5-24-2008

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Connexin expression and gap junctional coupling in human cumulus cells: contribution to embryo quality

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Abstract

Gap junctional coupling among cumulus cells is important for oogenesis since its deficiency in mice leads to impaired folliculogenesis. Multiple connexins (Cx), the subunits of gap junction channels, have been found within ovarian follicles in several species but little is known about the connexins in human follicles. The aim of this study was to determine which connexins contribute to gap junctions in human cumulus cells and to explore the possible relationship between connexin expression and pregnancy outcome from *in vitro* fertilization (IVF). Cumulus cells were obtained from IVF patients undergoing intracytoplasmic sperm injection (ICSI). Connexin expression was examined by RT-PCR and confocal microscopy. Cx43 was quantified by immunoblotting and gap junctional coupling was measured by patch-clamp electrophysiology. All but five of 20 connexin mRNAs were detected. Of the connexin proteins detected, Cx43 forms numerous gap junction-like plaques but Cx26, Cx30, Cx30.3, Cx32, and Cx40 appeared to be restricted to the cytoplasm. The strength of gap junctional conductance varied between patients and was significantly and positively correlated with Cx43 level, but neither was correlated with patient age. Interestingly, Cx43 level and intercellular conductance were positively correlated with embryo quality as judged by cleavage rate and morphology, and were significantly higher in patients who became pregnant than in those who did not. Thus, despite the presence of multiple connexins, Cx43 is a major contributor to gap junctions in human cumulus cells and its expression level may influence pregnancy outcome after ICSI.

Keywords: gap junction, conductance, connexin43, pregnancy

Introduction

Gap junctions are clusters of intercellular membrane channels that allow direct exchange of small molecules, including nutrients, metabolites, and second messengers, between cells [1,2]. An individual gap junction channel is formed when two hemichannels, one from each cell, dock end-to-end to form an intercellular channel. Hemichannels are called connexons and each is a hexamer of subunits called connexins (Cx). In mammals, connexins are encoded by a multigene family with 20 or more members. Individual connexins are distinguished by their sizes: Cx43, for example, is a ~43 kD protein whereas Cx40 is a ~40 kD protein. Gap junction channels composed of different connexins differ in their permeability to specific signaling molecules, properties that are assumed to underlie the physiological roles played by gap junctions in different cell types [3, 4].

Ovarian folliculogenesis requires complex regulatory mechanisms involving both endocrine and intra-ovarian signaling pathways. In developing follicles, gap junctions couple the growing oocyte and its surrounding granulosa cells into a functional syncytium allowing amino acids, glucose metabolites, and nucleotides to be transferred to the oocyte [5]. In addition, signals that regulate meiotic maturation of fully grown oocytes (including Ca^{2+} and cAMP) are thought to pass through the oocyte-granulosa cell gap junctions [6, 7]. Recent findings from gene expression studies in several species and gene targeting in mice have implicated gap junctional intercellular communication (GJIC) in follicular development and have suggested its involvement in female infertility [8].

Multiple connexins have been identified in ovarian follicles in several species [8]. In the mouse, the specific functions of individual connexins have been confirmed by targeted gene ablation. Cx43 is the pivotal connexin expressed in mouse granulosa cells, where it plays an indispensable role: granulosa cells from Cx43 knockout mice do not show evidence of gap junctional coupling, follicular growth is impaired, and the oocytes fail to achieve meiotic competence [9-11]. In contrast, Cx37 is the connexin that forms the gap junctions coupling the oocyte with surrounding granulosa cells. Loss of this connexin abolishes oocyte-granulosa cell coupling resulting in oocyte loss and premature luteinization of the follicles [12]. Therefore, analysis of mouse connexin knockouts has clearly shown that impairment of gap junctional coupling within the developing follicle is associated with diminished oocyte quality. Whether connexins play such an important role in human oogenesis remains unknown.

As a first step in answering this question, we sought to determine which connexins contribute to gap junctional coupling in human cumulus cells. To date, other than Cx43 and the mRNAs encoding Cx37 and Cx45 [13, 14], the connexins in human follicles have not been identified. We then went on to study the localization of connexins in human cumulus cells to determine which connexin(s) is the predominant one for contributing to gap junctions. Having identified a promising candidate, we tested the hypothesis that clinical outcome from in vitro fertilization is related to the level of expression of this connexin and to the extent of gap junctional coupling among the cumulus cells.

Materials and methods

Patients

Patients in this study were undergoing treatment in the Reproductive Endocrinology and Infertility Program at the London Health Sciences Centre, London, Ontario, Canada. The study design was approved by the Health Sciences Research Ethics Board of the University of Western Ontario and all patients gave informed consent. The standard long agonist protocol was used for ovarian stimulation. Briefly, pituitary downregulation was achieved with GnRH agonist (nafarelin acetate; Pfizer, San Juan, PR) treatment for 2 weeks, followed by stimulation of follicular growth with recombinant FSH until 4 to 5 leading follicles were 1.8 to 2.0 cm in diameter. Oocyte maturation was then triggered with recombinant choriogonadotropin (Ovidrel; EDM Serono, Rockland, MA), followed by retrieval 36 hours later. Cumulus granulosa cells were collected from oocytes being prepared for ICSI with day 3 embryo transfer. Clinical data, including mature oocyte rate (MII rate), fertilization rate, transferable rate (% embryos with more than 5 blastomeres and good morphology on day 3), implantation rate (ratio of number of fetuses to number of embryos transferred), and pregnancy outcome (determined by ultrasound 40 days after oocyte retrieval) were obtained by clinical staff, but the research team were blind to these outcomes until all data had been collected for all patients. A total of 115 women donated their cumulus cells for this study. All cumulus cells from each patient's oocytes were considered as one sample. Eleven samples were used for RT-PCR, 26 samples for immunofluorescence, 81 samples for western blotting, and 42 samples for gap junctional coupling assay (some samples were used for more than one type of analysis).

All products for this study were purchased from Invitrogen Canada Inc. (Burlington, ON, Canada) unless specially mentioned.

