의학박사 학위논문

Expressions of aquaporin family in human luteinized granulosa cells and their correlations with IVF outcomes

황체화된 과립막 세포내 아쿠아포린계의 발현과 체외수정시술 결과와의 연관성

2017 년 8 월

서울대학교 대학원
의학과 산부인과 전공
이 희 준

A thesis of the Docdor’ s degree

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이희준의 의학박사 학위논문을 인준함

2017 년 8 월

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Abstract

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Objective: The purpose of this study was to investigate the association between mRNA for specific aquaporins (AQPs) expressed in human luteinized granulosa cells (GCs) and their expression levels, and in vitro fertilization (IVF) outcomes.

Methods: Prospective observational study involving 111 women undergoing a stimulated IVF cycle was performed. Luteinized GCs were obtained at the time of oocyte retrieval. The mRNA expressions of AQP0-12 were explored by RT-PCR in GCs from another 27 women, and mRNAs for AQP0, 8 and 10 were not detected. Real-time quantitative RT-PCR (qRT-PCR) was performed to quantify mRNA level of AQP1-7, 9, 11, and 12. The mRNA for luteinizing hormone receptor (LHR) and steroidogenic acute regulatory protein (StAR) were also quantified by qRT-PCR.

Results: In samples from 111 women, retrieved oocyte number was negatively associated with the mRNA levels of AQP1, 4, 6, and 11 and LHR ($r = -0.311$, $r = -0.233$, $r = -0.203$, $r = -0.194$, and $r = -0.202$, respectively, $p<0.05$ for each), however, after adjustment for woman's age and serum anti-müllerian hormone (AMH) levels, only correlation with AQP1 was found ($r = -0.299$, $p<0.05$). Body mass index was negatively associated (after adjustment of age) with the mRNA level of AQP7 ($r = -0.259$, $p<0.05$). The fertilization rate was

positively associated with the mRNA level of AQP7 ($r=0.269$, $p<0.05$). The number or quality of embryos or clinical pregnancy was not associated with the mRNA levels of any of ten AQP subtypes. The mRNA levels for the ten AQP subtypes were correlated positively with LHR expression but negatively with StAR expression. Amongst high responders (oocyte number ≥14), the mRNA levels of AQP11 (1.4 ± 0.7 versus 1.7 ± 0.6) and LHR (1.3 ± 0.7 versus 1.7 ± 0.7) were significantly lower in the group with PCOS than in the non-PCOS group.

Conclusions: The mRNA of AQP1-7, 9, 11, and 12 was expressed in human luteinized GCs and mRNA level of AQP1 was negatively associated with retrieved oocyte number and mRNA level of AQP7 was positively associated with fertilization rate. AQP1 may be one of the factors that modulate individual ovarian response to exogenous gonadotropin. The mRNA level of AQP7 was positively associated with fertilization rate, which is a surrogate marker of oocyte competence, thus expression of AQP7 could be a marker for adequate folliculogenesis and healthy oocytes.

Key words: Aquaporins, granulosa cell, in vitro fertilization, polycystic ovary syndrome

CONTENTS

LIST OF TABLES AND FIGURES

Introduction

During folliculogenesis, follicular diameter dramatically increases and this event is accompanied by enlargement of the oocyte, proliferation of granulosa and theca cell layers, and formation of the fluid-filled antrum. The expansion of the fluid-filled antrum is believed to be secondary to massive fluid transport from the vasculature of the theca layer into the antrum. The exact mechanisms for fluid transport from the vasculature into the avascular granulosa cell (GC) layers are unclear. However, it is known that GCs produce hyaluronan and chondroitin sulfate proteoglycan versican, and therefore follicular fluids (FF) accumulate because of the generated osmotic gradient (Rodgers and Irving-Rodgers, 2010). It has been reported that the water permeability of an ovarian antral follicle is predominantly transcellular and is mediated by aquaporins (AQPs) (McConnell et al., 2002). AQPs are known to be present in GCs and could be actively involved in the transport of water into the follicle (Rodgers and Irving-Rodgers, 2010; Thoroddsen et al., 2011; Sales et al., 2013).

The AQPs are a family of small (25-34 kDa), hydrophobic and integral membrane channel proteins that facilitate rapid passive movement of water (Agre and Kozono, 2003). To date, 13 isoforms of AQPs (AQP0-12) have been identified in mammals (Agre et al., 2002; Huang et al., 2006), and at least nine AQP isoforms (AQP1-9) have been demonstrated in various compartments of the uterus-oviduct of the rat, mouse, pig, and human (Huang et al., 2006). Several mammalian AQPs, such as AQP0-2, 4-6 and 8, appear to be highly selective for the passage of water, whereas AQP3, 7, 9 and 10 (recently termed aquaglyceroporins) transport not only water but neutral solutes, including glycerol, urea, and other small non-electrolytes (Ishibashi et al., 1994; Rojek et al., 2008; Maeda et al., 2009).

AQP 5 and 7-9 (McConnell et al., 2002; Starowicz et al., 2014) and AQP7 and 8 (West-Farrell et al., 2009) have been detected in GCs in the rat and mouse, respectively. In pigs, AQP5 and 9 have been shown to be expressed in GCs of developing follicles while AQP1 was mainly expressed in the capillary endothelium (Skowronski et al., 2009). Over-expression of AQP1 in the Chinese hamster ovary has been shown to increase cell water permeability and enhance the rate of cell death (Jablonski et al., 2004). AQP4-knockout mice showed fewer antral follicles/corpora lutea and lower rates of pregnancy and decreased litter sizes (Sun et al., 2009). It has been demonstrated that AQP8 is an important determinant of mouse GC apoptosis and follicular maturation (Su et al., 2010). The same researchers also reported mRNA expression of AQP5, 7, 8, 11 and 12 in mouse GCs, as well as the increased occurrence of multi-oocyte follicles in the ovaries of AQP8-knockout mice (Su et al., 2013).

In humans, AQP1-4 has been shown to be expressed in both the GCs and theca cells of ovulating follicles (Thoroddsen et al., 2011). It was reported that AQP9 mRNA levels were shown to be significantly lower in the GCs of women with polycystic ovary syndrome (PCOS) compared to controls, and its expression was correlated with follicular androgen level (Qu et al., 2010).

There have been no studies concerning the possible role of AQPs in human infertility. Since AQPs are expressed in GCs and theca cells during folliculogenesis and the ovulation process, their expression could be indicative of GC function. Moreover, there are no reports investigating the differences in the expression of full AQP family between women with and without PCOS. Because women with PCOS often show an exaggerated response to ovarian stimulation in IVF cycles, the expression pattern of AQP subtypes would be different between women with and without PCOS. The

main purpose of this study was to investigate a possible association between the expression level of specific AQP subtypes and IVF outcomes. The expression patterns of AQPs are also explored between women with and without PCOS.

