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Expression of aquaporin 1 and 5 and their regulation by ovarian hormones, arachidonic acid, forskolin and cAMP during implantation in pigs

Agnieszka Skowronska[1], Patrycja Mlotkowska[2], Mariusz Majewski[1], Soren Nielsen[3] and Mariusz T. Skowronski[2,4]

[1]Department of Human Physiology, Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland

[2]Department of Animal Physiology, Faculty of Biology and Biotechnology, University of Warmia and Mazury in Olsztyn, Olsztyn, Poland

[3]Department of Health Science and Technology, Faculty of Medicine, Aalborg University, Aalborg, Denmark

[4]Institute of Veterinary, Poznań University of Life Sciences, Poznań, Poland

Address correspondence to:

Agnieszka Skowronska, MVD, PhD

University of Warmia and Mazury in Olsztyn

Department of Human Physiology

Warszawska 30, 10-082 Olsztyn, Poland

phone +48 89 5245304, fax +48 89 5245307

e-mail: agnieszka.skowronska@uwm.edu.pl

Summary

Aquaporin proteins (AQPs) are a family of channels expressed in numerous mammalian tissues, where they play a fundamental role in regulating water transport across cell membranes. Based on reports that AQPs are present in the reproductive system and participate in reproductive processes, our aim was to investigate the effect of progesterone (P_4), estradiol (E_2), oxytocin (OT), arachidonic acid (AA), forskolin (FSK) and cyclic adenosine monophosphate (cAMP) on AQP1 and AQP5 expression at mRNA and protein levels in porcine uterine explants from Days 14-16 of gestation in order to determine if they play a role in implantation period in pigs. Quantitative real time PCR and Western-blot analysis revealed that the uterine explants treated with FSK and cAMP produce delayed, but long-term effects on AQP1 abundance (24 h) while AQP5 had a rapid and sustained response to FSK and cAMP in protein content (3 and 24 h). AA increases gene and protein content of AQP1 after longer exposition whereas AQP5 increases after 3 h only at the protein level. Both AQPs potentially remains under control of steroid hormones. OT has been shown to increase *AQP1*, and decrease *AQP5* mRNA, without visible changes in protein content. P_4, E_2, AA, FSK and cAMP caused the appearance of AQP5 expression in the basolateral plasma membrane of the epithelial cells. The staining represents most likely AQP5 functioning mechanism for both absorption and reabsorption across the glandular epithelium.

Keywords: aquaporins, uterus, implantation, pig

Introduction

The uterine endometrium undergoes morphological and physiological changes to a state supporting the implantation. Dramatic reduction in the intrauterine fluid takes place at the time of embryonic implantation (Zhang *et al*. 2012) as well as glandular secretions and fluid shifts across endothelial and epithelial compartments (Liu *et al*. 2014). Water homeostasis during fetal development is of crucial physiological importance throughout pregnancy. The presence of aquaporins suggest that water moves across the cell, not only through membrane lipid bilayer but also through water channels proteins (Agre *et al.* 2002) The aquaporins (AQPs) are family of small (25-34 kDa), hydrophobic, integral membrane proteins that facilitate the rapid transcellular movement of water and small neutral solutes in response to osmotic/hydrostatic pressure gradients. Accumulating evidence indicates that these proteins were found to be involved in embryo and uterine fluid homeostasis during pregnancy (Huang *et al.* 2006, Kobayashi and Yasui 2010, Sha *et al.* 2011, Zhu *et al.* 2015). The presence of AQP1, 4 and 5 was found in the mouse uterus on Day 1-8 of pregnancy (Richard *et al.* 2003) while *AQP1, 3, 4, 5, 7, 8, 9* and *11* mRNA expression was observed in the human chronic villi between 10th and 14th weeks of gestation (Escobar *et al*. 2012). It has been shown that increased expression of AQP5 occurs at the time of the embryo implantation in the rat uterus (Lindsay and Murphy 2007). Further, multiple aquaporins are expressed in the early stage human embryos and AQP3 and AQP7 may play a role in pre-implantation mouse embryo development (Xiong *et al.* 2013) . Implantation of the developing embryo in the uterus is regulated by multiple effectors, such as steroid hormones, growth factors, cytokines and cyclooxygenase-derived prostaglandins (Bazer *et al*. 2009, Geisert *et al.* 2012). Unlike human and mouse, the pig has a non-invasive implantation (Bazer 1975, Kaufmann and Burton 1999) and release of embryonic estrogen serves as the signal for maternal recognition of pregnancy (Keys and King 1990, Jaeger *et al.* 2001). Furthermore, estrogen also regulates the expression of a variety of genes in the uterus that support implantation and conceptus development (Johnson *et al*. 2009). It has been found that, besides estrogens, the endometrium and trophoblast synthesize elevated amounts of prostaglandin E_2 (PGE_2) (Akinlosotu *et al.* 1986, Christenson *et al.* 1994). There is also evidence that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin E_2 are required for conceptus development and implantation (Kraeling *et al.* 1985, Ziecik *et al.* 2011). Some studies also revealed that ovarian steroid hormones (E_2 and P_4), arginine vasopressin, forksolin, cAMP, relaxin and insulin may be involved in the regulation of AQPs expression in the reproductive system, for