Cumulus cell culture

Cumulus cells were washed twice with culture medium consisting of DMEM/F12 (1:1) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin. The cells were grown on glass coverslips treated with 0.358 mg/mL collagen (BD Biosciences, Mississauga, ON) and cultured at 37°C, 5% CO_2 in air for no more than 48 hours.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA from cumulus cells was extracted using RNeasy® Mini Kit (Qiagen, Mississauga, ON) according to the manufacturer's instructions. Before reverse transcription (RT), the total RNA was digested with DNase I to remove genomic DNA. The first-strand cDNA was synthesized with Superscript II reverse transcriptase and oligo (dT) as primer. As internal controls for RT, samples without RNA or without reverse transcriptase were prepared in parallel. PCR reaction conditions were optimized for each set of primers (Table 1), with cycle phases as follows: denaturation, 45 sec at 94°C; annealing and extension, 45-60 sec at 72°C. All PCR reactions were performed in a final volume of 25 μl containing 2 μl of the first strand cDNA, 200 μmol/L dNTPs, 1U Taq polymerase, the appropriate volume of 50 mM $MgCl_2$, and 10 pmol of each primer. As negative controls for PCR, samples without first-strand cDNA or without Taq enzyme were used.

Immunofluorescence microscopy

Cells grown on glass coverslips were fixed with pre-chilled methanol/acetone (4:1) at 4°C for 20 min and then rinsed with phosphate-buffered saline (PBS) and prepared for immunostaining as previously described [15]. Briefly, the cells were blocked with washing buffer containing 3% BSA (w/v) for 1 h, immunolabeled with primary antibody for 1 h, washed with PBS, and immunolabeled with appropriate secondary antibody for 1 h in the dark. For double-immunolabeling of connexins, cells were treated with the first primary antibody for 1 h and then with Texas Red-conjugated secondary antibody for 1 h, followed by treatment with the second primary antibody for 1 h and finally by an Alexa Fluor®-conjugated secondary antibody. Several washes were interposed between the different antibody incubations. The data for primary and secondary antibodies are listed in Table 2. Cells were washed in PBS and the nuclei stained with 0.1% Hoechst for 10 min followed by washes with PBS and double distilled H_2O. The coverslips were mounted on slides with Airvol (Air Products & Chemicals, Inc., Allentown, PA) before storage at 4°C. The cells were imaged using a Zeiss (Thornwood, NY) LSM 510 META confocal microscope. Fluorescent signals were captured after excitation with 488, 543, or 730 nm laser lines. Digital images were prepared using Zeiss LSM and Adobe Photoshop 7.0 software.

Western blotting

Whole cell proteins were extracted with lysis buffer containing 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L sodium chloride, 0.02% sodium azide, 100 μg/mL phenylmethylsulfonyl fluoride, 1% NP-40, 0.1% SDS, 1 μg/mL aprotinin and 0.5% sodium deoxycholate. Samples were used for two or three experiments depending on

the number of cells obtained from the patient. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfout, Buckinghamshire, England). The membrane was blocked with 5% nonfat milk (w/v) in TBST for 1 h, and subsequently probed with anti-Cx43 antibody (1:5,000; Sigma, Oakville, ON) overnight at 4°C followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000; Biolynx Inc., Brockville, ON) for 1 h. Antibody binding was detected by ECL™ Western Blotting Detection Reagent (Amersham Biosciences, Little Chalfout, Buckinghamshire, England). The membrane was then stripped and re-probed with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:400; Chemicon International Inc., Temecula, CA) and anti-vimentin antibody (1:500; Sigma) for 1 h respectively then incubated with HRP-conjugated secondary antibody (1:5,000) for detection with the Amersham ECL™ Reagent. The relative intensity of Cx43 bands was determined by reference to the GAPDH and vimentin bands, and quantified using Quantity One software (Bio-Rad Laboratories (Canada) Ltd, Mississauga, ON).

Gap junctional conductance measurement

Single-electrode whole cell patch-clamp recording was used to measure cumulus cell membrane capacitance and gap junctional conductance as described [11]. Briefly, pipettes were made from borosilicate glass capillaries using a two-stage pipette puller (PP-83; Narishige, Tokyo, Japan). The intracellular pipette solution contained 70 mM KCl, 70 mM CsCl, 2 mM EGTA, 4 mM $MgCl_2$, 5 mM TEA-Cl^-, and 10 mM HEPES,

pH 7.3, and pipettes had a resistance of 3–5 MΩ. Cells on coverslips were transferred to a 2-ml recording chamber mounted on the stage of an inverted microscope (Olympus IMT-2). They were bathed in solution containing 140 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 20 mM HEPES, pH 7.4. Voltage clamp for whole-cell recordings was carried out with an Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA). Voltage clamping was applied to a single cell in a cluster with 15-20 cells. A depolarization voltage pulse (10 mV, 120-ms duration) was used to generate a transient capacitive current. The peak current and the steady-state current were measured. Currents were high-cut filtered at 10 kHz and digitized at 100 kHz. The experiment was repeated for at least four times for every cumulus cell sample. The estimated conductance between the patched cell and its surrounding cells was calculated. Data acquisition and analysis were performed using the Digidata 1200A interface and pClamp6 software (Axon Instruments).

Statistical analysis

Relative levels of Cx43 protein normalized to GAPDH or vimentin were calculated and compared with gap junctional coupling strength as determined by conductance assay. Similarly, relative Cx43 levels were compared with pregnancy outcome based on ultrasound. Overall, 35 patients in this study became pregnant and 46 did not, with the age of the former group being 31.2 ± 0.71 and that of the latter group being 34.5 ± 0.69 (mean ± SEM). To carry out these comparisons, the patients were divided into two groups based either on whether their mean Cx43 or intercellular conductance measurement fell above or below the population mean, or whether they became

pregnant. Age of patients was one of factors analyzed in this study, and all patients were divided into three age groups (30 and under, 31-35, 36 and above) to look for any association between Cx43 or conductance level and patient age. Statistical analysis (one-way ANOVA) was performed using the Statistical Package for Social Science (SPSS 13.0 for Windows; SPSS Inc., Chicago, IL). $P < 0.05$ was considered to be significant.

Results

Detection of connexin mRNAs

RT-PCR was used to survey cumulus cells for the presence of mRNAs encoding 20 connexins (Cx25, Cx26, Cx30, Cx30.2, Cx30.3, Cx31, Cx31.1, Cx31.9, Cx32, Cx36, Cx37, Cx40, Cx40.1, Cx43, Cx45, Cx46, Cx47, Cx50, Cx59, and Cx62). A representative gel illustrating the PCR products is shown in Figure 1. All but five connexin mRNAs (Cx30.2, Cx31.9, Cx40.1, Cx46, and Cx47) were detected, and this result was consistent for all 11 samples surveyed by RT-PCR. No specific bands were found in negative control samples, and all PCR products were confirmed by sequencing (data not shown).

