발생과 생식 제 8권 제 1호, 49~55 (2004)
Dev. Reprod. Vol. 8, No. 1, 49~55 (2004)



# Expression of Aquaporin-4 and -8 Genes in Mouse Uterus during the Estrous Cycle

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# 발정주기 동안 생쥐 자궁에서의 Aquaporin-4와 -8 유전자의 발현

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**ABSTRACT** : Aquaporins(AQPs) are a family of transmembrane water channel proteins that are widely distributed in various tissues throughout the body and play a major role in transcellular and transepithelial water movement. Uterine endometrium undergoes recurrent uterine stromal edema in response to hormonal stimuli, however, the mechanism regulating the fluid transport during the estrous cycle has not been fully understood. To investigate the possible role of AQPs in water movement in uterus during the estrous cycle, expression patterns of AQP - 1, - 3, - 4, - 5, - 8, and - 9 mRNA in mouse uterus were analyzed by using semiquantitative reverse transcription-polymerase chain reaction(RT-PCR). We employed a combination of laser capture microdissection(LCM) and RT-PCR to examine the expression patterns in specific uterine cell types luminal epithelial cells(LE) and stromal cells(S). Our results showed that the level of AQP-4 mRNA was significantly increased while the level of AQP-8 mRNA was significantly decreased during the proestrus through the estrus stage. In addition, LCM revealed that AQP-4 and - 8 mRNAs were highly expressed in LE compared with S. Taken together, these results suggest that AQPs may have an important function in physiological changes of mouse uterus during the estrous cycle.

**Key words** : Aquaporins, Estrous cycle, LCM, Mouse, Uterus.

**요 약** : Aquaporins(AQPs) 유전자는 다양한 조직의 상피세포와 내피세포에 존재하며 다량의 물 이동을 조절하는 막성 단백질로서, 세포간 또는 세포막 사이의 물 이동에 중요한 역할을 하는 것으로 알려져 있다. 발정기의 생쥐 자궁에서는 자궁내막세포의 증식과 함께 수화되는 특징을 보이며, 자궁내강으로 물이 이동되어 자궁내액의 점성이 낮아지는 현상이 나타난다. 따라서, 본 연구에서는 생쥐의 발정주기 동안 자궁에서의 형태학적인 변화와 관련하여 AQP 유전자가 물 이동의 매개체로서 중요한 역할을 할 것으로 추측하여, 생쥐 발정주기 동안에 AQP 유전자의 발현 양상을 역전사 중합효소 연쇄반응을 통하여 관찰하였다. 또한, laser capture microdissection(LCM)을 이용하여 자궁내 세포의 종류에 따른 AQP 유전자의 발현 양상을 조사하였다. AQP-4 전령체는 발정주기의 proestrus와 estrus 시기에 발현량이 유의하게 증가하는 반면, AQP-8 전령체는 동일한 시기에 유의하게 감소하는 것으로 관찰되었다. 또한, LCM 기법을 통해서 AQP-4와 -8 전령체가 자궁기질세포보다 자궁내막세포에서 강하게 발현이 유도됨을 관찰하였다. 결과적으로, 생쥐 자궁에서 AQP-4와 -8 유전자가 발정주기에 따라 발현량이 변화하는 것으로 보아 난소내 호르몬인 estrogen과 progesterone에 의해 조절될 가능성이 있으며, proestrus와 estrus 시기에 자궁내강으로 자궁내액을 수송하는데 중요한 역할을 할 것으로 사료된다.

## INTRODUCTION

In mammals, the uterus of a mature female undergoes a cyclic trophy-atrophy alternation. This is a steroid-driven event called estrous cycle in rodents, reflecting the cyclic change of the uterine endometrium in preparation for reproduction. The ovarian hormones, estrogen($E_2$) and progesterone($P_4$) which are secreted in variant during the estrous cycle, serve as inducer or repressor

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in cellular physiology(Jacob & Monod, 1961) and lead to the morphological and functional changes of uterus(Kim et al., 1982). Absorption and secretion of uterine luminal fluids are important for sperm capacitation, embryo development, and implantation (Paria et al., 2000). Although it has been suggested that uterine vascular permeability was increased to response with estrogen or certain vasoactive mediators in the mouse, the mechanisms that regulate fluid transport during the estrous cycle are not fully understood.