a review see Hua *et al.* (2013). It has also been postulated that pig endometrium secretes oxytocin (Trout *et al.* 1995), possesses its receptors (Whiteaker *et al.* 1994) and regulates $PGF_{2\alpha}$ secretion (Uzumcu *et al*. 1998).

In previous studies (Skowronski *et al*. 2009, Skowronski, 2010), we provided an anatomical basis for AQP 1, 5 and 9 expression in the porcine uterus during different stages of the estrous cycle and pregnancy. AQP1 expression was present in the endothelial cells of the blood vessels, AQP5 in the cells of myometrium and the luminal and glandular epithelium. However, the AQPs gene expression and AQPs regulation controlling uterine fluid homeostasis has not been examined in porcine uterine tissue. Very recently, an *in vitro* study of corresponding cyclic uterus gene/protein expression has been performed by Skowronska *et al.* (2015). There is little information available about water transport mechanism during implantation in the pig. Based on these data, we have designed an *in vitro* experiment to elucidate whether the steroid hormones, progesterone (P_4) and estradiol (E_2), and other factors: oxytocin (OT), arachidonic acid (AA; substrate for prostaglandins synthesis) as well as forskolin (FSK; adenylate cyclase activator) and cAMP (second messenger, cyclic adenosine monophosphate) may impact AQPs expression during implantation (Days 14-16 of gestation). Consequently, the primary aim of this study was to: (i) examine the changes in AQP1 and AQP5 at mRNA and protein levels in porcine uterine explants in the presence of P_4, E_2, OT, AA, FSK and cAMP; (ii) determine the effect of exposure time to the experimental factors on AQP1 and 5 expression in uterine explants*;* (iii) examine the localization of AQP1 and 5 in uterine explants after a certain incubation period with examined factors.

Materials and Methods

Animals and collection of uterine tissue

The experiment was performed in accordance with Animal Ethics Committee, University of Warmia and Mazury in Olsztyn, Poland (AEC approval No. 66/2010/DTN) and conducted at the University of Warmia and Mazury. Gilts (Large White × Polish Landrace) were observed daily for estrus behavior, and they were used in the study during their third consecutive normal estrous cycle. They were naturally bred on the second day of estrus. The animals were slaughtered (n=5) at a local abattoir, and tissue samples were recovered from mature cross-bred gilts on Day 14-16 of pregnancy which corresponds to the period of implantation in pigs. Pregnancy was confirmed by the presence of embryos after flushing uterine horns with 20 ml

of sterile saline. The uteri were placed immediately in ice-cold phosphate-buffered saline (PBS), supplemented with 100 IU/ml penicillin (Polfa, Poland) and 100 μg/ml streptomycin (Polfa, Poland) and transported to the laboratory on ice within 1 h for *in vitro* tissue culture.