Materials and Methods

Subjects

This study included a total of 111 women undergoing IVF/ICSI at a single infertility center during the study period (January 2013 - December 2013). The inclusion criteria were two factors; less than 43 years of age and normal or high responders with six mature oocytes or more. Initially 153 women were recruited, but ten women disagreed with the study, two women were excluded due to faulty injection of hCG, and 30 women were excluded due to poor ovarian response and old age. Finally 111 women were included in this study (Fig. I). All subjects were less than 43 years of age (mean age = 35.2 ± 4.4 years), and were normal or high responders with six mature oocytes or more. The indications for IVF among 111 women included unexplained (n = 35), tubal (n = 27), ovulatory (n = 25), male (n = 17), and uterine (n = 7). None of the women had taken oral contraceptive pills for three months prior to IVF. The Institutional Review Board of the Seoul National University Bundang Hospital approved the use of patients' medical records and assays of human GCs.

Among 111 women, 17 women were considered as PCOS by the Rotterdam Consensus (Rotterdam EA-SPcwg, 2004) because two or more among the following three criteria were present: (1) oligoovulation or anovulation, (2) clinical and/or biochemical signs of hyperandrogenism, (3) polycystic ovaries on ultrasonography. Seventeen women were previously diagnosed as PCOS at

other hospitals and then referred to this infertility center for performing IVF. Thus their detailed hormonal statuses (17-alpha-hydroxyprogesterone, cortisol, insulin, and/or insulin resistance) were absent at the time of participation. Even if present, the values were measured several months or even several years ago, thus not included in this study. Although detailed hormonal values were absent, 17 women fulfilled the Rotterdam criteria.

IVF protocol and collection of human luteinized granulosa cells

Ovarian stimulation was performed by recombinant follicle-stimulating hormone (FSH; Gonal-F®; Merck-Serono, Geneva, Switzerland) (n = 16) or recombinant FSH with human menopausal gonadotropin (hMG; Menopur®; Ferring, Malmo, Sweden) add-back (n = 95). The pituitary was suppressed by flexible multiple-dose protocol of gonadotropin-releasing hormone (GnRH) antagonist (Cetrotide® 0.25 mg/d; Merck-Serono) (n = 56) or mid-luteal long protocol of GnRH agonist (Decapeptyl® 0.1 mg/d; Ferring) (n = 55). When leading follicle(s) reached 18 mm in diameter, urinary human chorionic gonadotropin (hCG; IVF-C® 5,000 IU; LG, Seoul, Korea) (n = 83) or recombinant hCG (Ovidrel® 250 μg; Merck-Serono) (n = 28) was administered, and oocytes were retrieved via the transvaginal route 36 hours later.

Oocytes were inseminated by conventional method (n = 17), split insemination (n=64), or intracytoplasmic sperm injection (ICSI) (n = 30) depending on sperm or oocyte quality. Up to three embryos were transferred three days (n = 85) or five days (n = 17) after oocyte retrieval. Embryo transfer was not performed in nine women due to the imminent risk of ovarian hyperstimulation syndrome. Therefore, data from 102 women were included when assessed the association with pregnancy outcome. Day 3 cleavage stage

embryos were classified as grade A-D and top quality embryos were defined as grade A (equal-sized blastomeres and no fragments without apparent morphologic abnormalities). Day 5 blastocyst stage embryos were classified according to Gardner's grading system and top quality embryos were defined as grade AA, AB, AC, BA, BB, or CA. Intramuscular progesterone (50 mg/d, Samil, Korea) was administered for luteal phase support. A clinical pregnancy was defined by the presence of an intrauterine gestational sac with pulsating fetal heartbeats 3-4 weeks after oocyte retrieval.

At the time of oocyte retrieval, oocyte-cumulus complexes were isolated and the remaining FF was centrifuged for 5 min at 1,200 rpm. The resulting cell pellets were diluted in TCM199 media containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). Red blood cells were removed by centrifuge with 50% Percoll gradient for 20 min at 3,000 rpm, and the remaining cells were incubated for 10 min with TCM199 containing 0.1% type III hyaluronidase and 0.1% type I collagenase (Sigma-Aldrich). After washing, the cell concentration was adjusted to approximately 10^6/mL, and the number and viability of luteinized GCs were determined by 0.4% Trypan blue stain (Sigma-Aldrich); samples with >85% viability were used in this study.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Before quantitative RT-PCR experiment, RT-PCR analysis was performed in GCs obtained from 27 women undergoing IVF, who were not part of 111 women. The mean age of 27 women was 34.8 ± 0.8 years (range: 28 – 44 years) and the mean number of retrieved oocyte was 15.4 ± 2.1 (range: 2 – 42); indications for IVF was unexplained (n = 7), tubal (n = 8), ovulatory (n = 8), male (n = 3), and uterine (n = 1). The purpose of this preliminary RT-PCR

analysis was to demonstrate whether AQP subtypes are expressed or not in human GCs, regardless of patient's or stimulation characteristics. Total RNA was extracted from the luteinized GCs by the TRIzol method and cDNAs were synthesized by the Suprime script RT premix (GeNet Bio, Daejeon, Korea) according to the manufacturer's instructions. Total RNA (3 μg) was reverse transcribed using Maxime PCR PreMix kit (iNtRON, Seongnam, Korea) and oligo dT primers. Reverse-transcription products (3 μL) were amplified by PCR with primers specific for AQP0-12 (Table I). mRNA for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The PCR was carried out in a 20 μL reaction mixture containing 2 μL 10 × PCR buffer (containing 2.5 mM Mg^{2+}), 2.5 mM dNTP, 1 μL sense and antisense primers (20 pM), 2.5 U i-Taq DNA Polymerase (iNtRON) and 3 μL cDNA sample. The PCR profiles used are shown in Table I. GAPDH was used as an internal control. The PCR products were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide.

Quantitative RT-PCR analysis

In samples from 111 women, total RNA was extracted using the Trizol method and cDNAs were synthesized by the Suprime script RT premix (GeNet Bio) according to the manufacturer's instructions. Real-time quantitative RT-PCR (qRT-PCR) was performed with a 7500 Real Time PCR system with SYBR Green (Bioneer, Daejeon, Korea). Real-time qRT-PCR was carried out in a 20 μL reaction volume containing 10 μL Accupower 2 x Greenstar qPCR Master Mix, 3 μL cDNA, and 5 μL distilled water. Sequences for specific primers for AQP1-7, 9, 11 and 12 and the conditions are listed in Table II. The mRNA for luteinizing hormone receptor (LHR) and steroidogenic acute regulatory protein (StAR), as a luteinization marker, were

also quantified by real-time qRT-PCR. Data were analyzed by the comparative cycle threshold method in all experiments. Real-time qRT-PCR was repeated twice using entirely different sets of GCs and the values were averaged. Each value was normalized against a corresponding relative amount of GAPDH mRNA and expressed as a value relative to the control.