Water movement across cells can occur either by diffusion through the lipid bilayer or through protein water channels termed aquaporins(AQPs). Structurally, the AQPs consist of six transmembrane spanning domains and two hemi-channels, each with a highly conserved NPA(asparagines, proline, and alanine) motif. These hemi-channels are found on loops B and E and form an hourglass shaped channel which may permit water transport (Heymann & Engel, 1999). In mammals, there are currently more than 10 isotypes of water channels, referred to as AQPs: AQP-0~AQP-9(Sui et al., 2001). AQPs can be divided into two major groups: whereas AQP-0, -1, -2, -4, -5, -6, and -8 are permeable to water but not to small organic and inorganic molecules, AQP-3, -7, and -9 are permeable to glycerol or urea as well as water(Borgnia et al., 1999). Previous studies have shown that specific AQP isotypes are expressed in both the male and female reproductive tissues in some mammalian species(Fisher et al., 1998; Gannon et al., 2000).

Even though a number of studies on AQPs, little is known about the expression patterns of AQP family genes during the estrous cycle in mouse. The purpose of the present study was to examine the expression profile of the AQP family genes in the mouse uterus during the estrous cycle. We performed the RT-PCR to examine the expression pattern of AQP family genes at mRNA level. In addition, laser capture microdissection(LCM) was conducted to determine the expression of AQP family genes in specific uterine cell types.

## MATERIALS AND METHODS

### 1. Animal and Uterus Preparation

ICR mice(Department of Life Science, Hanyang University) were maintained under the controlled environment with 14L:10D photoperiod and provided with food and water *ad libitum*. Vaginal smears were daily checked and the stage of the estrous cycle was determined based on the characteristic features of specific stages of estrous cycle, as described earlier(Rugh, 1980). Half of the uterus was immediately frozen in liquid nitrogen for total RNA isolation and the remaining tissue was embedded in Tissue-Tek(Sakura Finetek, Torrance, CA) for LCM. These samples were stored at −70℃ until use.

### 2. Total RNA Extraction

All solutions used in this study were prepared with distilled water treated with 0.1% diethylpyrocarbonate(DEPC; Sigma, St. Louis, MO). Total RNA extraction was performed by using TRIZOL reagent(InVitrogen, Carlsbad, CA) modified from guanidium thiocyanate-phenol-chloroform RNA extraction protocol (Chomozynski & Sacchi, 1987). The resuspended RNA was then treated with ribonuclease(RNase)-free deoxyribonuclease(DNase; Takara, Shiga, Japan) to remove any contaminating DNA. Concentration and purity were evaluated by the ratio of optical density ($OD_{260}$:$OD_{280}$) with spectrophotometer(Ultraspec 2100 pro, Amersham Pharmacia Biotech, Cambridge, United Kingdom).

### 3. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

One μg of total RNA in DEPC-treated distilled $H_2O$ was incubated at 95℃ for 5 min. RT was carried out with 20 μl reaction mixture of 4 μl 25 mM $MgCl_2$(Takara), 2 μl 10× PCR buffer(100 mM Tris-HCl, 500 mM KCl, pH 8.3), 2 μl 10 mM dNTPs(Gibco BRL, Rockville, MD), 1 μl Oligo(dT) 15 primer(Promega, Madison, WI, USA; 2.5 μM), 1 μl RNasin (Applied biosystems, Foster, CA), 1 μl Murine Leukemia Virus reverse transcriptase(MuLV; Applied biosystems), and DEPC-treated distilled $H_2O$. The reaction was carried out in the PCR thermal cycler(Takara PCR Thermal cycler 480) by using a program with the following parameter: 42℃ for 60 min; 99℃ for 5min. After reaction was completed, samples were either used directly for PCR or stored at −20℃.