Detection and localization of connexin proteins

Based on our detection of multiple connexin mRNAs by RT-PCR, we used available antibodies to explore the expression of nine of the cognate proteins (Cx26, Cx30, Cx30.3, Cx31, Cx32, Cx37, Cx40, Cx43, and Cx45) by immunofluorescence. The specificity of the antibodies, with the exception of Cx30.3 and Cx31, had been

confirmed using HeLa cells engineered to overexpress the proteins. Cx26, Cx30, Cx30.3, Cx32, Cx40, and Cx43 were detected in cumulus cells of all patient samples, but Cx31, Cx37, and Cx45 were not detected despite the presence of their mRNAs (Fig. 2). Cx43 formed a large number of gap junction-like plaques between the cells (arrows in Fig. 2). In contrast, Cx26, Cx32, and Cx40 mainly localized in the cytoplasm with few membrane plaques being found, while Cx30 and Cx30.3 were restricted to the cytoplasm. Negative controls without primary antibody did not produce positive signals (data not shown).

To explore the possibility of connexin co-localization, we used double-labelled immunofluorescence. The results of these experiments were consistent with those shown in Figure 2, in that Cx26 and Cx32 were restricted to the cytoplasm while Cx43 formed numerous plaques between the cells (Fig. 3). At least two of Cx26, Cx32, and Cx43 could be found in the same cells, but Cx26 and Cx32 did not co-localize with Cx43. Interestingly, Cx26 and Cx32 co-localized in the cytoplasm (Fig. 3).

Quantification of Cx43

Cx43 was detected in all 81 samples tested. A representative western blot is shown as Figure 4A. The relative amount of Cx43 protein was determined by reference to two internal controls, vimentin and GAPDH, revealing variation in Cx43 expression level between cumulus cells of different patients. The relative Cx43 protein levels determined from the two internal controls were fairly consistent between patients (Fig. 4B).

Quantification of gap junctional conductance

Patch clamp electrophysiology provides a sensitive and quantifiable means of measuring electrical conductance between cells. A 10 mV depolarizing voltage pulse in a voltage-clamped single cumulus cell resulted in a current transient characterized by a rapid onset to reach peak current, followed by a rapid decay to steady state current that was almost identical to the holding current. The changes in decay time constant and steady-state current in a cluster of interconnected cumulus cells provide a quantitative measure of conductance due to gap junctional coupling of the cells [11]. The estimated conductance was taken as a measure of the total gap junctional conductance between the cells. This conductance varied between patients, although most patients showed conductance above 80 nS (Fig. 5).

Relation between Cx43 and gap junctional conductance

Given that Cx43 was the only connexin detected that formed numerous gap junction-like plaques between the cumulus cells, we sought to determine whether the strength of gap junctional conductance is related to the level of Cx43. In Figures 6A and 6B, gap junctional conductance is plotted against the Cx43 level normalized to vimentin and GAPDH, respectively, for each patient. Linear regression analysis revealed a weak but positive influence of Cx43 level on conductance for both plots. Despite the weakness of this influence, gap junctional conductance was significantly greater in cumulus cell samples whose normalized Cx43 level was greater than the mean of all samples (Fig. 6C). Conversely, the normalized level of Cx43 was significantly greater in those cumulus cell samples whose conductance was greater than the mean of all samples (Fig. 6D).

Relation between Cx43 or gap junctional conductance and patient age

Because patient age is an important factor for pregnancy outcome in IVF treatment, we looked for an association between age and Cx43 or conductance level. The results showed that, in our patient population, neither Cx43 nor conductance level differed significantly between age groups (Fig. 7A,B). Thus, the mean age of patients in the high Cx43 or conductance group was equal to that in the low Cx43 or conductance group (Fig. 7C).

Relation between Cx43 or gap junctional conductance and clinical data

Since Cx43 level in cumulus cells correlates with gap junctional conductance, and given the demonstrated importance of the latter for folliculogenesis in mutant mice, we explored the possibility that clinical outcomes from ICSI are related to Cx43 level. Patients were partitioned into two groups based on whether their cumulus cell Cx43 expression was above or below the mean for all patients (Fig. 8A,B). Oocyte maturation (MII) rate and fertilization rate in the high Cx43 group were not different from those in the low Cx43 group, although the MII rate in the high Cx43 group was slightly higher than that in the low Cx43 group using GAPDH as the standard. On the other hand, higher Cx43 level was significantly associated with higher transferable rate and implantation rate. Comparison of vimentin and GAPDH band intensities on the western blots did not reveal any difference between patients who became pregnant and those who did not (Fig. 8C). Pregnancy outcome was then used to partition the 81 patients for which we had determined relative Cx43 level into two groups, and the mean relative intensity of the Cx43 band, normalized to vimentin or GAPDH, for the

two groups was compared. Regardless of which protein was used as the internal standard, the mean relative Cx43 level was significantly higher for samples taken from patients who became pregnant ($P < 0.01$) (Fig. 8D). Correspondingly, for vimentin-normalized samples, the pregnancy rate in the higher Cx43 group (more than the mean) was 57.1% while the pregnancy rate in the lower Cx43 group (less than the mean) was only 28.2%. For GAPDH-normalized samples the corresponding difference was 71.9% versus 24.5%.

We also examined the relationship between gap junctional conductance, measured by single patch voltage clamp, and clinical outcome. Figure 9A shows that, as with Cx43 level, there was no relationship between conductance and either MII rate or fertilization rate, but high conductance (above the population mean) was positively associated with higher transferable embryo rate, implantation rate, and pregnancy rate. Correspondingly, cumulus cells from patients who became pregnant after ICSI exhibited significantly higher gap junctional conductance (Fig. 9B).

Discussion

Multiple functions have been proposed for the gap junctions that couple the oocyte and surrounding somatic cells within growing and maturing follicles. For example, animal experiments have shown that gap junctions are important for coordinating the functions of granulosa cells and for permitting communication of the developing oocyte with the surrounding cumulus cells [8, 16, 17]. Gap junctional coupling among the granulosa cells is required to maximize their proliferative response to GDF9, an

oocyte-derived paracrine factor, perhaps by propagating downstream cell growth signals throughout the population [18]. Furthermore, granulosa cells utilize the gap junctional communication pathway to maintain oocyte pH, support its oxidative metabolism, and regulate its progression through meiosis [19-22].

