Menopause: The Journal of The North American Menopause Society
Vol. 15, No. 4, pp. 648-654
DOI: 10.1097/gme.0b013e31816086ef

Decreased expression of endometrial vessel AQP1 and endometrial epithelium AQP2 related to anovulatory uterine bleeding in premenopausal women

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Abstract

Objective: Aquaporins (AQPs) may be involved in the occurrence of abnormal uterine bleeding as the mediators between ovarian steroids and cyclic endometrial changes. The aim of the present study was to investigate the characteristics of endometrial AQPs in women with anovulatory uterine bleeding and explore the relationship between endometrial AQPs and ovarian steroids.

Design: Sixty-one women with premenopausal anovulatory uterine bleeding and 108 women with normal cycles were involved in this study. Endometrial biopsies were obtained from the women with anovulatory uterine bleeding and normal control women. Serum estradiol and progesterone concentrations were measured with enzyme-linked immunosorbent assay on the same day as an endometrial biopsy was performed. AQP1 and AQP2 mRNA expression was evaluated using reverse-transcriptase polymerase chain reaction. Immunohistochemistry was used to localize AQP1 and AQP2 in the endometrium, and their expression was quantified by an image analysis/measuring system.

Results: AQP1 was located in the endothelium of small vessels, whereas AQP2 was mainly found in luminal and glandular epithelium. The expression levels of AQP1 and AQP2 mRNA and protein were higher in the secretory phase than those in the proliferative phase ($P < 0.01$) in normal endometrium, and their expression was related to serum steroid hormones ($P < 0.01$). However, the expression of AQP1 and AQP2 decreased in the endometrium in anovulatory uterine bleeding comparing with normal endometrium ($P < 0.01$). The correlation between AQP expression and ovarian steroids vanished ($P > 0.05$) in anovulatory uterine bleeding.

Conclusions: Our findings indicate that cyclic expression of endometrial AQP1/AQP2 correlated with steroid hormone levels may be essential to normal endometrial function and decreased AQP1/AQP2 expression in endometrial vessels or epithelium may be involved in the occurrence of anovulatory uterine bleeding.

Key Words: Aquaporin – Estradiol – Progesterone – Anovulatory uterine bleeding – Endometrium.

Dysfunctional uterine bleeding is a common gynecologic disorder that can affect any woman during her reproductive years. Most patients with dysfunctional uterine bleeding have an underlying etiology of chronic anovulation with unopposed estrogen stimulation of the endometrium, and it may be associated with disturbed endometrial angiogenesis,[1] increased vascular fragility,[2] and loss of the integrity of the endothelial, epithelial, and stromal supporting structures.[3]

The aquaporins (AQPs) are a family of proteins that facilitate rapid passive movement of water.[4] Water transport across an epithelial barrier can occur by transcellular or paracellular means.[5] The presence of tight junctions between the epithelial cells in endometrium indicates that water crosses this layer through transcellular pathways.[6,7] AQPs exhibit a greater capacity for water movement compared with simple diffusion alone[8] and may be the chief controller of fluid movement in the endometrium.[4] Cyclic variations of menstruation necessitate well-controlled, dynamic movement of fluids across cell membranes. Therefore, regular expression of AQPs is important for endometrial fluid balance and normal cyclic menses. Furthermore, angiogenesis is an essential component of endometrial repair and regeneration

Received August 30, 2007; revised and accepted October 30, 2007.

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Funding/support: This work was supported by National Basic Research Program of China (no. 973: 2006CB944006 and 2006CB504004), National Natural Science Foundation of China (no. 30670789 and 30700273), and Key Research Program of Science and Technology Department of Zhejiang Province, China (no. 2006C13078 and no. 2005C23021).

Financial disclosure: None reported.

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TABLE 1. *Characteristics of anovulatory uterine bleeding and normal menstrual cycle groups*

Group	N	Age, y	BMI	Hypertension, %	Diabetes, %	Coronary heart disease, %	Participation rates, %
Anovulatory uterine bleeding	51	40.1	23.8	5.3	7.3	5.9	93.8
Normal cyclic	88	40.5	23.5	5.7	6.9	5.3	95.6

N, number of samples; BMI, body mass index.

after menses. Perturbation of this process is associated with abnormal uterine bleeding. It has been shown that AQP1 plays a role in angiogenesis,[9] suggesting that natural angiogenesis and menses might require regular AQP1 expression.