Prior to use cDNA for PCR reactions of target AQP genes, each uterine RNA sample obtained via vaginal smear check was validated by assaying for up-regulated or down-regulated genes which are previously known to be changed during the estrous cycle in the mouse uterus. These include lactoferrin(LF), calcium-binding protein D9k(Calbindin-$D_{9K}$), complement C3(C3), and

**Table 1. Primers used for RT-PCR**

| Genes | | Primer sequences | Product size(bp) | GenBank No. |
|---|---|---|---|---|
| Lactoferrin | F | 5'-AGGAAAGCCCCCCTACAAAC-3' | 276 | D88510 |
| | R | 5'-GGAACACAGCTCTTTGAGAAGAAC-3' | | |
| Calbindin-$D_{9k}$ | F | 5'-ATGTGTGCTGAGAAGTCTCCTGCAGAAATG-3' | 239 | AF136283 |
| | R | 5'-CATTGTGAGAGCTTTTTGAAGAAAGCTTCG-3' | | |
| Complement C3 | F | 5'-GTGAGGGGCAGGTCAATAGC-3' | 242 | NM009778 |
| | R | 5'-CAGGAGGGCGTAGGATGTGG-3' | | |
| Factor B | F | 5'-CTGTGGCATGGTGTGGGAGC-3' | 369 | NM008198 |
| | R | 5'-GTGCAGGGGAGACAGATGGG-3' | | |
| rpL7 | F | 5'-TCAATGGAGTAAGCCCAAAG-3' | 246 | BC051261 |
| | R | 5'-CAAGAGACCGAGCAATCAAG-3' | | |
| AQP-1 | F | 5'-CATTCTCTCGGGCATCACCT-3' | 481 | BC007125 |
| | R | 5'-TCTATTTGGGCTTCATCTCC-3 | | |
| AQP-3 | F | 5'-TTGAACCCCGCTGTGACCTT-3' | 262 | BC027400 |
| | R | 5'-CGCTGTGCCTATGAACTGAT-3' | | |
| AQP-4 | F | 5'-TGGAGCCGGCATCCTCTACC-3' | 480 | U88623 |
| | R | 5'-TCCACTTGGCTCCGGTTGTC-3' | | |
| AQP-5 | F | 5'-CCCTCATCTTCGTCTTCTTTG-3' | 307 | NM009701 |
| | R | 5'-TTGCCTGGTGTTGTGTTGTTG-3' | | |
| AQP-8 | F | 5'-TCATTGCTACCTTGGGGAAC-3' | 409 | AF018952 |
| | R | 5'-CAGGCTCCAGAGATGCTACC-3' | | |
| AQP-9 | F | 5'-CCCAAAACCATTTGTATCCG-3' | 542 | BC024105 |
| | R | 5'-GCTGGTTCTGCCTTCACTTC-3' | | |

F, forward primer; R, reverse primer.

Factor B(BF). Using these genes, validated uteri were selected and carried out RT-PCR with target AQP genes.

PCR was carried out in 1.6 μl 25 mM $MgCl_2$, 2 μl 10× PCR buffer(100 mM Tris-HCl, 500 mM KCl, pH 8.3), 1.6 μl 2.5 mM dNTPs(Takara), Ex *Taq* polymerase(Takara), 0.5 μl 10 pmol each of the appropriate forward and reverse primers, 12.7 μl of DEPC-treated distilled $H_2O$, and 1 μl of cDNA. After mixing all components in a 200 μl tube(BIO-RAD thin wall tube, BIO-RAD, Hercules, CA, USA), the PCR reaction was carried out in the PCR thermal cycler. Following an initial denaturation at 94℃ for 5 min, optimized cycles of amplification were performed at 94℃ for 30 sec, each annealing temperature(60～65℃) for 30 sec, 72℃ for 30 sec. The final extension reaction was performed at 72℃ for 10 min and cooled down to 4℃. The sequences of the primers used in RT-PCR were listed in Table 1.