Multiple connexins have been identified in ovarian follicles from different mammalian species, including Cx26, Cx32, Cx30.3, Cx37, Cx40, Cx43, Cx45, and Cx60 [8]. In the mouse ovary, Cx43 is very abundant and appears to be the only connexin contributing to the gap junctions between granulosa cells of growing follicles [10, 11], but Cx37 is restricted to the gap junctions linking cumulus cells with the oocyte [23]. The ovarian phenotypes of mice lacking individual connexins illustrate the importance of gap junctional communication for female fertility. Loss of gap junctional communication can result in a reduction in the number of follicles present at birth [24] and can impair follicular growth and development of oocyte meiotic competence [9, 12, 25]. Follicle deficiency and impairment of folliculogenesis are both hallmarks of premature ovarian failure [26]. However, we know little about connexins in human ovarian follicles and their roles in human folliculogenesis. It was thus considered important to explore the expression of connexins in human ovarian follicles and their possible involvement in fertility.

Using RT-PCR, we detected 15 of the 20 connexin mRNAs in human cumulus cells. Interestingly, a similar result was obtained from ovarian cancer cells, where 11 connexin mRNAs (Cx26, Cx30, Cx30.3, Cx31, Cx31.1, Cx32, Cx37, Cx43, Cx45, Cx46, and Cx50) were detected [27]. Six of the nine connexin proteins looked for in

our cumulus cell samples were detected by immunostaining but only Cx43 was primarily localized in the membrane where it forms gap junction-like plaques between the cells. The other connexins detected were mainly restricted to the cytoplasm of the cells where Cx26 and Cx32, at least, co-localize. To date, only Cx37 and Cx43 have been proved to form gap junctions in granulosa cells despite the presence of other connexins [8]. The expression of multiple connexins could reflect other functions, besides the formation of gap junctions, being served by connexins in human follicles [28].

Two factors point to a dominant role for Cx43 in forming the gap junctions coupling human cumulus cells. First is the fact that this connexin alone among all the connexins examined in this study is localized mainly in membrane plaques. Secondly, the level of Cx43 in the cumulus cells of different patients is significantly correlated with the strength of gap junctional coupling as revealed by conductance measurement. Although other connexins may contribute to cumulus cell gap junctions, their individual contributions are likely to be less important.

In the present study, we found that the Cx43 level in cumulus cells varies between patients, and that those patients whose cumulus cells fell within the higher Cx43-expressing group had higher transferable and implantation rates and were more likely to have a successful pregnancy outcome. Likewise, the mean relative Cx43 level in cumulus cells from pregnant patients was significantly higher than that in non-pregnant patients. Despite the fact that patient age is one of factors that affect pregnancy outcome from IVF, Cx43 and conductance levels were not correlated with

patient age in our study. We therefore propose that the observed variation between patients reflects differences in oocyte quality since, together with gap junctions between the oocyte and cumulus cells, the cumulus cell gap junctions allow sharing of molecules with the oocyte as it grows within the follicle, thus influencing oocyte metabolism [29]. Furthermore, in mutant mouse models where gap junctional coupling within developing follicles has been genetically ablated, oocyte quality is restricted [9, 12]. Cx43 level and the strength of gap junctional coupling among cumulus cells retrieved from follicles for assisted conception procedures may reflect the situation before ovulation, when oocytes were growing and oocyte quality was being determined. Cumulus cell Cx43 can thus be added to the list of markers of oocyte and embryo developmental competence and Cx43 level in cumulus cells can be considered one factor influencing pregnancy outcome after ICSI. It should be kept in mind, however, that the patients in our study underwent suppression prior to stimulation, a hormonal regime that is expected to alter gene expression and metabolism within the follicle. Thus our results may not apply equally to women undergoing single follicle aspiration.

Our finding that Cx43 expression level and the strength of intercellular coupling among cumulus cells does not correlate with oocyte maturation or fertilization rate, but does influence embryo quality post-fertilization, may indicate a temporal effect of oocyte metabolic deficiency. Such a temporal effect could arise, for example, if a low level of intercellular coupling among the cumulus cells reduces the oocyte's store of one or more essential nutrients. While the remaining supply of such a nutrient may be

sufficient to support early events like the first meiotic division and fertilization, as development proceeds its depletion might eventually affect embryo quality. Pyruvate might be an example of this hypothetical nutrient since it is supplied to the growing oocyte by the surrounding cumulus cells via the gap junctions connecting the two cell types [29] and is oxidatively metabolized for ATP generation in the oocyte mitochondria [30]. An insufficient flux of pyruvate moving through the cumulus cell layers and into the growing oocyte would result in a reduced supply of ATP to support post-fertilization development, possibly resulting in reduced developmental competence. Indeed, Van Blerkom et al [31] reported that cohorts of human oocytes with lower ATP content, though able to be fertilized and to develop normally leading up to the time of embryo transfer, were less likely to generate a pregnancy.

Our results are in contrast with those published recently by Hasegawa et al [32]. In that study as in ours, Cx43 level was measured in relation to GAPDH in cumulus cells from patients undergoing IVF by ICSI. While their data did indicate a lack of correspondence between Cx43 level and either the fertilization rate or the ability of the zygotes to cleave, Cx43/GAPDH ratio was *negatively* correlated with embryo morphology (>7 blastomeres with <10% fragmentation) on day 3 after insemination; implantation and pregnancy rates were not reported and intercellular gap junctional coupling was not measured. This discrepancy remains unresolved.

In conclusion, our data indicate that Cx43 is a major contributor to gap junctions in human cumulus cells, but the presence of additional connexins may reflect other functions for these proteins during human folliculogenesis. Cx43 level in cumulus cells

is related to intercellular coupling and pregnancy outcome, implicating it as a factor in pregnancy outcome in assisted conception.

Acknowledgements

We thank Dr. Qing Shao for providing the anti-Cx30 antibody and Jessica Lori Riley for providing the rabbit anti-Cx26 antibody. We also gratefully acknowledge the advice and assistance of Kevin Barr, Lynda Hughes, Dr. Tony Li, and Lesley Mok. This work was funded as part of the “Healthy Gametes and Great Embryos” Strategic Initiative of the Canadian Institutes of Health Research, Institute of Human Development, Child and Youth Health.

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Figure legends

Figure 1. Example of an RT-PCR survey of human cumulus cells for connexin mRNAs. Primers and amplification conditions were optimized for each of 20 connexin sequences. All PCR products were run on a 1.2% agarose gel. All but Cx30.2, Cx31.9, Cx40.1, Cx46, and Cx47 mRNAs were detected.

Figure 2. Expression of Cx26, Cx30, Cx30.3, Cx32, Cx40, and Cx43 in human cumulus cells was confirmed by immunostaining. Cx31, Cx37, and Cx45 were not detected. Hoechst dye was used for nucleus staining. The same magnification was used in all pictures, as shown in the scale bar.