Statistical Analysis

Statistical calculations were performed with SPSS version 20.0 (SPSS, Chicago, IL, USA) and the results were considered statistically significant at p-values of <0.05. Before any statistical analysis, a normal distribution was checked for all data. Because all of data did not show a normal distribution, a non-parametric analysis was always used in the present study. Non-parametric Spearman correlation test was performed to demonstrate an association between two numerical variables. For comparison of means between two groups, the non-parametric Mann-Whitney U test was used but the data were expressed as mean ± SD. The chi-squared test was used to compare proportions between two groups. Data of AQPs, LHR and StAR were expressed as log scale.

Figure I. CONSORT flowchart

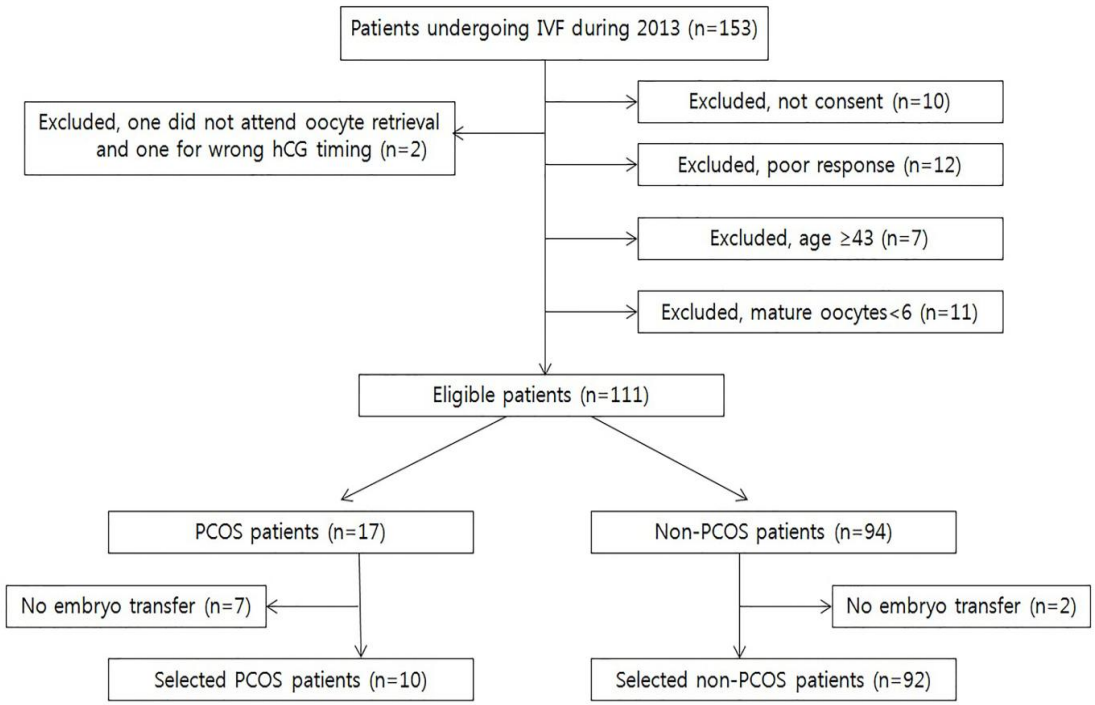


Table I. Primers for aquaporin family and their conditions for RT-PCR analysis

Gene	Primer forward (5'-3')	Primer reverse (5'-3')	Product size (bp)	Annealing temperature (℃)	Cycles
AQP0	CTT TGC TCC TGC CAT TCT CA	AGG TTC CCC TGT GAC CTC TG	202	69	35
AQP1	GTC CAG GAC AAC GTG AAG GT	ACC CTG GAG TTG ATG TCG TC	656	64	40
AQP2	TGA GCT GTC CCA TAC TCC CA	ACC ACT GCC AAG TTG TGG AA	265	64	40
AQP3	GTC ACT CTG GGC ATC CTC AT	GGC CAG CTT CAC ATT CTC TT	654	64	40
AQP4	GGT GGC CTT TAT GAG TAT GT	GCT TTC AGT GCG ATC TTC	261	55	45
AQP5	CCA CCC TCA TCT TCG TCT TC	TCA CTC AGG CTC AGG GAG TT	643	64	40
AQP6	GAG GAA GGG AGG TTT GAG	GAT TTT GGT AGT CTT TCA GAG G	291	55	45
AQP7	CAG CTG TGT CTC TTC GCC AT	TTG CTG AAG ACC TGT TTG CC	200	69	35
AQP8	CAC GCT GGG GAA TAT CAG TG	GAT GAT CTC TGC CAC CAA CG	247	55	30
AQP9	GGG CAC GTT CAT CTT GAT TG	TAA ATG CCA AAG ACG GTT GC	279	55	30
AQP10	TAC CAG GAC TCA GGC TTC T	TTC CCC ACC CCT AAC TAC	297	55	45
AQP11	CAT CAC CTT TTT GGT CTA TG	GAA AAA GCT GAA CAT CAA AA	175	55	35
AQP12	GCC TTT TGT TTC CAT CTG	CTT GTT CTT CTG GCC GTA	300	52	45
GAPDH	ACC ACA GTC CAT GCC ATC AC	TCC ACC ACC CTG TTG CTG TA	556	55	35

AQP: aquaporin, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Table II. Primers for aquaporin family and their conditions for qRT-PCR analysis