Therefore, disturbance of AQPs expression may play a role in the occurrence of anovulatory uterine bleeding. To date, studies undertaken to elucidate the roles of AQPs in human endometrium are still quite limited. A microarray experiment found AQP1 and AQP2 mRNA present in human endometrium.[10] Recently, several immunohistochemical studies demonstrated the localization of AQP1 and AQP2 in endometrium.[11-13] In addition, there has been only one report on AQP in endometrium pathophysiology published to date.[13] Therefore, the purpose of the present study was to determine whether the expression of AQP1 and AQP2 in anovulatory uterine bleeding endometrium changed compared with that in the normal menstrual cycle. We also investigated whether expression of AQPs in human endometrium was regulated by ovarian steroids.

METHODS

Tissue samples

Ethical approval for this study was obtained from the Ethics Committee of the School of Medicine of Zhejiang University, and informed consent was received from each woman. Endometrial samples for reverse-transcriptase polymerase chain reaction (RT-PCR) were obtained from 30 women, including 10 women with anovulatory uterine bleeding (median age, 35 y) and 20 control women (median age, 31 y). The endometrial samples of the anovulatory uterine bleeding group were collected when the women underwent dilatation and curettage. The control women had normal menstrual cycles (28-32 d) and attended our hospital for in vitro fertilization embryo transfer treatment because of male factor infertility. Endometrial samples in these patients were obtained by a biopsy catheter (Pipelle, Laboratoire CCD, Paris, France) during the proliferative or secretory phase of the spontaneous menstrual cycle before in vitro fertilization embryo transfer treatment. Women were excluded if they had received exogenous hormones in the past 3 months. Endometrial tissues were rinsed in sterile saline, snap frozen in liquid nitrogen, and stored at −70°C for RNA extraction shortly after collection.

For immunohistochemistry, 139 women were involved in this study, comprising the anovulatory uterine bleeding group and control group (normal menstrual phase group). The anovulatory uterine bleeding group included 51 endometrial samples from premenopausal women (median age, 41 y) who underwent hysterectomy or endometrial biopsy for anovulatory uterine bleeding. The diagnosis of anovulatory uterine bleeding was confirmed by history, low serum progesterone, and results of endometrial sampling. Eighty-eight women who had normal menstrual cycles and had undergone a biopsy for infertility or hysterectomy for cervical diseases constituted the control group. The control endometrial samples were collected during the menstrual period on days 6 to 23. According to the Noyes pathologic diagnosis,[14] endometrial samples in the control group were divided into two subgroups: proliferative phase endometrium group (48 women; median age, 43 y) and secretory phase endometrium group (40 women; median age, 42 y). Women were excluded if exogenous hormones were administered in the past 3 months. There was no significant difference between the two groups in participation rates, medical history, and general characteristics (Table 1). Blood samples were obtained for measurement of serum estradiol (E_2) and progesterone (P_4) concentration on the same day that the endometrial biopsy was performed in both groups.