Figure 3. Co-expression of Cx26, Cx32, and Cx43 in human cumulus cells. Cells were doubly labelled with primary antibodies against different connexins and the bound primary antibodies detected using different fluorescently tagged secondary antibodies. Hoechst dye was used for nucleus staining. The magnifications are shown by the scale bars.

Figure 4. Quantification of Cx43 in human cumulus cells by western blotting. (A) A representative blot showing Cx43 protein levels in different patients. The three electrophoretic variants of Cx43 represent the three phosphorylation states of the protein typically seen in granulosa cells and other tissues [15]. (B) Quantification of Cx43 protein in different patients using GAPDH and vimentin as internal controls.

Figure 5. Variation of gap junctional conductance among cumulus cells from different patients. The patient numbers correspond to those in Figure 4B. One cumulus cell from a small cluster of cells was patched in whole-cell configuration. A voltage pulse was applied through the patch pipette and the resulting capacitative current transient was analyzed to obtain the initial peak current and the final steady-state current. These values were used to calculate the junctional conductance between the patched cell and its surrounding cells as described in Tong et al [11].

Figure 6. Relationship between Cx43 level and gap junctional conductance in cumulus cells from different patients. (A,B) Scatter plots and regression analysis illustrating the positive relationship between Cx43 level (normalized to vimentin and GAPDH, respectively) and gap junctional conductance. r^2 indicates goodness-of-fit. (C) Difference in mean gap junctional conductance between cumulus cell samples partitioned by Cx43 level (relative band intensity above or below the population mean as determined by reference to either vimentin or GAPDH). (D) Difference in mean Cx43 level (relative band intensity as determined by reference to either vimentin or GAPDH) between cumulus cell samples partitioned by gap junctional conductance (above or below the population mean). Different letters above the bars indicate significant differences ($P < 0.05$ by one-way ANOVA).

Figure 7. Relationship between Cx43 or conductance and patient age. (A) Mean Cx43 level in three age groups (30 and under, n=30; 31-35, n=26; 36 and above, n=25). (B) Mean conductance in three age groups (30 and under, n=16; 31-35, n=13; 36 and above, n=13). (C) Relationship between patient age and Cx43 or conductance level. Number of patient is listed in Table 3. None of the differences was significant.

Figure 8. Relationship between Cx43 level and clinical data. (A) Comparison of Cx43 level, determined with reference to vimentin, and clinical outcomes. Oocytes were evaluated for nuclear maturity and graded as metaphase II (MII), metaphase I, or prophase I. Fertilization was considered have occurred when two clear pronuclei were present after 16-18 h insemination. Embryo transferability was estimated on day 3 post-insemination according to a grading system, with embryos having more than 6 blastomeres and good morphology being considered as transferable. Implantation rate is the ratio of fetuses (determined by day 40 ultrasound) to embryos transferred. (B) Comparison of Cx43 level, determined with reference to GAPDH, and clinical outcomes. (C) Confirmation that cumulus cell vimentin and GAPDH levels are comparable between pregnant and non-pregnant patients. Lysis buffer volumes were adjusted to account for differing cell numbers obtained from different patients. The mean vimentin or GAPDH level for each sample was determined from at least two measurements. (D) Relationship between Cx43 level and pregnancy outcome (determined by day 40 ultrasound): difference between cumulus cell sample groups, partitioned by pregnancy outcome, in relative level of Cx43 (relative band

intensity as determined by reference to either vimentin or GAPDH). In all cases, different letters above the bars indicate significant differences ($P < 0.05$ by one-way ANOVA). In this study 35 patients became pregnant while 46 did not.

Figure 9. Relationship between gap junctional conductance and clinical data. (A) Comparison of gap junctional conductance with MII rate, fertilization rate, transferable embryo rate, implantation rate, and pregnancy rate. Pregnancy rate is the ratio of number of pregnant patients to number of total patients in each group. (B) Comparison of conductance level between pregnant patients and non-pregnant patients. In this study 20 patients became pregnant while 22 did not. Different letters above the bars indicate significant differences ($P < 0.05$ by one-way ANOVA).

Figure 1.

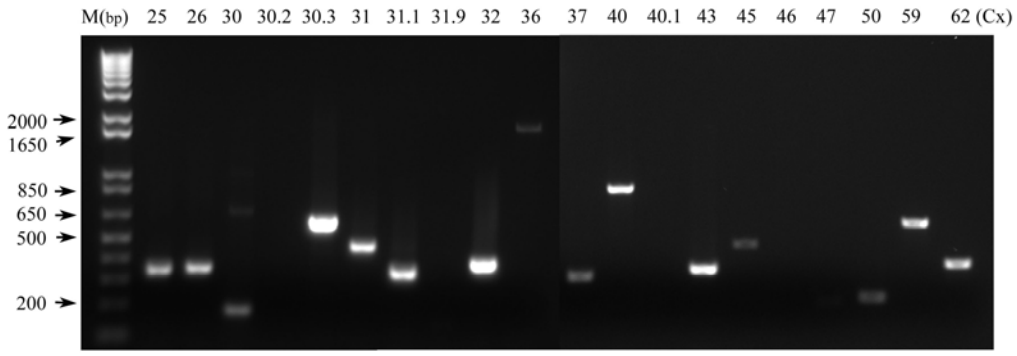
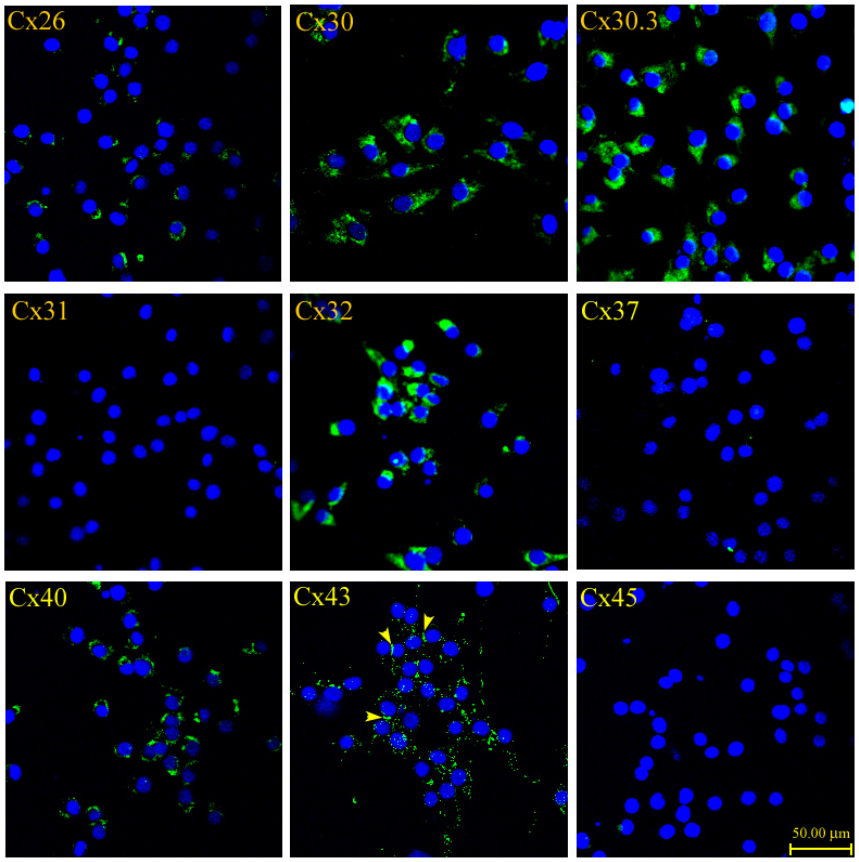