Subsequently, reaction product was analyzed using 1% agarose gel(Seakem LE agarose, FMC Bioproducts, Rockland, MA, USA) electrophoresis at 100V for 30 min, stained with ethidium bromide(EtBr, 0.5 g/ml), and photographed under UV transilluminator. The intensity of each band was quantified by densitometric scanning followed by BioID image analysis software (GS-700, BIO-PROFIL, Vilber Lourmat, Cedex, France) and the expression levels were normalized against the density of the corresponding ribosomal protein L-7(rpL7) PCR product as an internal control.

### 4. Laser Capture Microdissection(LCM) and Semiquantitative RT-PCR

LCM system allows the isolation of specific uterine cell types without contamination from other cell types. Green et al.(2003) showed that each specific cell type could be isolated with LCM system and mRNA from each cell type could be successfully

extracted with high purity. Thus, we isolated luminal epithelial cells and stromal cells respectively and carried out RT-PCR. Uterine horns were embedded in Tissue-Tek compound and frozen in liquid nitrogen. Tissue samples were cut in 6 μm thickness and mounted onto poly-L-lysine coated clean glass slides. Identification of specific cell types were judged from parallel hematoxylin stained sections. After staining with Harris hematoxylin, each population of uterine cells was isolated using P.A.L.M. Robot-Micro-beam version 4.0(P.A.L.M. Microlaser Technologies AG, Berniried, Germany). For each cell, an average of 120 laser shots were transferred onto 0.5 ml tube cap and stored at −70°C until utilizing for total RNA extraction(Hong et al., 2004). Total RNA extraction step was initiated with 300 μl TRIZOL reagent and following step was same above. RT-PCR was carried out using total RNA and the ratio of each specific gene against rpL7 gene was calculated.

### 5. Statistical Analysis

In quantitative PCR study, each bar represented mean±SD. Student's *t*-test was used to determine the statistical significance of mRNA expression level. $P<0.05$ was considered statistically significant.

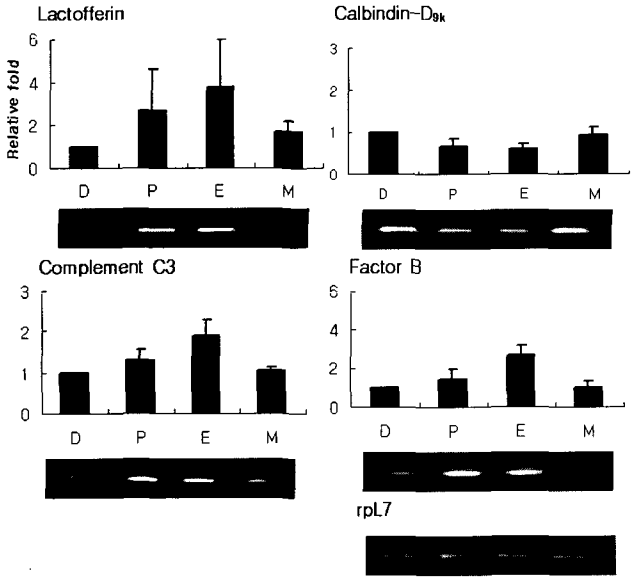


**Fig. 1. RT-PCR analysis to validate the responsiveness of estrogen in mouse uterus using well-characterized marker genes.** Values of each band were normalized to rpL7 for the same sample. D, diestrus; P, proestrus; E, estrus; M, metestrus. Each value represents the mean±SD from triplicated experiments.