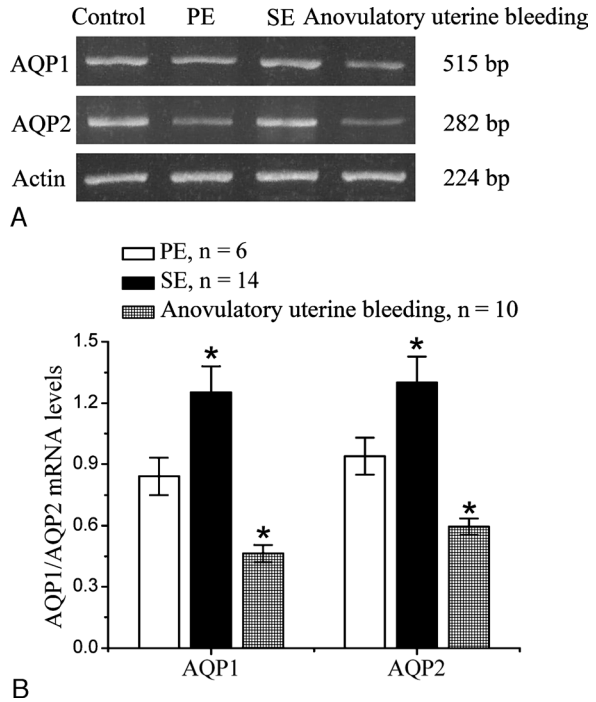

FIG. 1. Aquaporin (AQP) mRNA expression assessed by reverse-transcriptase polymerase chain reaction in human endometrium. **A:** AQP1 and AQP2 mRNAs were detected in human endometrium throughout the menstrual cycle and in the endometrium in anovulatory uterine bleeding. Kidney tissue was used as a positive control. **B:** Differential expression of AQP1 and AQP2 averaged data from six samples of proliferative phase endometrium (PE), 14 samples of secretory phase endometrium (SE), and 10 samples of endometrium in anovulatory uterine bleeding. The mRNA samples for each of the AQP1 and AQP2 were expressed as ratios with the corresponding values for β-actin. $^*P < 0.05$ versus PE.

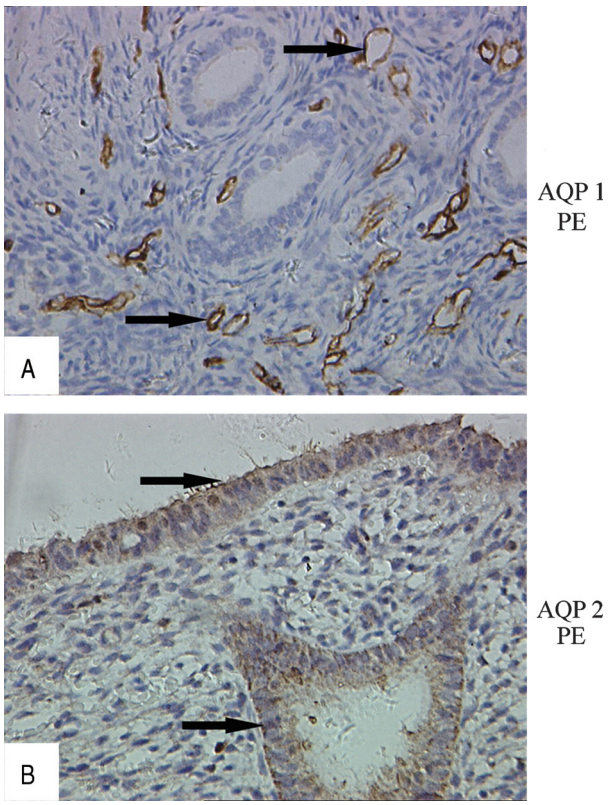

FIG. 2. Immunohistochemistry images of human endometrium showing location of aquaporin (AQP) 1 and AQP2. AQP1 locates in the endothelium of the capillaries and small blood vessels (**A**, arrow). AQP2 staining is mainly in luminal and glandular epithelium (**B**, arrow). Magnification ×400. PE, proliferative phase endometrium.

RT-PCR

Total RNA was extracted from endometrium using TRIzol Reagent (Invitrogen, Carlsbad, CA) followed by DNase treatment (Invitrogen) according to manufacturer's instructions. The cDNA was reverse-transcribed from total RNA in 20 μL of the mixture containing RT buffer, 1 mM of deoxynucleotide triphosphate, 1.25 μM random primers, 25 U of ribonuclease inhibitor, and 100 U of RT (all from Toyobo, Osaka, Japan). RNA (1 μg) was added to each RT reaction, and samples were incubated at 30°C for 10 minutes, at 42°C for 60 minutes, and at 85°C for 5 minutes. Two microliters of RT products were amplified by PCR. The primer sequences used in the present study were derived from published research[15] and synthesized by Shanghai Sangon Corporation (Shanghai, China). PCR was performed in a thermal cycler (PTC-200 DNA Engine; MJ Research, Waltham, MA) with initial denaturation at 94°C for 5 minutes. Amplifications were performed for 35 cycles of denaturing at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds, followed by a final extension period at 72°C for 10 minutes. RT-PCR products were visualized in 2% agarose gels. Kidney tissue[16] was used as the positive control in RT-PCR.