Uterine explants culture

Sections of the middle part of uterine horn collected from pigs were opened longitudinally on the mesometrial surface. Uteri were washed three times in sterile PBS then carefully cut into small slices (400 mg weight) and then washed three times in medium M199 (Sigma, USA). Individual uterine slices were placed in culture vials containing 2 ml Medium 199 supplemented with 0.1% BSA (Sigma), 20 μg nystatin (Sigma) and 20 μg gentamicin (Krka, Novo Mesto, Slovenia) and then incubated in a shaking water bath at 37° in a humidified atmosphere of 95% O_2 and 5% CO_2 for 18 h (Franczak *et al.* 2006). After preincubation, the culture medium was replaced with fresh medium, and the explants were treated with vehicle (control) or P_4 (10-5 M; Sigma), E_2 (10-9 M; Sigma), OT (10-7 M; Sigma), AA (10-5 M; Sigma), FSK (10 μg/mL; Sigma) and cpt-cAMP analog (200 μM; Sigma) and incubated for an additional 3 or 24 h. Concentrations for the treatments were previously determined (Yang *et al*. 2003, Franczak *et al*. 2006). All treatments were performed in triplicates in five independent experiments. Furthermore, uterine tissue explants were snap-frozen in liquid nitrogen (for RNA and protein extraction) and stored at -80°C until further use.

Total RNA isolation, cDNA synthesis and quantitative real-time PCR analysis

Total RNA was extracted, using the total RNA Prep Plus kit (A&A Biotechnology, Poland) according to the manufacturer`s protocol, from uterine explants collected after *in vitro* culture (Skowronska *et al.* 2015). Total RNA quality and quantity were determined with spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Wilmington, DE, USA). Total RNA samples were transcribed to cDNA using an Enhanced Avian HS RT-PCR Kit it (Sigma) and a mix of dNTPs and random hexamers as primers. Real-Time PCR was performed in duplicate for each sample using a 7300 Real-Time PCR system and SYBR®Green PCR Master Mix (Life Technologies, Grand Island, NY, USA). Real-Time PCR reaction included 12.5 μl SYBR Green PCR master mix, 1 μM forward and reverse primers each and reverse transcribed cDNA (3.5 μl of diluted RT product) supplemented with water to a volume of 25 μl. The conditions of the thermal cycling for each gene were: initial denaturation for 10 min at 95°C, denaturation for 15 sec at 95°C, primer annealing for 1 min at 60°C. Specific primers for *AQP1* and *AQP5* (Table 1) were designed with the Primer

Express 3.0 software (Life Technologies) and their specificities were confirmed by comparison of their sequences with the sequence of *AQP1* and *AQP5* deposited in a database and calculation of the statistical significance of the match was performed using the Basic Local Alignment Search Tool (BLAST). For the specificity control, non-template controls and dissociation curve analysis of the amplified products were used for each amplification. The specificity of amplifications was further validated with electrophoresis of the putative amplicons in a 2% agarose gel and, after extraction from gel, automated sequencing using 3730xl DNA Analyzer (Life Technologies). Levels of gene expression were calculated using the ΔΔ Ct method and normalized using the geometrical means of reference genes expression levels, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and *18S rRNA*.

SDS-PAGE and Western Blot analysis

The tissues were placed in ice-cold dissection buffer (0.3 M sucrose, 25 mM imidazol, 1 mM EDTA in ddH_2O, pH 7.2) containing 8.4 μM leupeptin and 0.4 mM pefabloc (Skowronski, 2010). The tissue samples were homogenized using an ultra Turrax T8 homogeniser (IKA Labortechnik, Staufen, Germany) and centrifuged at 4,000×g for 15 min at 4°C. The supernatant was diluted in SDS buffer contained a final concentration of 62 mM Tris (hydroxymethyl)-aminomethane, 0.1 M sodium dodecyl sulphate (SDS), 8.7% glycerol, 0.09 mM bromophenol blue and 0.04 M dithiothreitol (DTT), pH 6.8. The protein samples were heated for 5 min at 90°C and stored in refrigerator for further analysis. Total protein amounts were determined with spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Wilmington, DE, USA). The samples warmed up to 37°C were loaded into 12.5% polyacrylamide gels and proteins were separated by electrophoresis. The proteins of studied gels were then electro-transferred onto nitrocellulose membranes (Hybond ECL RPN3032D, Amersham Pharmacia Biotech, Little Chalfont, UK) for 1 h at 100V. The membranes were blocked with 5% milk in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.5 and 0.1% vol/vol Tween 20) for 1 h. After washing, the membranes were incubated overnight at 5°C with anti-AQPs or β-actin antibodies. Thereafter, the membranes were washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (P448, diluted 1:3,000, Dako A/S, Glostrup, Denmark) in PBS-T for 1 h. After washing with PBS-T, the sites of antibody-antigen reaction were visualized with an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Little Chalfont, UK) and exposure to photographic film (Hyperfilm ECL, RPN3103K, Amersham Pharmacia Biotech, Little Chalfont, UK). The results of Western blotting were quantified by densitometric