## RESULTS

### 1. Validation of Marker Genes Regulated by Estrogen in Mouse Uterus

To validate correctness of collected uteri via vaginal smear check, RT-PCR was carried out by assaying several marker genes which are known to be regulated by estrogen during the estrous cycle in mouse uterus. RT-PCR result showed that the expression of LF, C3, and BF are increased at the proestrus and estrus stage, whereas Calbindin-$D_{9k}$ is increased at the metestrus and diestrus stage. These results were in accordance with those of other previous studies(Walmer et al., 1992; Das et al., 1998; Nie et al., 2000; Li et al., 2002). The expression patterns and the relative ratios of each gene are shown in Fig. 1.

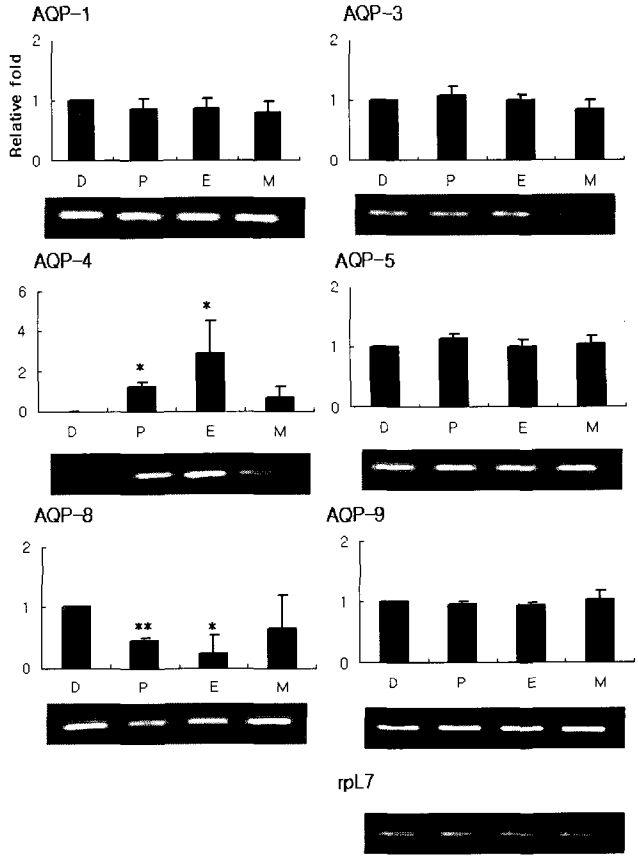


**Fig. 2. Expression patterns of AQP-1, -3, -4, -5, -8, and -9 mRNAs in mouse uterus during the estrous cycle using semiquantitative RT-PCR.** Values of each band were normalized to rpL7 for the same samples. D, diestrus; P, proestrus; E, estrus; M, metestrus. Each value represents the mean±SD from triplicated experiments(*, $p<0.05$; **, $p<0.001$).

### 2. Screening of AQP mRNAs Expression in Whole Uterus during the Estrous Cycle

RT-PCR was performed to investigate the expression patterns of AQPs mRNA(AQP-1, -3, -4, -5, -8, and -9) in uteri.

Whereas the expression levels of AQP-1, -3, -5, and -9 mRNA were not changed, AQP-4 and -8 mRNAs were changed during the estrous cycle(Fig. 2). The expression level of AQP-4 mRNA was significantly increased at the proestrus($p<0.05$) and estrus stage($p<0.05$), but decreased at the metestrus and diestrus stage. This change is paralled with that of LF. In contrary with AQP-4, the expression of AQP-8 mRNA was significantly decreased at the proestrus($p<0.001$) and estrus stage($p<0.05$) compared to the metestrus and diestrus stage.

### 3. Analysis of AQP-4 and -8 Expression Patterns in Specific Uterine Cell Types

RT-PCR data in Fig. 2 shows the expression pattern of AQP mRNAs in whole uterus including all uterine cell types - luminal epithelial, stromal, and muscle cells. Therefore, we isolated luminal epithelial and stromal cells using the LCM technique and carried out RT-PCR to investigate the expression in uterine specific cell types. Fig. 3 shows a microdissections of specific uterine cells-stromal cells and luminal epithelial cells. LCM revealed that AQP-4 and -8 mRNAs were predominantly expressed in luminal epithelial cells compared with stromal cells (Fig. 4). While AQP-4 mRNA was strongly activated in luminal epithelial cells at the proestrus and estrus stage(Fig. 4A), AQP-8 mRNA was showed the highly expressed in luminal epithelial cells at the diestrus stage(Fig. 4B). Quantitative RT-PCR data clearly showed that some members of AQP genes were differentially regulated during the estrous cycle in specific uterine cell types.