Gene	Primer forward (5'-3')	Primer reverse (5'-3')	Product size (bp)	Annealing temperature (℃)	Cycles
AQP1	GACCACGAGGCTGATTCCTCTC	CTCCCCTCCATCACAACTCTCC	95	59	40
AQP2	CTCTTTTGTGCTTGTTTCCGGTAGTT	AGATTGTGCGGGACTCATCAAAG	94	59	40
AQP3	CCTTCACGATCCACCCTTTCA	TGCTATTTGGGCAAGGTCCAG	99	59	40
AQP4	GTGGTGCATGTGATTGACGTTG	TGTCTGCTTTCAGTGCGATCTTC	107	59	40
AQP5	CGCGCTCAACAACAACACAA	AGTGGAGGCGAAGATGCAGAG	91	67	40
AQP6	GAAGCCGTCTCCAGGCTCTCT	CTTAGTTTCCTGGGTCCCTCCTG	102	69	40
AQP7	GGGTGTCCCTTGGCATGAAC	CCCATTGCTGAAGACCTGTTTG	110	65	40
AQP9	CGTCTTTGGCATTTACTATGATGGACT	AATGTGTGCTGTTGCATTTTCTCC	85	60	40
AQP10	TGCCCTCCATAGCCTGTC	CACCCCTAACTACCCTCTTGG	105	59	40
AQP11	GCTGCAACTGCTGAGCGAAC	TGGACGTGCCCACCAGAG	104	60	40
AQP12	CCCGCTGTGGCTCTGT	GTAAGGTGTGTCCCGAGCA	118	69	40
LHR	CTTGGAGGATGGCTCTTTTCT	CATGGGGAAGCAAATACTGAC	90	60	40
StAR	GCCCAAGAGCATCATCAAC	TTCAACACCTGGCTTCAGAG	107	60	40
GAPDH	ACGTGTCAGTGGTGGACCTGA	AGCCCAGGATGCCCTTGA	111	59	40

AQP: aquaporin, LHR: luteinizing hormone receptor, StAR: steroidogenic acute regulatory protein, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

Results

In a preliminarily RT-PCR experiment using GCs obtained from another 27 women, AQP1 mRNA was detected in 13, AQP2 mRNA in 23, AQP3 mRNA in 19, AQP4 mRNA in 23, AQP5 mRNA in 14, AQP6 mRNA in 22, AQP7 mRNA in 22, AQP9 mRNA in 19, AQP11 mRNA in 24, and AQP12 mRNA in 13 women. GAPDH mRNA was detected in all samples from 27 women. The mRNAs for AQP0, 8, and 10 were not detected in the samples from 27 women (Fig. II). Thereafter, qRT-PCR analyses were performed for the quantification of mRNA level of AQP 1-7, 9, 11, and 12; representative band images are shown in Fig. II.

Overall distribution of mRNA levels (log scale) of ten AQPs, LHR and StAR are depicted as a Box-Whisker plot (Fig. III). In human luteinized GCs, mRNA for AQP7 and 9 was relatively strongly expressed.

As shown in Table III, body mass index (BMI) was negatively associated with the mRNA level of AQP7 only (before and after adjustment of age). A significant negative association was found between retrieved oocyte number and the mRNA levels of AQP1, 4, 6, and 11 and LHR. Because retrieved oocyte number depends on woman's age and serum level of anti-müllerian hormone (AMH), an adjustment for woman's age and serum AMH level was performed. The retrieved oocyte number was associated only with the mRNA level of AQP1. A scatter diagram showed a negative association between retrieved oocyte number and the mRNA level of AQP1 (Fig. IV-A).

Fertilization rate was positively associated with the mRNA level of AQP7 (Table III) (Fig. IV-B). In the sub-group analysis, the fertilization rate was associated with the mRNA level of AQP7 only in conventional method group (n = 17), and no association was found in ICSI group (n = 30) and split insemination group (n = 64) (data not shown). The mRNA levels of ten AQPs,

LHR and StAR were not correlated with the absolute number or the percentage of top quality embryos (Table III).

The mRNA level of LHR was positively associated with the mRNA levels of ten AQP subtypes, but the mRNA level of StAR was negatively associated with them (Table IV). Meanwhile, the mRNA levels of ten AQP subtypes were significantly positively correlated with each other ($p < 0.05$ for all, data not shown). A significant negative association was found between mRNA levels of LHR and StAR (Table IV). Multiple regressions also revealed that mRNA level of AQP1 are still negatively associated with retrieved oocyte number, when adjusting the potential confounding factors, for example, LHR and StAR (data not shown).

When variable parameters were compared between pregnant and non-pregnant women, the only significant factor was woman's age (Table V). There were no significant differences in mRNA levels of ten AQP subtypes, LHR and StAR between pregnant and non-pregnant women. There were no significant different according to the causes of infertility and IVF stimulation protocols such as GnRH agonist or GnRH antagonist cycle (data not shown).

Women with PCOS (n = 17) were younger, had higher serum AMH levels and obtained more retrieved oocytes, when compared with the non-PCOS group (n = 94) (Table VI). In the PCOS group, the mRNA levels of AQP1 and 11 and LHR were significantly lower than in the non-PCOS group. Because all women with PCOS were high responders (≥14 oocytes) and the mRNA level of AQP1 was related to the retrieved oocyte number, subgroup analysis was performed in cases with retrieved oocyte number ≥14. As shown in Table VI, mRNA levels of AQP11 and LHR were significantly lower in the PCOS group (n = 17) than in the non-PCOS group (n = 34).

Figure II. Representative band images by RT-PCR. AQP0, 8 and 10 was not detected in all samples from 27 women. AQP1-7, 9, 11 and 12 was detected in all or a part of examined samples.

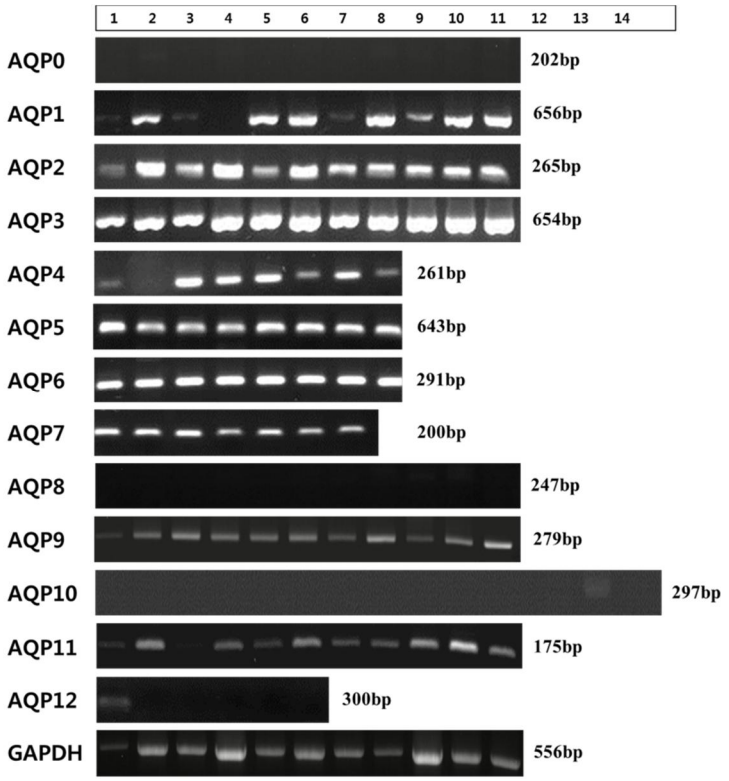


Figure III. Box-Whisker plot showing the distribution of mRNA levels of ten aquaporin (AQP) subtypes, luteinizing hormone receptor (LHR), and steroidogenic acute regulatory protein (StAR) in human luteinized granulosa cells (expressed as log scale).