M(bp)
25
26
30
30.2
30.3
31
31.1
31.9
32
36
37
40
40.1
43
45
46
47
50
59
62 (Cx)
2000
1650
850
650
500
200

Figure 2.



615

Figure 3.

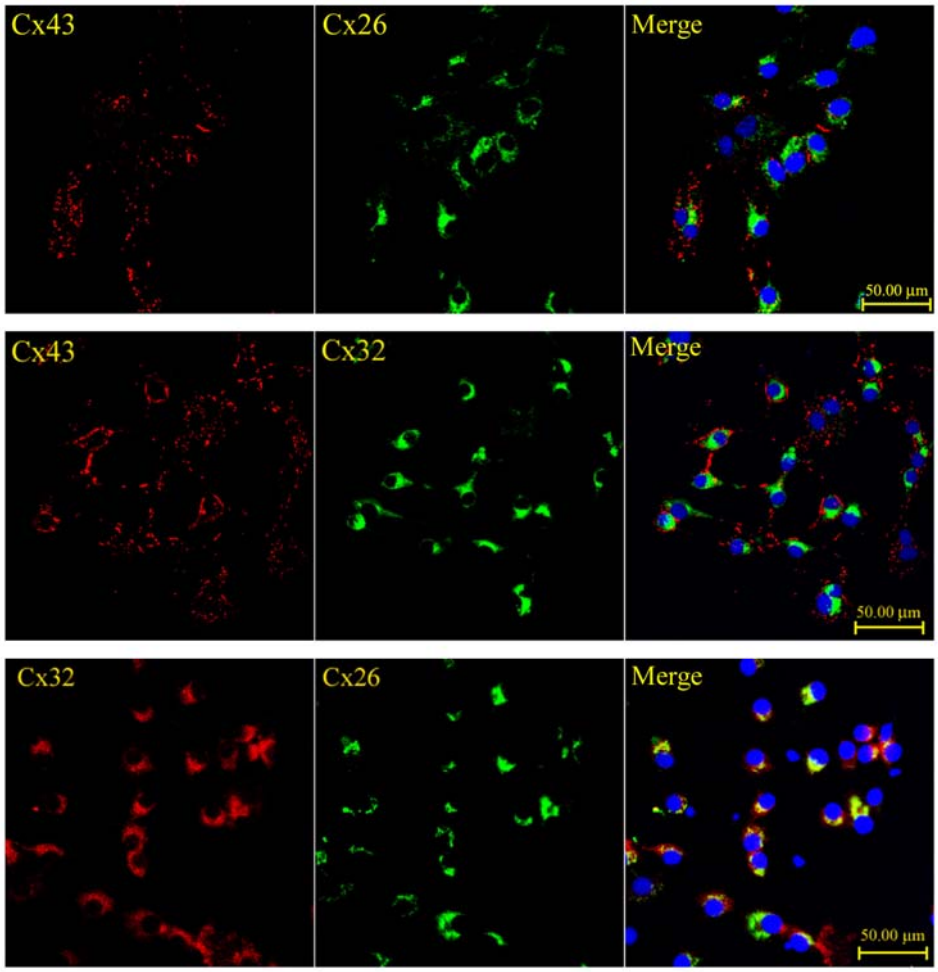
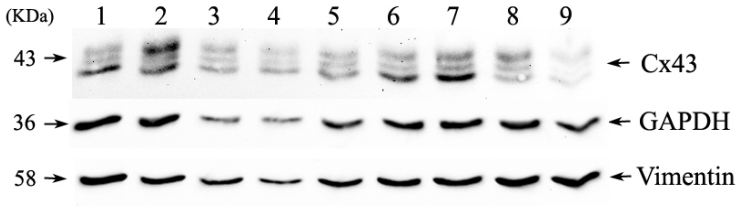

Cx43
Cx26
Merge
50.00 µm
Cx43
Cx32
Merge
50.00 µm
Cx32
Cx26
Merge
50.00 µm

Figure 4.