scanning of immunoblots with GelScan for Windows ver. 1.45 software (Kucharczyk, Poland). Data were expressed as a ratio of AQP proteins relative to actin protein in OD units.

Immunohistochemistry

For preparation of paraffin-embedded tissue sections (4-µm thickness), the tissues were dehydrated in ethanol followed by xylene and finally embedded in paraffin (Skowronski *et al.* 2007). The staining was carried out using indirect immunoperoxidase. The sections were dewaxed and rehydrated. For immunoperoxidase labeling, endogenous peroxidase was blocked by 0.5% H_2O_2 in absolute methanol for 10 min at room temperature. To reveal antigens, the sections were submerged in 1 mM Tris solution (pH 9.0) supplemented with 0.5 mM EGTA and heated in a microwave oven. After the treatment, the sections were left for 30 min in the buffer for cooling. Nonspecific binding of IgG was eliminated by incubating the sections in 50 mM NH_4Cl for 30 min, followed by blocking in PBS supplemented with 1%BSA, 0.05% saponin and 0.2% gelatin. The sections were incubated overnight at 4°C with primary antibodies (see below) diluted in PBS supplemented with 0.1% BSA and 0.3% Triton X-100. The sections were rinsed with PBS supplemented with 0.1% BSA, 0.05% saponin and 0.2% gelatin, and then incubated with horseradish peroxidase-conjugated secondary antibody (Dako A/S, Glostrup, Denmark). Labeling was visualized by 0.05% 3,3 diaminobenzidine tetrahydrochloride (DAB). The microscopy was carried out using an Olympus light microscope (BX51, Japan).

Primary Antibodies

Antibodies to AQP1 and AQP5, used in Western blot analysis and immunocytochemistry, were previously characterized, respectively by Terris *et al.* (1996) and Nielsen *et al.* (1997). All polyclonal antibodies were affinity-purified (SulfoLink Kit, Pierce, Rockford, IL).The anti-β-actin antibody was used (cat. no. A2066; Sigma-Aldrich, St Louis, MO). In our previous study, we demonstrated that anti-AQP1 and anti-AQP5 antibodies preincubated with the immunizing peptide prevented labeling in the pig uterus (Skowronski, 2010). In addition, immunoglobulins from non-immunized rabbit were used as a negative control (Figure 5 c and Figure 6 o-p).

Statistical Analysis

All numerical data were analyzed by one-way ANOVA and least significant difference (LSD) post hoc test and reported as the means ± S.E.M. from five separate experiments (pigs), each

performed in triplicates. Statistical analyses were performed using the Statistica program (StatSoft Inc., Tulsa, USA). Values for $p<0.05$ were considered statistically significant.

Results

Effects of P_4, E_2, OT, AA, FSK, and cAMP on AQP1 and AQP5 mRNA expression in uterine tissue explants

