum at 37°C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂. All oocytes underwent intracytoplasmic sperm injection (ICSI).

Embryo quality assessment

Three days after insemination, embryo quality (EQ) was investigated under a microscope with a micro-manipulator. EQ was categorized as: good morphology group if the

number of blastomeres was greater than seven cells with less than 10% fragmentation; or other groups.

RNA preparation and reverse transcription, and real-time quantitative TaqMan PCR

Total RNA was extracted from cumulus cells using phenol-chloroform methods. The cDNAs were reverse-transcribed using Takara RNA PCR kit (Takara, Shiga, Japan) with 500 ng total RNA, Oligo dT-Adaptor Primer and AMV Reverse Transcriptase according to manufacturer's recommendation. RT thermal profile were 55 and 99°C for 30 and 5 min respectively. Real-time quantitative PCR was performed using QuantiTect Multiplex PCR Kit (QIAGEN, Hilden, Germany) in using 20 μ l reaction containing 2 μ l cDNA, 2×QuantiTect Multiplex PCR Master Mix and 0.4 mM of each primer by ABI PRISM 7900 (Perkin-Elmer Applied Biosystems, CA, USA). The primer for PCR reactions was designed based on human Cx43 cDNA sequences which is available in the Genome Database. The upstream primer (5'-ACTTGCCTTTTCATTT TACTTC-3') and the downstream primer (5'-CCTGGGCACCACTCTTTT-3') predict a 88 bp DNA fragment. GAPDH cDNA fragments were amplified as positive controls. Two RT-PCR reactions were performed with each sample.

Statistics

Comparisons between the groups were made by Mann-Whitney's *U* test. The data were entered into a computerized data analysis program (StatView for windows, SAS Institute, Inc., Cary, NC). For all analyses, $p < 0.05$ was considered statistically significant.

Results

A total of 105 samples of cumulus cells in each follicle from the 29 patients were analysed for Cx43 expression. In these samples, 64 (61.0%) were fertilised; 59 (92.2%) of fertilised oocytes cleaved. Seventeen (58.6%) patients became pregnant after embryo transfer.

Quantitative RT-PCR

The baseline expression of cx43 and PR mRNA in cumulus cells was detected in all samples. Amplification of increasing amounts of plasmid (diluted 1:10, 100, 1,000, 10,000), containing the target sequence was performed. Fluorescence intensity was plotted against the logarithm of the plasmid concentration to construct a standard curve.

Table 1 Correlation between Cx43/GAPDH and fertilisation, cleavage, day 3 morphological embryo quality assessmentData: mean±standard error, ():n, *p*-value: Mann-Whitney's *U* test.

Cx43/GAPDH	Fertilised (64)	Unfertilised (41)	<i>p</i> value
	0.42±0.13	0.54±0.13	0.079
Cx43/GAPDH	Cleaved (59)	Not cleaved (5)	<i>p</i> value
	0.46±0.010	0.49±0.12	0.190
Cx43/GAPDH	Good embryo (18)	Others (41)	<i>p</i> value
	0.21±0.18	0.54±0.20	0.035

The mRNA integrity was controlled by amplification of the GAPDH housekeeping gene.

Cx43 and embryo development

Relationship between expression of Cx43 in cumulus cells and embryo development is shown in Table 1. Expression of Cx43 was not significantly different between the fertilised group and the unfertilised group. The expression of Cx43 was not significantly different between the group that cleaved, and the group that did not cleave. Cx43 expression was significantly lower in the good morphology group than the other group.

Discussion

Previous studies suggested that gap junction communications between the oocyte and cumulus cells might play an important role in regulating meiotic resumption and cytoplasmic maturation [8, 9]. Evidence has emerged to support the involvement of various locally produced factors as co-regulators of folliculogenesis and oocyte nuclear and cytoplasmic maturation in addition to extrinsic regulation by pituitary gonadotropins and metabolic hormones [9]. The oocyte not only receives signals from the somatic compartment but also transmits molecules necessary for the growth and development of these cells [10–13].

In the mouse, successful fertilisation was correlated with the quantity and quality of the expanded cumulus mass [14]. In the mare, cumulus expansion in oocytes retrieved from excised ovaries of slaughtered mares has been related to granulosa cell apoptosis with no relation to follicle size. Expanded oocytes issuing from apoptotic follicles show increased meiotic competence, but not increased activation rate after ICSI [15]. Optimal expansion of the cumulus mass may appear to be essential for oocyte and embryo maturation. In this study, Cx43 down regulation was strongly correlated with embryo quality compared to fertilisation and cleavage. Expansion of cumulus cells and down regulation of Cx43 might more affect cytoplasmic maturation than regulating meiotic resumption.

It is suggested that Cx43 was involved in germ cell proliferation from early stages of folliculogenesis [5]. Previous studies demonstrated an increase in the amount of Cx43 in the large antral follicles in a stage at which serum concentrations of FSH are relatively elevated [16, 17]. On the other hand, the preovulatory surge of serum concentrations of LH is followed by a drop at the level of Cx43 mRNA and reduction in the amount of its corresponding protein [3, 16–19]. In equine, porcine and rat cumulus cells, initiation of meiotic resumption is associated with reduction of Cx43 protein levels [7, 20, 21]. In this study, we demonstrated human good embryos were retrieved from COC whose Cx43 expressions were low. In response to the preovulatory surge of LH, cell-to-cell communication in the follicle is interrupted, the oocyte resumes meiosis, the mature oocyte is released, and the follicle undergoes luteinization [22, 23]. It has been recognized that cAMP transferred from the cumulus cells via gap junction is an inhibitory factor involved in the meiotic resumption and the regulation of meiotic progression beyond the metaphase I (MI) stage [24, 25]. The progesterone bound newly synthesized PR in cumulus cells was associated with reduced proliferative activity of cumulus cells and COC expansion, and closed the gap junctional communication with in cumulus cells [26–29]. In our previous study, although PR expression in cumulus cells at the time of oocyte collection was neither significantly different between the fertilised group and the unfertilised group nor between the group which cleaved and group which did not cleave, PR expression was lower in the day 3 good morphology group than in the other group [30]. In view of present results and previous studies, we suggest that full reduction of PR and Cx43 expression in cumulus cells at the time of oocyte collection may be essential to acquire developmental competence of the human oocyte, and that oocyte maturation is controlled by the expression and the reduction of PR and Cx43.

In conclusion, day 3 good embryos were retrieved from COC whose Cx43 expressions were lower than the others. Our findings are first report in human ICSI cycle and suggest that the full reduction of total Cx43 expression is essential to acquire developmental competence.

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