620 **A**



B

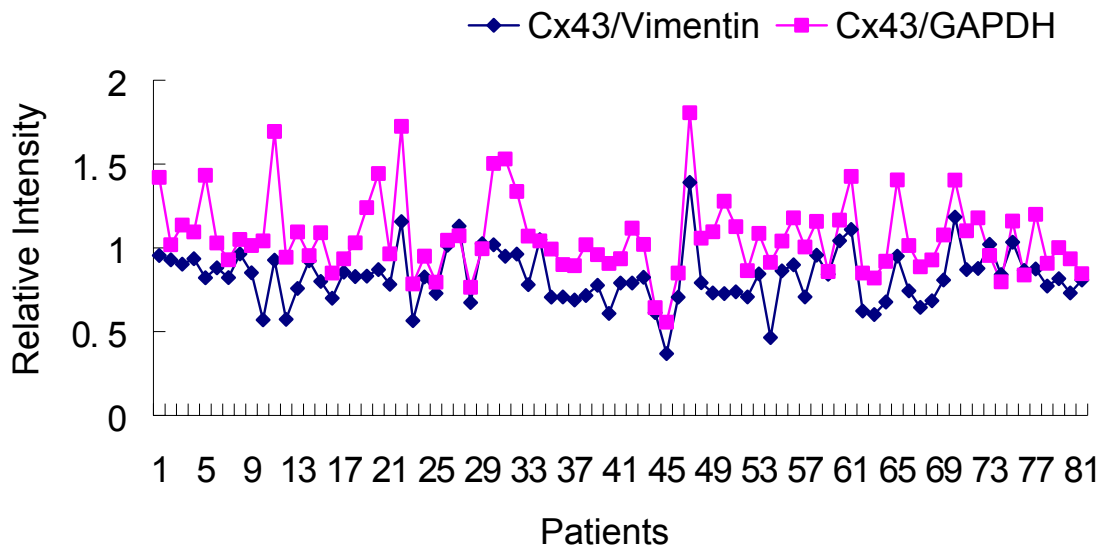
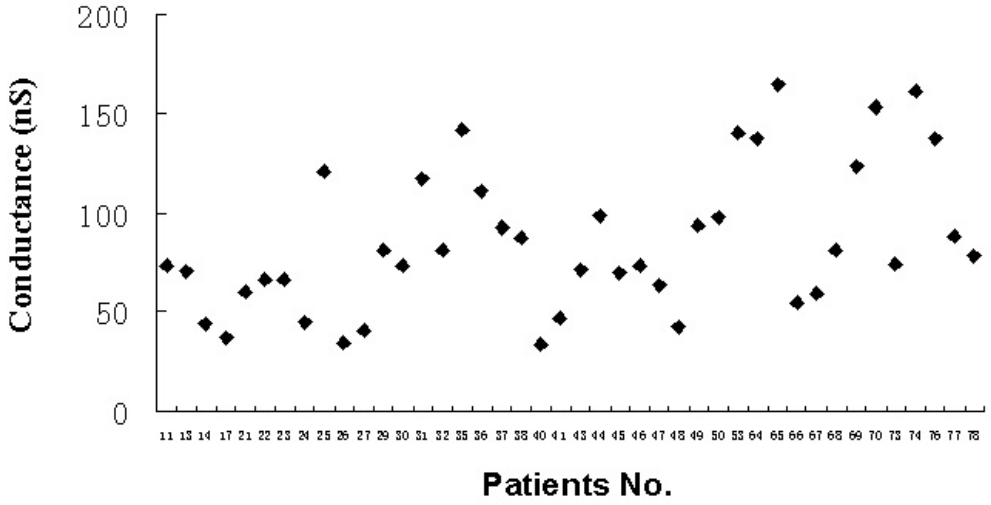


Figure 5.


200
150
100
50
0
Conductance (nS)
11 13 14 17 21 22 23 24 25 26 27 29 30 31 32 35 36 37 38 40 41 43 44 45 46 47 48 49 50 53 64 65 66 67 68 69 70 73 74 76 77 78
Patients No.

625

Figure 6.

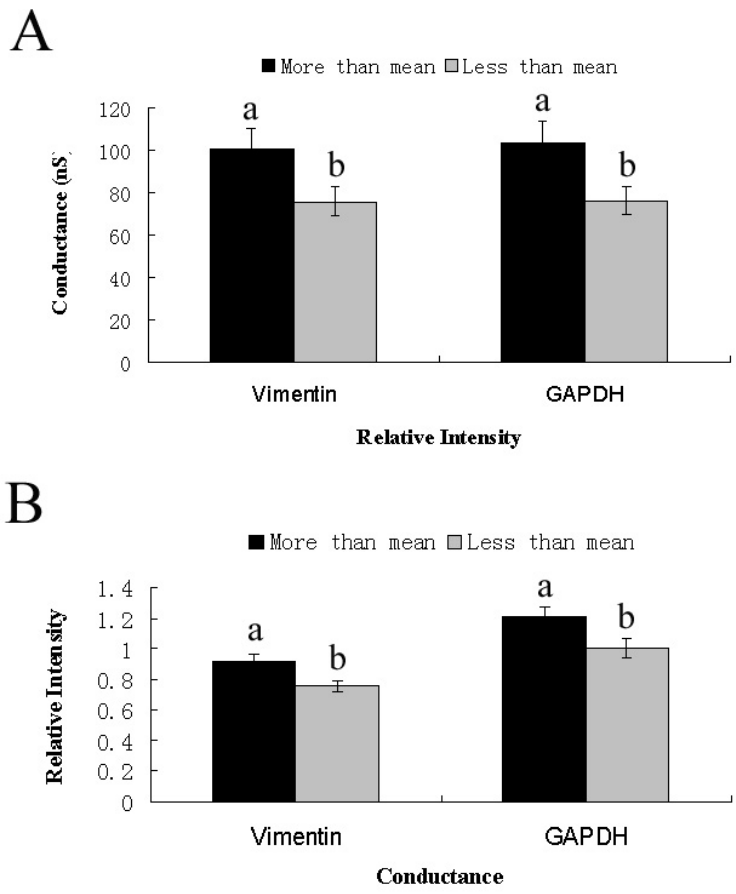

A
More than mean
Less than mean
120
100
80
60
40
20
0
a
b
a
b
Vimentin
GAPDH
Relative Intensity
B
More than mean
Less than mean
Relative Intensity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
a
b
Vimentin
GAPDH
Conductance

Figure 7.