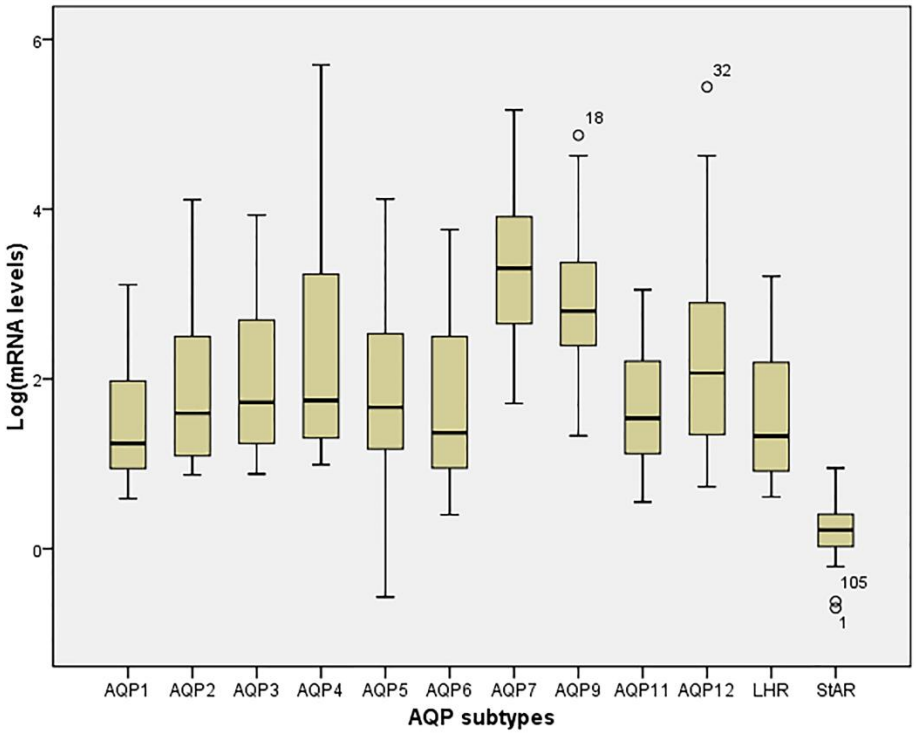


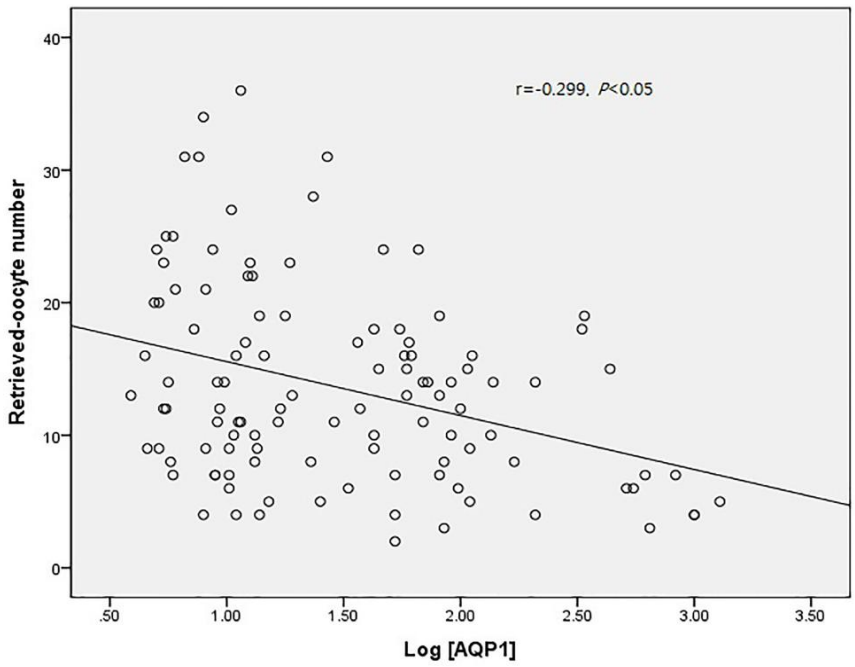
Table III. Correlation coefficient to show association of IVF outcomes with mRNA expression level of ten AQP subtypes, LHR and StAR (log scale) in total 111 women

	BMI		Retrieved oocyte number		FR	Number of top quality embryo	Percentage of top quality embryo
	(before Adjust*)	(after Adjust*)	(before Adjust*)	(after Adjust**)			
Log [AQP1]	-0.003	-0.104	-0.311‡	-0.299†	0.117	-0.173	-0.038
Log [AQP2]	-0.049	-0.129	-0.155	-0.102	0.075	-0.156	-0.056
Log [AQP3]	-0.034	-0.177	-0.144	-0.135	0.172	-0.153	-0.062
Log [AQP4]	0.004	-0.116	-0.233†	-0.221	0.165	-0.184	-0.092
Log [AQP5]	0.049	-0.049	-0.128	-0.138	0.094	-0.142	-0.056
Log [AQP6]	-0.051	-0.145	-0.203†	-0.219	0.151	-0.150	-0.046
Log [AQP7]	-0.230†	-0.259†	-0.106	-0.061	0.269†	-0.199	-0.183
Log [AQP9]	-0.020	-0.135	-0.001	-0.031	0.070	-0.133	-0.109
Log [AQP11]	-0.007	-0.130	-0.194†	-0.147	0.077	-0.164	-0.036
Log [AQP12]	0.082	-0.015	-0.185	-0.177	0.118	-0.180	-0.068
Log [LHR]	-0.006	-0.144	-0.202†	-0.145	0.166	-0.158	-0.070
Log [StAR]	-0.001	0.009	0.022	0.035	-0.013	-0.061	-0.080

Spearman correlation test.
†p <0.05, ‡p <0.01.
AQP: aquaporin, LHR: luteinizing hormone receptor, StAR: steroidogenic acute regulatory protein, BMI: body mass index, Adjust: adjustment, FR: fertilization rate.
Adjustment* was made by patient's age.
Adjustment** was made by patient's age and serum anti-müllerian hormone level.