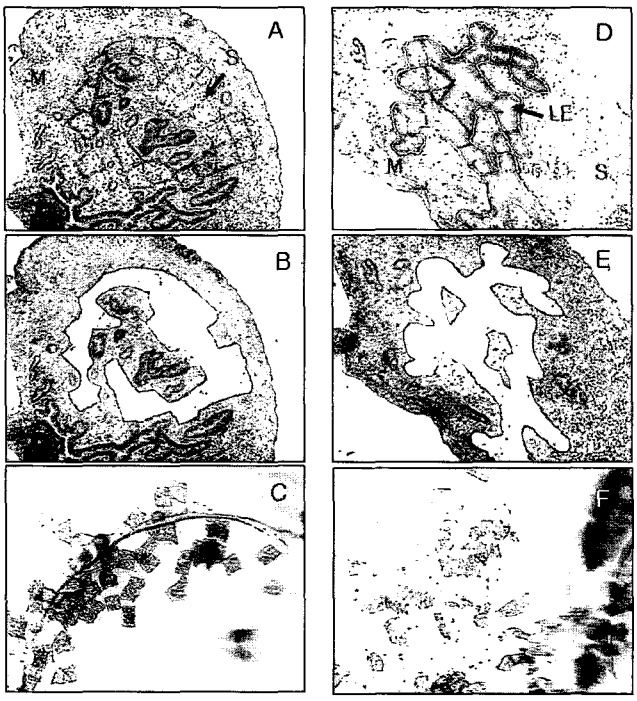


**Fig. 3. Laser capture microdissection(LCM) procedure.** Uterine sections were stained with hematoxylin before LCM(A and D). Remained sections after capture of stromal cells(B) and luminal epithelial cells(E) from A and D. Captured cells on the cap of the tube(C and F). LE, luminal epithelial cells; S, stromal cells; M, muscle cells.

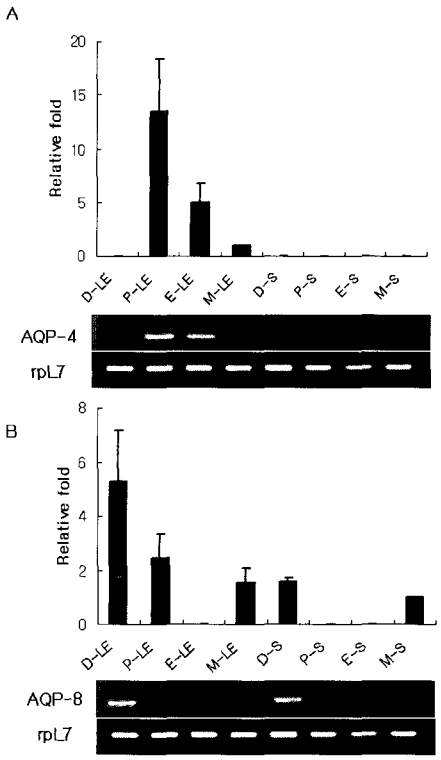


**Fig. 4. Expression patterns of AQP-4(A) and -8(B) mRNAs in specific uterine cell types during the estrous cycle.** Values of each band were normalized to rpL7 for the same samples. D, diestrus; P, proestrus; E, estrus; M, metestrus. LE, luminal epithelial cells; S, stromal cells. Each value represents the mean±SD from triplicated experiments.