Immunohistochemistry and image analysis

Immunohistochemical staining was performed using anti-AQP1-specific antibody (Santa Cruz Biochemical; B-11, sc-25287) and anti–AQP2-specific antibody (sc-9882) as the primary antibodies. Detection of the primary antibodies of AQP1 was performed with PowerVision Two-Step Histostaining Reagent (PV-6001) and AQP2 with Streptavidin/Peroxidase Histostain-Plus kits (SP-9003) supplied by Beijing Zhongshan Biotech Company (Beijing, China). The endometrial samples were sectioned at 4-μm intervals, blocked in 1% bovine serum albumin, and incubated with primary antibody at a 1:100 dilution for 2 hours at room temperature. Tissue

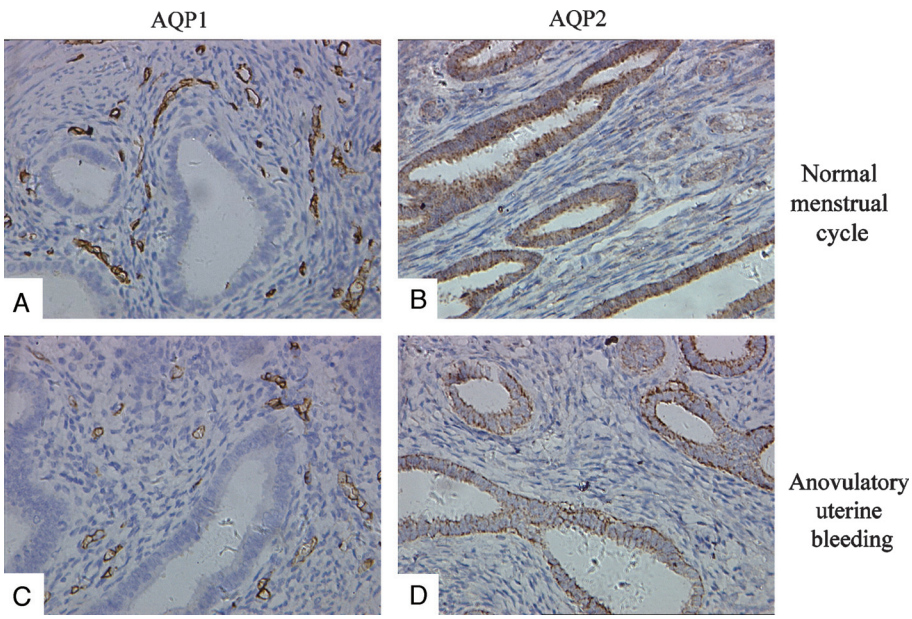

FIG. 3. Expression of aquaporin (AQP) 1 and AQP2 in normal cyclic endometrium (**A** and **B**) and anovulatory uterine bleeding (**C** and **D**). Expression levels of endometrial AQP1 and AQP2 are lower in anovulatory uterine bleeding group than those in normal menstruation group. Magnification ×400.

TABLE 2. *AQP immunostaining score in normal cyclic and anovulatory uterine bleeding endometrium (mean ± SE)*

		AQP1			AQP2		
Group	N	IOD	Area	Density	IOD	Area	Density
Proliferative phase endometrium	48	1,196 ± 77	13,683 ± 652	0.0847 ± 0.0036	7,466 ± 418	77,875 ± 2,949	0.0941 ± 0.0030
Secretory phase endometrium	40	1,626 ± 87[a]	13,946 ± 510	0.1135 ± 0.0042[a]	9,946 ± 599[a]	77,557 ± 2,515	0.1291 ± 0.0025[a]
Anovulatory uterine bleeding	51	859 ± 50[a]	12,861 ± 719	0.0671 ± 0.0017[a]	5,253 ± 307[a]	71,188 ± 3,226	0.0743 ± 0.0025[a]