Figure IV. Scatter diagrams show the association between retrieved oocyte number and AQP1 mRNA levels (A), and between fertilization rate and AQP7 mRNA levels (B).

A.



B.

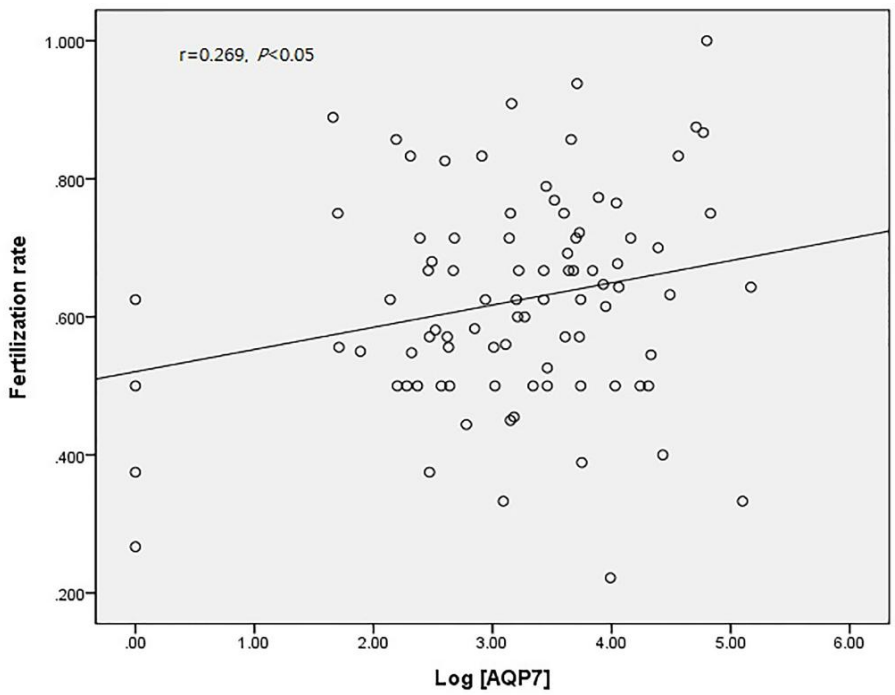


Table IV. Association of mRNA levels of ten AQP subtypes with mRNA levels of LHR and StAR (log scale)

	Log [LHR]		Log [StAR]	
	r	**p**	**r**	**p**
Log [AQP1]	0.873	<0.01	-0.421	<0.01
Log [AQP2]	0.904	<0.01	-0.448	<0.01
Log [AQP3]	0.936	<0.01	-0.468	<0.01
Log [AQP4]	0.957	<0.01	-0.536	<0.01
Log [AQP5]	0.879	<0.01	-0.491	<0.01
Log [AQP6]	0.925	<0.01	-0.556	<0.01
Log [AQP7]	0.749	<0.01	-0.461	<0.01
Log [AQP9]	0.748	<0.01	-0.307	<0.01
Log [AQP11]	0.906	<0.01	-0.480	<0.01
Log [AQP12]	0.837	<0.01	-0.471	<0.01
Log [LHR]			-0.513	<0.01

Spearman correlation test.
AQP: aquaporin, LHR: luteinizing hormone receptor, StAR: steroidogenic acute regulatory protein.

Table V. Comparison of variable parameters between pregnant and non-pregnant group

	Pregnant group (n=43)	Non-pregnant group(n=59)	p
Age	33.9 ± 3.9	36.2 ± 4.7	0.016
No. of parous women	3 (7.0%)	8 (13.6%)	NS
No. of IVF cycle	1.4 ± 0.6	1.6 ± 1.0	NS
BMI	21.3 ± 2.5	21.0 ± 2.9	NS
Basal serum FSH (mIU/mL)	6.3 ± 3.3	7.5 ± 2.8	NS
Serum AMH level (ng/mL)	3.7 ± 2.0	3.5 ± 2.7	NS
Indication of IVF			NS
Unexplained	16	17	
Tubal	12	15	
Ovulatory	6	12	
Male	7	10	
Uterine	2	5	
Pituitary suppression			
GnRH agonist	24 (55.8%)	32 (54.2%)	NS
GnRH antagonist	18 (41.9%)	28 (47.5%)	NS
No. of retrieved oocyte	13.4 ± 5.7	12.4 ± 7.6	NS
No. of mature oocyte	9.8 ± 4.3	8.9 ± 6.3	NS
No. of ICSI use	33 (76.7%)	52 (88.1%)	NS
Fertilization rate (%)	67.2 ± 0.2	73.7 ± 0.2	NS
No. of embryo transferred	2.4 ± 0.5	2.3 ± 0.5	NS
No. of top quality embryo	0.9 ± 1.5	0.6 ± 0.9	NS
%top quality embryo	13 ± 23	10 ± 19	NS
Day of embryo transfer			NS
Day 3	33 (76.7%)	52 (88.1%)	
Day 5	10 (23.3%)	7 (11.9%)	
Log [AQP1]	1.5 ± 0.6	1.5 ± 0.6	NS
Log [AQP2]	2.0 ± 0.9	1.8 ± 0.9	NS
Log [AQP3]	2.1 ± 0.9	2.0 ± 0.8	NS
Log [AQP4]	2.4 ± 1.2	2.3 ± 1.2	NS
Log [AQP5]	2.0 ± 0.8	1.8 ± 0.9	NS
Log [AQP6]	1.9 ± 0.9	1.8 ± 0.9	NS
Log [AQP7]	3.2 ± 1.0	3.2 ± 1.0	NS
Log [AQP9]	2.9 ± 0.7	2.9 ± 0.7	NS
Log [AQP11]	1.7 ± 0.6	1.7 ± 0.6	NS
Log [AQP12]	2.4 ± 1.0	2.2 ± 1.0	NS
Log [LHR]	1.7 ± 0.8	1.6 ± 0.7	NS
Log [StAR]	0.2 ± 0.3	0.2 ± 0.3	NS

Mean ± SD. Mann-Whitney U test. NS: not significant ($p > 0.05$). AMH: anti-müllerian hormone, ICSI: intracytoplasmic sperm injection, BMI: body mass index.