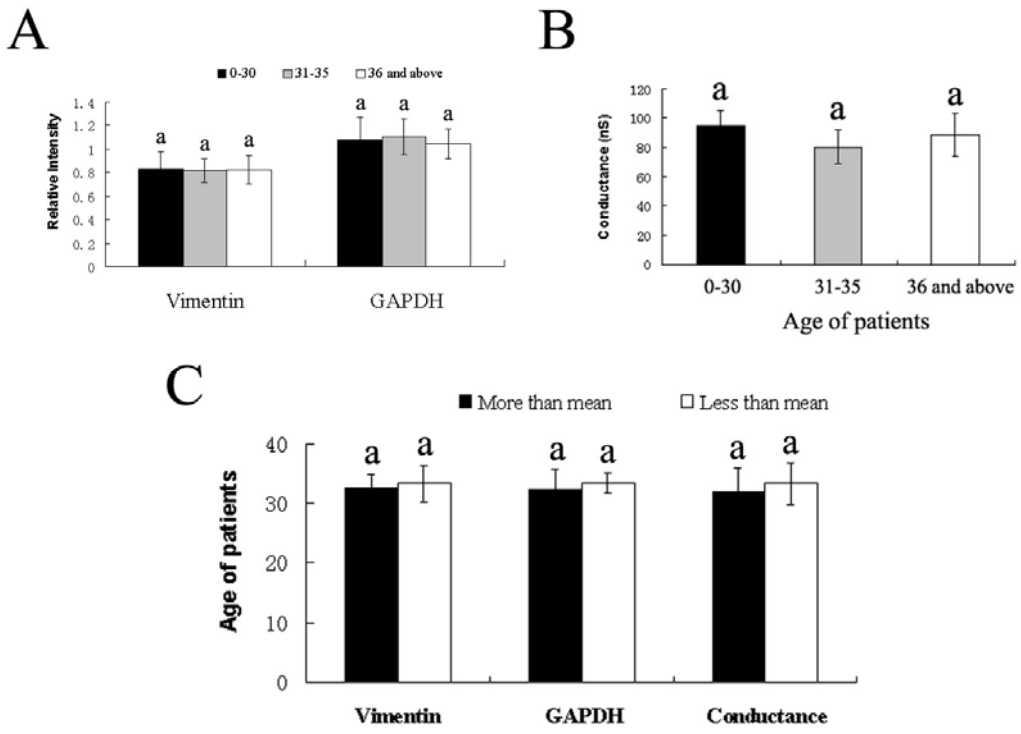

A
0-30
31-35
36 and above
Relative intensity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
a
a
a
a
Vimentin
GAPDH
B
Conductance (nS)
120
100
80
60
40
20
0
a
a
a
0-30
31-35
36 and above
Age of patients
C
More than mean
Less than mean
Age of patients
40
30
20
10
0
a
a
a
a
a
a
Vimentin
GAPDH
Conductance

Figure 8.

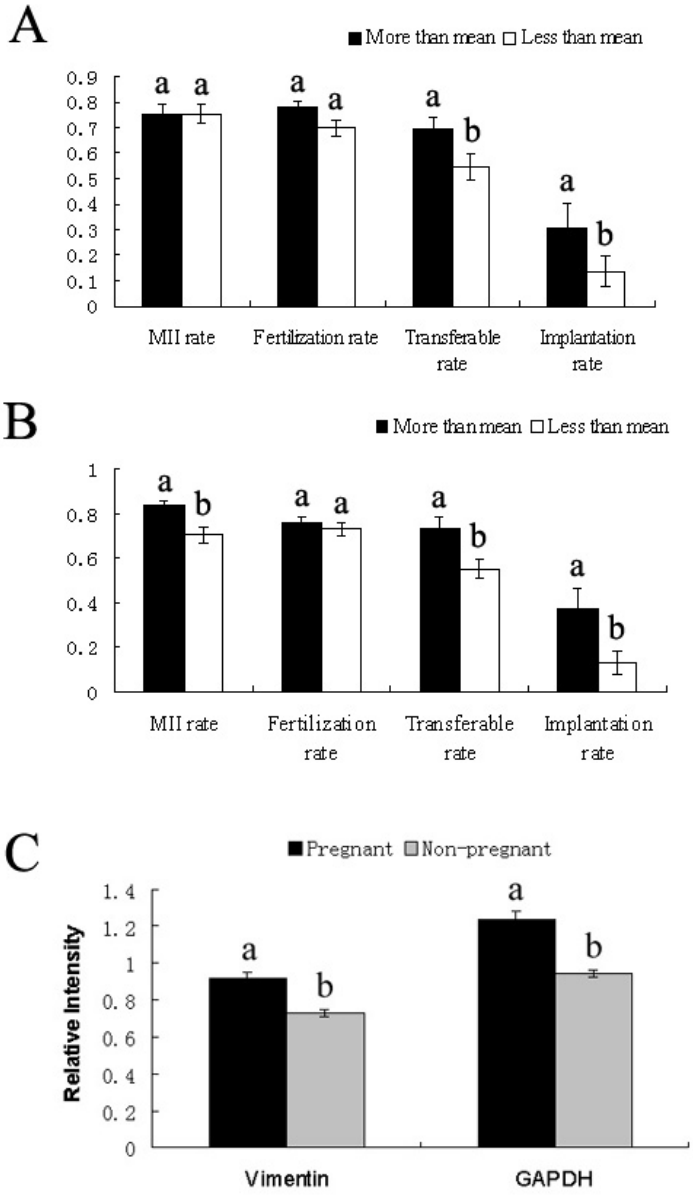
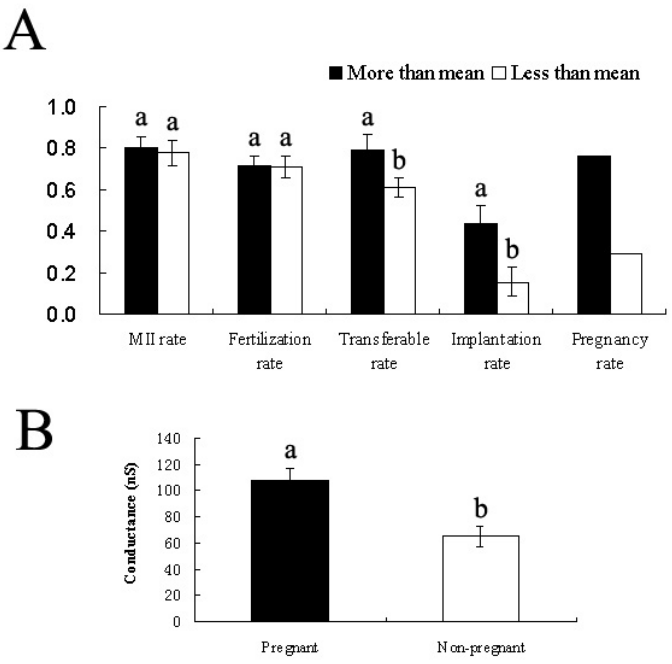

A
More than mean
Less than mean
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a a
a a
a b
a b
MII rate
Fertilization rate
Transferable rate
Implantation rate
B
More than mean
Less than mean
1
0.8
0.6
0.4
0.2
0
a b
a a
a b
a b
MII rate
Fertilization rate
Transferable rate
Implantation rate
C
Pregnant
Non-pregnant
Relative Intensity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a b
a b
Vimentin
GAPDH

Figure 9.


A
More than mean
Less than mean
1.0
0.8
0.6
0.4
0.2
0.0
a a
a a
a b
a b
MII rate
Fertilization rate
Transferable rate
Implantation rate
Pregnancy rate
B
Conductance (nS)
140
120
100
80
60
40
20
0
a
b
Pregnant
Non-pregnant

635