AQP, aquaporin; N, number of samples; IOD, integrated optical density.
[a] $P < 0.01$ versus proliferative phase endometrium.

sections were then washed with phosphate-buffered saline and incubated with secondary antibody for 30 minutes. After washing again, the sections were reacted with diaminobenzidine (Beijing Zhongshan Biotech Company) and counterstained with hematoxylin, dehydrated, and mounted in distrene dibutylphthalate xylene. Rat renal tissue was used as a positive control. Immunostaining of the negative control was carried out omitting the primary antibody.

Immunostained sections were characterized quantitatively by digital image analysis using the Image Pro-Plus 6.0 with the method introduced by Wang-Tilz et al.[17] Images were obtained with an Olympus FM10 microscope fitted with a microimage video camera. A series of 10 random images on several sections was taken for each immunostained slide to obtain a mean value. Staining was defined by color hue and intensity, and the area with color hue and intensity in a certain range was chosen as the positive area. The positive area choosing regimen was then applied equally to all images, and created preview images and measurements were obtained. The intensity of the labeling was determined by the computer program and gave a gray value ranging from zero (black) to 256 (white). Immunohistochemical parameters assessed in the area detected include (1) the sum integrated optical density, which indicates the total amount of staining material in one image; (2) total stained area, which indicates the total area of staining in one image; and (3) the mean intensity, which indicates the mean staining material in each pixel in the stained area of one image.

Serum E_2 and P_4 measurements

The concentrations of E_2 and P_4 in the sera of the patients were measured with double antibody radioimmunoassay (Diagnostic Products, Los Angeles, CA) as previously described.[18]

Statistical analysis

All results are expressed as mean ± SE. Multiple comparisons were made using one-way analysis of variance (Bonferroni as the post hoc test) with SPSS 13.0 for Windows. Linear regression was used to analyze the correlation between AQP protein expression and serum hormone levels. The level of significance was $P < 0.05$.

RESULTS

Identification and location of AQP family members in human endometrium

To identify expression of AQPs in human endometrium, RNA samples of anovulatory uterine bleeding and of control endometrium were assessed for AQP1 and AQP2 mRNA. RT-PCR detection confirmed that AQP1 and AQP2 mRNA was expressed in the human endometrium in both anovulatory uterine bleeding and in the normal menstruation cycle (Fig. 1A).

Immunohistochemistry was used to localize AQP1 and AQP2 in paraffin sections of human endometrium. AQP1

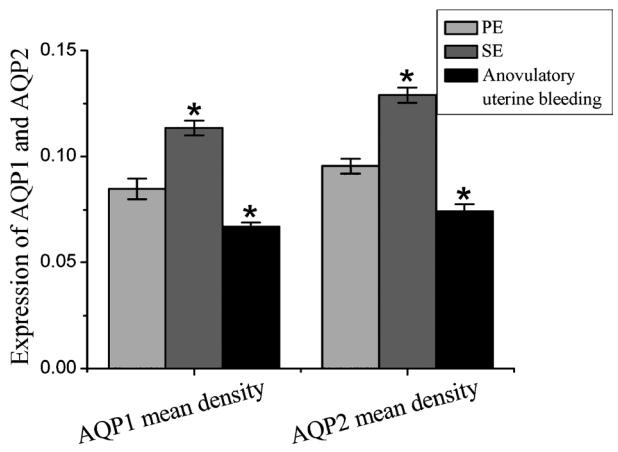

FIG. 4. Density of aquaporin (AQP) 1 and AQP2 staining in the endometrium in anovulatory uterine bleeding and the normal menstrual cycle. Expression of endometrial AQP1 and AQP2 increased in SE group and decreased in anovulatory uterine bleeding group compared with that in PE group. PE, proliferative phase endometrium; SE, secretory phase endometrium. * $P < 0.01$ compared with PE.