Table VI. Comparison of variable parameters between PCOS and non-PCOS group

	[A] PCOS group	[B] Non-PCOS group (overall)	P [A-B]	[C] Non-PCOS group (oocyte ≥14)	P [A-C]
No. of patients	17	94		34	
Age	32.9 ± 4.0	35.6 ± 4.4	0.014	34.1 ± 3.5	NS
No. of parous women	1 (5.9%)	11 (11.7%)	NS	11 (32.4%)	NS
No. of IVF cycle	1.3 ± 0.6	1.5 ± 0.9	NS	1.4 ± 0.7	NS
BMI	21.4 ± 2.1	21.2 ± 2.3	NS	21.3 ± 2.0	NS
Basal serum FSH (mIU/mL)	6.8 ± 2.0	7.1 ± 3.1	NS	5.9 ± 2.2	NS
Serum AMH level (ng/mL)	8.3 ± 5.5	3.3 ± 2.3	<0.01	3.9 ± 2.2	<0.01
Pituitary suppression			NS		NS
GnRH agonist	10 (58.8%)	45 (47.9%)		24 (70.6%)	
GnRH antagonist	7 (41.2%)	49 (52.1%)		10 (29.4%)	
No. of retrieved oocyte	24.5 ± 6.1	11.6 ± 5.8	<0.01	17.9 ± 3.9	<0.01
No. of mature oocyte	19.3 ± 5.5	8.3 ± 4.8	<0.01	12.7 ± 4.5	<0.01
No. of ICSI use	13 (76.5%)	77 (81.9%)	NS	27 (79.4%)	NS
Fertilization rate (%)	70.5 ± 18.2	71.5 ± 19.1	NS	68.1 ± 17.9	NS
No. of embryo transferred	2.0 ± 0.1	2.4 ± 0.5	<0.01	2.2 ± 0.4	<0.01
No. of top quality embryo	0.8 ± 1.1	0.7 ± 1.2	NS	1.1 ± 1.5	NS
%top quality embryo	5 ± 7	11 ± 22	NS	13 ± 20	NS
Day of embryo transfer			NS		NS
Day 3	6 (60%)	79 (85.9%)		21 (63.6%)	
Day 5	4 (40%)	13 (14.1%)		12 (36.4%)	
Clinical pregnancy rate	2/10 (20.0%)	41/92 (44.6%)	NS	19/33 (57.6%)	NS
Log [AQP1]	1.2 ± 0.5	1.5 ± 0.6	0.027	1.5 ± 0.6	NS
Log [AQP2]	1.5 ± 0.7	1.9 ± 0.9	NS	1.9 ± 0.8	NS
Log [AQP3]	1.7 ± 0.6	2.1 ± 0.8	NS	2.1 ± 0.7	NS
Log [AQP4]	1.9 ± 0.9	2.4 ± 1.2	NS	2.3 ± 1.0	NS
Log [AQP5]	1.6 ± 0.6	1.9 ± 0.9	NS	2.0 ± 0.8	NS
Log [AQP6]	1.4 ± 0.8	1.8 ± 0.9	NS	1.8 ± 0.8	NS
Log [AQP7]	3.2 ± 0.8	3.2 ± 1.1	NS	3.4 ± 0.9	NS
Log [AQP9]	2.7 ± 0.6	2.9 ± 0.7	NS	3.0 ± 0.7	NS
Log [AQP11]	1.4 ± 0.7	1.7 ± 0.6	0.024	1.7 ± 0.6	0.04
Log [AQP12]	1.8 ± 0.9	2.3 ± 1.0	NS	2.3 ± 0.9	NS
Log [LHR]	1.3 ± 0.7	1.7 ± 0.7	0.045	1.7 ± 0.7	0.042
Log [StAR]	0.2 ± 0.3	0.2 ± 0.2	NS	0.2 ± 0.2	NS

Mean ± SD. Mann-Whitney U test. NS: not significant ($p > 0.05$).

AMH: anti-müllerian hormone, ICSI: intracytoplasmic sperm injection, AQP: aquaporin, LHR: luteinizing hormone receptor, StAR: steroidogenic acute regulatory protein, BMI: body mass index.

Discussion

This study showed that mRNAs of AQP1-7, 9, 11, and 12 are expressed in human luteinized GCs from infertile women undergoing a stimulated IVF cycle. Currently, mRNA of AQP1-4 and AQP9 has been reported to be present in human GCs (Qu et al., 2010; Thoroddsen et al., 2011). Among thirteen AQP members, mRNAs for AQP0, 8 and 10 in human luteinized GCs were not found in this study. It has been shown that mRNA of AQP8 exists in GCs from the mouse and rat (McConnell et al., 2002; West-Farrell et al., 2009; Starowicz et al., 2014) and that AQP8 appears to be important in preventing formation of multi-oocyte follicles in the mouse (Su et al., 2010; Su et al., 2013). Use of highly viable GC fraction (>85% by Trypan blue stain) in this study could be one of the reasons that not all AQP subtypes were detected; it was demonstrated that AQP8 is an important determinant of mouse GC apoptosis during follicular maturation (Su et al., 2010) and therefore, AQP8 expression may be tenuous in highly viable GC fraction.

Although ten AQP subtypes in human luteinized GCs were detected in this study, it is still unknown whether whole AQP members are expressed in antral or growing follicles before ovulation is triggered. Nonetheless, it is encouraging that two aquaglyceroporins (AQP7 and AQP9) were highly expressed in human luteinized GCs, because these AQPs can transport not only water but also neutral solutes, including glycerol, urea, and other small non-electrolytes (Huang et al., 2006). The expression of these two isoforms of AQPs in human GCs would facilitate the rapid transport of small neutral molecules, which is important in follicle expansion and development (Huang et al., 2006).

Furthermore, the mRNA level of AQP7 was found to be positively associated with fertilization rate, which is a surrogate marker of oocyte competence.

Thus expression of AQP7 could be a marker for adequate folliculogenesis and healthy oocytes. It is largely unknown why only AQP7 was correlated with fertilization rate among same aquaglyceroporin family (AQP 3, 7, 9, and 10). It is assumed that the expression levels of specific AQP subtype could be different according to specific cell types.