## DISCUSSION

The mammalian uterus undergoes extensive morphological changes during the estrous cycle. To better understand the me-

chanisms that underlie uterine edema during the estrous cycle, we examined the expression of AQP water channels during the estrous stage. We investigated the mRNA expression levels of AQP-1, -3, -4, -5, -8, and -9 during the estrous cycle by RT-PCR analysis. Whereas AQP-1, -3, -5, and -9 mRNAs were not changed, the expression levels of AQP-4 and -8 mRNAs were dramatically changed compared to other genes during the estrous cycle. We measured the AQPs' mRNA levels in whole uterus using the RT-PCR. In addition, LCM system make it possible that could verify the localization of AQP mRNAs whether in endometrium or in myometrium. By using LCM based RT-PCR, we identified AQP-4 and -8 mRNA expressions in mouse uterus and clearly showed their cell-type specific expression patterns. So far, histological approaches, such as *in situ* hybridization and immunohistochemistry, have been the unique way to clarify cell-specific localization of genes within tissues, containing the uterus. It is extremely difficult to perform *in situ* hybridization and/or immunohistochemistry for follow-up experiments of enormous numbers of uterine genes from cDNA microarrays. Recently developed LCM approach coupled with RT-PCR is a new and easy tactic to investigate cell-specific localization of numerous numbers of genes(Green et al., 2003; Hong et al., 2004).

In our preliminary study, we performed the localization of AQP-4, -5, and -8 proteins in mouse uterine section by immunohistochemistry(data not shown). Recently, Jablonski et al.(2003) could not found the expression of AQP-4 and -5 proteins in either $E_2$-injected or oil-injected mouse. However, Richard et al.(2003) showed that mRNAs and proteins of AQP-4 and -5 were existed in the periimplantation uterus. In the present study, we showed the existence of AQP-4 and -5 mRNAs in mouse uterus during the estrous cycle. Although the reasons underlying these variations are not clear, they might be attributed to the different strains of mouse used in experiment, differences in experimental procedures; for example, ovariectomized and steroid supplemented mice(Jablonski et al., 2003) vs. mice at periimplantation period(Richard et al., 2003). The present study showed that AQP-4 mRNA was strongly expressed in luminal epithelial cells compared with stromal cells by LCM and this mRNA was prominently increased at the proestrus and estrus stage. Fata et al.,(2001) measured the nonmanipulated serum levels of $P_4$ and 17β-estradiol at a window that represents the initial onset each estrous stage. They observed a 40% increase in the average serum concentration of 17β-estradiol from proestrus (47.3 ± 2.1 pg/ml) to estrus(66.0 ± 3.2 pg/ml). Thus, we suggested that the significant increased expression of AQP-4 in these stages may reflect the effects of estrogen at this stage, causing the uterus to imbibe water into lumen.

AQP-8 was the only isotype found in the uterine stromal cells, where supporting the confluent distribution of water in this region(Jablonski et al., 2003), and our result correlate with this data in protein level(data not shown) and laser captured sample mRNA. Thus, we suggest that water imbibition into the uterine tissue from the capillaries to the spaces surrounding the stromal cells, occurs through increased diffusion, resulting from the increased hyperemia and capillary permeability. These AQP genes may be important in limiting this imbibed water to the stromal layer while protecting the myometrial layer from edema. However, to the contrary with AQP-4, AQP-8 mRNA expression was increased during the metestrus and diestrus stage, thus its function might be imbibition of water from lumen to capillary cell.

In summary, the expression of AQP genes are differentially regulated during the estrous cycle in mouse uterus. It is also possible that these AQP genes are regulated under ovarian steroid hormones during the estrous cycle. Therefore, these results may enable better understanding of the mechanisms underlying the cyclic alteration of uterine trophy-atrophy that occurs in preparation for sperm capacitation and endometrial receptivity after mating.

## ACKNOWLEDGEMENTS

We thank Prof. Hee Gyoo Kang for contributing to several experiments and helpful suggestions in Department of Biomedical Laboratory Science, Seoul Health College. We are also grateful to Hee Joung Lim for the excellent technical assistance of laser capture microdissection.

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