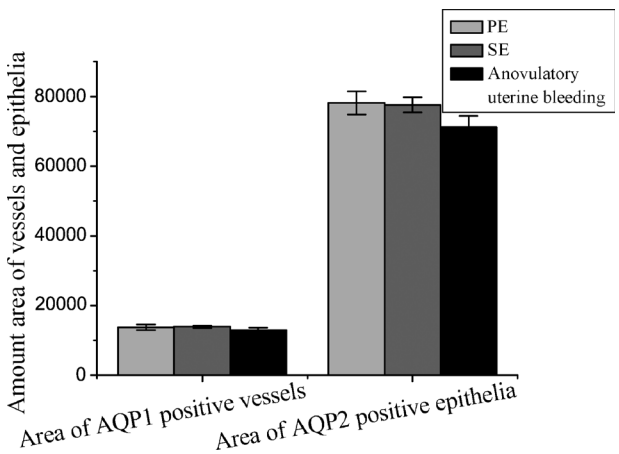

FIG. 5. Total area of aquaporin (AQP) 1 and AQP2 staining of the endometrium in anovulatory uterine bleeding and in the normal menstrual cycle. PE, proliferative phase endometrium; SE, secretory phase endometrium. No significant differences were observed among each group.

TABLE 3. *Serum concentrations of steroid hormones in women with normal menstrual cycle or anovulatory uterine bleeding (mean ± SE)*

Group	N	E_2 (pmol/L)	P_4 (pmol/L)
Proliferative phase endometrium	23	220.85 ± 17.49	2.13 ± 0.19
Secretory phase endometrium	20	616.81 ± 40.06[a]	46.55 ± 2.98[a]
Anovulatory uterine bleeding	30	216.09 ± 15.75	1.17 ± 0.058

N, number of samples; E_2, estradiol; P_4, progesterone.
[a] $P < 0.01$ versus proliferative phase endometrium and anovulatory uterine bleeding.

was detected in the endothelium of the capillaries and small blood vessels. AQP2 mainly stained in the luminal and glandular epithelium throughout the cycle (Fig. 2). A small number of endometrial vessels demonstrated staining (Fig. 3). There was also very faint staining of stromal cells. The endothelium showed intense membrane and cytoplasmic immunostaining but no nuclear staining.

Differential expression of AQP1 and AQP2 in the endometrium in normal menstruation and anovulatory uterine bleeding

Semiquantitative analysis by RT-PCR showed that AQP1 and AQP2 mRNA expression increased in the secretory phase endometrium and decreased in women with anovulatory uterine bleeding compared with the proliferative phase endometrium (Fig. 1B). As seen in Table 2 and Figures 3 and 4, quantification of the immunostaining of both AQP1 and AQP2 of both revealed higher expression (integrated optical density and density) in the secretory phase endometrium group than in the proliferative phase endometrium group ($P < 0.001$), which is consistent with the results of our previous research.[11] The expression (integrated optical density and density) of AQP1 and AQP2 decreased more in the anovulatory uterine bleeding group ($P < 0.01$) than the in control group. The total area of AQP1 positive vessels and AQP2 positive epithelium (area) showed no significant differences between anovulatory uterine bleeding and normal control groups (Fig. 5) ($P > 0.05$).

Correlation between the expression levels of AQPs in human endometrium and the serum levels of E_2 and P_4

Serum E_2 and P_4 concentrations and the endometrial AQP protein levels were higher in the secretory phase endometrium group than in the proliferative phase endometrium PE group ($P < 0.01$). The levels of E_2 and P_4 decreased significantly in the anovulatory uterine bleeding group than in the secretory phase endometrium group ($P < 0.01$) (Table 3). There was a positive correlation between the levels of