A negative association between some AQP members (especially AQP1) and retrieved oocyte number is one of the intriguing findings from the present study. This study's finding suggests that AQP1 may be one of the factors that modulate individual ovarian response to exogenous gonadotropin. An earlier report indicated that over-expression of AQP1 may be harmful because it increased cell water permeability and enhanced the rate of cell death in Chinese hamster ovary (Jablonski et al., 2004). It was reported that AQP1 expression abruptly increased after follicular rupture, and therefore that AQP1 may act on the process relating to transition of the follicle into a corpus luteum (Thoroddsen et al., 2011; Sales et al., 2013). Since AQP1 may be a surrogate marker of corpus luteum function, decreased expression of AQP1 suggests weaker corpus luteum activity in high responders (Sales et al., 2013),. In the present study, mRNA levels of LHR and StAR, a well-known GC luteinization marker, were also assessed. Interestingly, mRNA levels for ten AQP subtypes were correlated positively with LHR mRNA levels but negatively with StAR mRNA levels. A significant negative association was also found between mRNA levels of LHR and StAR. StAR is a key regulatory protein of steroid hormone biosynthesis (Clark et al., 1995; Clark et al., 1997; Stocco and Clark, 1997; Chen et al., 1999). Both mRNA and protein of StAR were found to be rich in functional corpus luteum whereas they were absent in regressed corpus luteum (Chen et al., 1999). Their expression can be enhanced by luteotropic hormones such as hCG, LH, and estradiol (Juengel et

al., 1995; Sandhoff and McLean, 1996; Townson et al., 1996). A negative association of StAR expression with AQP or LHR expression may indicate that earlier well-luteinized corpus luteum expresses less AQP and less LHR. The expression of AQPs could be modulated by several sex steroid hormones (Jablonski et al., 2003; Richard et al., 2003; He et al., 2006; Lindsay and Murphy 2006), however, the relationship is unknown because sex steroid hormones in patient's serum or FF were not measure in this study. Nonetheless, a negative association of StAR expression with LHR expression was consistent with the result by Tsuchiya et al. who demonstrated that injection of an ovulatory dose of hCG caused an increase of StAR mRNA levels and a temporary loss of LHR mRNA levels in follicular cells (Tsuchiya et al., 2003).

In the present study, GCs from women with PCOS showed lower mRNA levels of AQP1, AQP11 and LHR compared to those from women without PCOS. After selecting high responders only (oocyte number ≥14), mRNA levels of AQP11 and LHR were still lower in the PCOS group. The lower mRNA level of AQP9 have been reported in GCs from women with PCOS than those from women without PCOS (Qu et al., 2010), but such a difference was not found in this study. Because women with PCOS have divergent phenotypic and hormonal profiles, further investigation would be needed to clarify the association of AQP expression with sex steroid hormone status in women with PCOS.

Interestingly, among ten AQP subtypes, the mRNA level of AQP7 only was negatively correlated with BMI. Previous two studies also showed lower expression of AQP7 in obese than lean women, but the AQP was measured in adipose tissue (Marrades et al., 2006; Prudente et al., 2007). Although the studied cell types are different, lower expression of AQP7 in obese women is

same with the result of present study, interestingly. The possible mechanisms whether AQP7 affect the pathophysiology of obesity should be explored further. In women with PCOS, BMI would be higher than those without PCOS, however, in the present study, BMI as well as mRNA level of AQP7 were not different between two groups. From previous two reports from Korea, obesity is very rare among the Korean women with PCOS (Kim et al., 2012; Lee et al., 2009), thus the obesity may be not the major component in the Korean women with PCOS.

Previous studies reported that LHR is over-expressed in GCs from small antral follicles (less than 10 mm) in women with PCOS (Willis et al., 1998; Jakimiuk et al., 2001). However, there were no reports concerning LHR expression after ovarian stimulation. Considering the results of Tsuchiya et al. who demonstrated temporary loss of LHR after hCG triggering (Tsuchiya et al., 2003), LHR-lowering action by hCG triggering might occur more profoundly in women with PCOS.

A relatively small number of subjects in the PCOS group would be the main limitation of this study. Thus a definite conclusion would be possible after a further large-scaled study. Certain markers should be verified by protein level to understand the role of the marker. Therefore, based on results in this study, AQP levels should be confirmed by western blotting and further functional analyses should be made.

In conclusion, this study showed that mRNAs of AQP1-7, 9, 11, and 12 are expressed in human luteinized GCs from infertile women undergoing a stimulated IVF cycle. The mRNA level of AQP1 was associated with retrieved oocyte number and the mRNA level of AQP7 was associated with fertilization rate. A different profile of AQP expression was observed in GCs from women with PCOS.

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국문 초록

서론: 본 연구에서는 황체화된 과립막 세포에서 분비되는 아쿠아포린계(aquaporins, AQPs)의 mRNA 발현 정도와 체외수정시술 결과와의 관련성을 규명하고자 하였다.

방법: 체외수정시술을 받은 111 명을 대상으로, 난자 채취 당일 황체화된 과립막 세포를 수집하였다. 미리 27 명을 대상으로 AQP0-12 의 mRNA 발현 양상을 RT-PCR 의 방법으로 조사하였고, 이 중에서 AQP0, 8, 10 은 발현되지 않았다. AQP1-7, 9, 11, 12 의 mRNA 발현양을 qRT-PCR 의 방법으로 조사하였다. Luteinizing hormone receptor (LHR)과 steroidogenic acute regulatory protein (StAR)의 mRNA 발현 양상도 qRT-RPC 로 조사하였다.

결과: 111 명의 연구 대상에서, 채취된 난자 갯수가 AQP1, 4, 6, 11 그리고 LHR 의 mRNA 발현 정도와 음의 상관관계가 있었다($r = -0.311$, $r = -0.233$, $r = -0.203$, $r = -0.194$, and $r = -0.202$, respectively, $p<0.05$ for each). 그러나 나이와 AMH 값으로 보정한 이후에는 AQP1 만이 음의 상관관계가 있었다($r = -0.299$, $p<0.05$). 나이로 보정 후 체질량지수는 AQP7 과 음의 상관관계가 있었다($r = -0.259$, $p<0.05$). 수정율은 AQP7 과 양의 상관관계가 있었다($r=0.269$, $p<0.05$). 배아의 질과 임신율은 AQP 의 발현

정도와 관련이 없었다. AQP 의 mRNA 발현 정도는 LHR 발현과 양의 상관관계가 있었고, StAR 발현과는 음의 상관관계가 있었다. 채취된 난자 갯수가 14 개 이상인 고반응군에서, AQP11 과 LHR 의 mRNA 발현 정도가 다낭성난소증후군 환자군에서 더 낮게 발현되었다.

결론: 황체화된 과립막 세포에서는 AQP 1-7, 9, 11, 12 의 열 가지 아형의 mRNA 이 발현되었다. AQP1 은 발현 정도는 체외수정시술의 채취된 난자 갯수와 음의 상관관계가 있었고, AQP7 의 발현 정도는 수정율과 양의 상관관계가 있었다. AQP1 은 외부 성선자극호르몬 주사에 대한 난소 반응을 조절하는 요소 중 하나일 가능성이 있으며, AQP7 은 난자 형성과 난자의 질을 나타내는 표지자로써의 가능성이 있겠다.

주요어: Aquaporin, 과립막 세포, 체외수정시술, 다낭성난소증후군

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