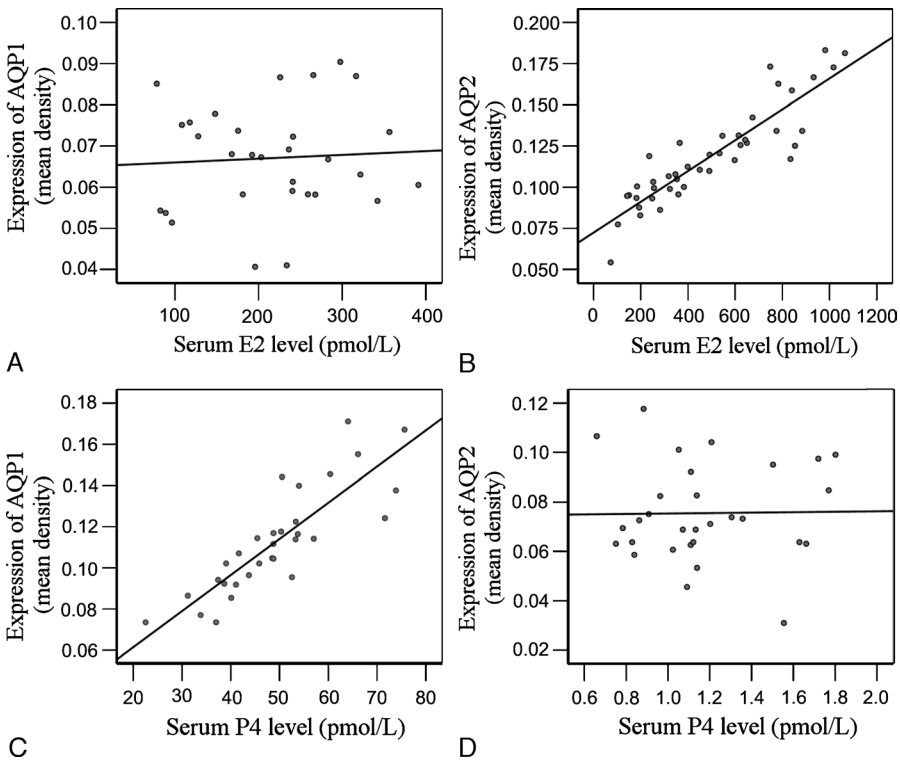

FIG. 6. Correlations between expression levels of aquaporin (AQP) in human endometrium and the serum levels of sex steroid hormones. **A:** The correlation between the densities of endometrial AQP1 and the serum estradiol (E_2) levels in women with anovulatory uterine bleeding is not significant ($r^2 = 0.04$, $P > 0.05$). **B:** There is a positive correlation between the densities of endometrial AQP2 and serum (E_2) levels in women with the normal menstrual cycle ($r^2 = 0.802$, $P < 0.01$). **C:** There is a positive correlation between densities of endometrial AQP1 and serum progesterone (P_4) levels in women with a normal menstrual cycle ($r^2 = 0.72$, $P < 0.01$). **D:** The correlation between the densities of endometrial AQP2 and the serum P_4 levels in women with anovulatory uterine bleeding is not significant ($r^2 = 2.17 \times 10^{-4}$, $P > 0.05$).

endometrial AQP protein expression and the serum hormone levels in the normal menstrual cycle ($P < 0.01$), but no significant correlation was found between AQP expression and serum ovarian steroids in anovulatory uterine bleeding ($P > 0.05$) (Fig. 6).

DISCUSSION

In the present study, we investigated the expression of AQP family members AQP1 and AQP2 in normal menstrual cycle and anovulatory uterine bleeding endometrium. We found that mRNA and protein of AQPs were present in human endometrium of normal menstrual cycle and anovulatory uterine bleeding, and the expression levels of endometrial AQP1 and AQP2 in the secretory phase were higher than those in the proliferative phase, suggesting the role of AQPs in menstruation cycle. There was a marked down-regulation of AQP1 and AQP2 in the endometrium of women with anovulatory uterine bleeding, indicating a probable relationship between decreased expression of AQPs and the occurrence of anovulatory uterine bleeding. Furthermore, there was a positive correlation between the levels of endometrial AQP protein expression and the levels of serum ovarian steroids in the normal menstrual cycle, but it vanished in anovulatory uterine bleeding, implying possible regulatory effects of steroids on the expression of AQPs.

Angiogenesis is an essential component of endometrial repair and regeneration after menses. It has been suggested that disturbances of the normal processes of angiogenesis in the endometrium during menstruation may lead to menorrhagia.[19] The vessels over the endometrial surface vary considerably in size, and some are much wider in diameter and thinner walled than normal.[20] These vessels are also much more fragile than normal and bleed easily in response to minor stress.[2,21] All these abnormalities are a reflection of disturbed angiogenesis. AQP1 plays an important role in angiogenesis. It is expressed in tumor microvessels[22] and is involved in impaired angiogenesis in tumors in both humans[23,24] and mice.[9] Our results provide evidence that AQP1 was mainly located in the endothelium of the capillaries and small blood vessels in human endometrium, and it might participate in angiogenesis within the uterus. It seems that decreased expression of AQP1 in the endometrium might be involved in disturbed endometrial vascular remodeling, which participates in the occurence of anovulatory uterine bleeding, whereas further investigation is required to prove this hypothesis.

Endometrial edema varies throughout the menstrual cycle, reaching maximum just after the window of implantation.[25] It has been suggested that AQP1 may play a role in uterine vascular permeability and fluid imbibition.[26] In the present study, AQP2 was shown to increase in the glandular epithelium during the secretory phase, coinciding with the increase in endometrial edema. It is possible that AQP2 plays a role in this process as well.[11] Stromal edema and increased blood flow are suggested to facilitate uterine growth by diluting or removing inhibitory factors.[27] The present study showed that both AQP1 and AQP2 expression decreased in the endometrium in anovulatory uterine bleeding compared with the controls, raising the possibility that decreased AQP1 and AQP2 expression may lead to deficient water movement in the endometrium and be associated with the occurrence of anovulatory uterine bleeding.

Endometrial growth and differentiation are under the overall control of estrogen and progesterone. However, it is unclear how these steroids specifically regulate menstrual changes. It has been shown that AQP1 and AQP2 are two water channels strongly regulated by estrogen.[26,28] Progesterone increases the immunohistochemical appearance of AQP1 in rats but not humans.[13,28-30] In our present study, there was a positive correlation between the levels of endometrial AQP protein expression and the serum hormone levels. It is possible that the high level of progesterone and E_2 during the secretory phase may be associated with the increased expression of AQP1 and AQP2 and further related to stromal edema and endometrial angiogenesis during this phase. The characteristic of anovulatory uterine bleeding is variable estrogen stimulation without sufficient progesterone. We found that not only the expression of endometrial AQPs but also the levels of serum E_2 and P_4 in the women with anovulatory uterine bleeding were significantly reduced compared with those in the control group in the secretory phase. It appears that ovarian hormones, which are decreased in the women with anovulatory uterine bleeding, are unable to support sufficient AQP expression and are associated with anovulatory uterine bleeding. Interestingly, the correlation between serum concentrations of ovarian steroids and endometrial levels of AQPs disappears in anovulatory uterine bleeding. Perhaps this is due to the irregular low concentrations of E_2 and P_4. Further study is needed to clarify this possibility.

Because the present research was a hospital-based case-control study and the sample size was relatively small, this may limit our results in interpreting the mechanisms underlying anovulatory uterine bleeding. Conversely, it is very difficult to get endometrial samples from normal women as controls. In this study, the control women were in the hospital for treatment and might not represent the true comparison population of healthy women. The results would be more convincing if a population-based control was used and more samples were obtained. Further studies should be proposed to elucidate the exact mechanisms of anovulatory uterine bleeding.

CONCLUSIONS

In summary, the results of the present study show that the expression of AQP1 and AQP2 in human endometrium is significantly decreased in anovulatory uterine bleeding, which may provide an explanation for the mechanisms underlying anovulatory uterine bleeding. Moreover, we demonstrate that serum ovarian steroid concentrations correlate positively with AQP1 and AQP2 levels in the regular menstrual cycle, suggesting the possibility that the AQP family may be one of the mediators between ovarian steroids and cyclic uterine changes. Our results may help to under-

stand the mechanism(s) underlying anovulatory uterine bleeding and to treat it further.

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