Using quantitative real time PCR, we have found the presence of the investigated AQP isoforms *AQP1* and *AQP5* mRNA in porcine uterine explants during the implantation period. The level of the *AQP1* and *AQP5* transcripts fluctuated in the tissue depending on the incubation time; 3- and 24-hours (Figure 1 and 2). In detail, in porcine uterine explants *AQP1* mRNA expression decreased significantly ($p<0.05$) after 3-h treatment with E_2, oxytocin, AA and cAMP when compared to the respective control. However, a significant increase in *AQP1* mRNA expression was seen after longer treatment (24-h) with oxytocin, AA, cAMP and FSK ($p<0.05$). In contrast, P_4 did not affect the expression of *AQP1* mRNA neither after short nor longer incubation (3- and 24-hours, respectively). The relative control abundance of *AQP1* transcript harvested in the uterine tissue was approximately 3-fold higher than *AQP5* after 3-hours incubation (Figure 1). Progesterone had no effect on *AQP5* mRNA level in the uterine porcine explants after 3 or 24 h incubation. Treatment of uterine explants with E_2, OT and AA resulted in decreased *AQP5* expression after 3 h, when compared to respective control values ($p<0.05$, Figure 2). The level of *AQP5* mRNA of uterine explants remained markedly low after longer incubation (24 h) with OT. On the contrary, *in vitro* treatment of uterine explants with cAMP caused an increase in uterine *AQP5* expression, but only after 24-h ($p<0.05$, Figure 2).

Effects of P_4, E_2, OT, AA, FSK, and cAMP on AQP1 and AQP5 protein expression in uterine tissue explants

Figures 3 and 4 show Western blot analysis of AQP1 and 5 protein in uterine tissue explants during implantation. It was demonstrated that both aquaporins were expressed in porcine uterine tissue explants at both exposure times. A band of AQP1 protein product of the expected size (29 kDa) was clearly detected in all studied tissues (Figure 3). A stimulatory effect on AQP1 expression was observed after treatment of explants with E_2 and P_4 during 3- and 24-h incubations, respectively (~1.5-2.5 fold, $p<0.05$). In turn, AA, FSK and cAMP

treatment for 3 h did not significantly affect AQP1 protein expression but after longer incubation (24 h) it stimulated its expression ($p<0.05$). In contrast, OT did not affect the protein expression of AQP1. The effect of studied factors on the expression of AQP5 protein is presented in Figure 4. A band of 28 kDa that represented AQP5 was detected. AQP5 protein expression in uterine explants representing implantation significantly increased after 3-h treatment with P_4, E_2, AA, FSK and cAMP ($p<0.05$) and remained elevated after 24-h ($p<0.05$), except AA. Similar to AQP1, in the presence of OT, AQP5 protein content in uterine explants was not affected during either incubation times. Treatment with AA resulted in an elevation of AQP5 protein only after 3-h incubation ($p<0.05$).

Immunohistochemical localization of AQP1 and AQP5 in uterine tissue explants during the implantation period

In the tissue sections of the pig uterus, control and after 3-h exposure to P_4, immunoperoxidase labeling for AQP1 was associated with uterine endothelial cells during the implantation period (Figure 5 a and b). Both the apical and basal plasma membranes exhibited stable AQP1 labeling (arrows) without changes in localization within the cells. In turn, in sections of the porcine uterus treated with control and chosen factors, immunoperoxidase labeling for AQP5 was associated with uterine epithelial cells (Figure 6 a-j) and smooth muscle cells (Figure 6 k-n). Following 3- and 24-h treatments of the tissue with P_4, E_2, FSK and cAMP as well as 3-h treatment with AA during the implantation period, a prominent AQP5 labeling was seen in both the apical and basolateral plasma membranes of the epithelial cells (Figure 6 b-j). In the smooth muscle cells, in contrast to the epithelial cells, changes in AQP5 localization within the cell membranes were not observed in control (Figure 6 k-l) and response to 3-h treatment with P_4 (Figure 6 m-n).

Discussion

In the present study, we have shown *in vitro* the effect of P_4 and E_2, oxytocin, AA, FSK and cAMP on AQP1 and AQP5 expression at mRNA and protein levels in porcine uterine explants from the implantation period. Implantation in pigs is characterized by trophoectoderm attachment of the blastocyst to the endometrial luminal epithelium, without promoting erosion of the maternal tissue (Bazer, 1975, Gray *et al.* 2001). The glandular epithelium is believed to secrete the majority of histotroph to nourish and support

development of the conceptus (Spencer *et al*. 2004). The luminal epithelium of the pig uterus is responsible for endometrial remodeling for non-invasive implantation as well as expresses specific factors that prevent invasion of porcine embryo (Wollenhaupt *et al.* 2011). Hence, the integrity of both the maternal and fetal tissues is not disrupted by the blood vessels that develop from the blastocyst and those from the uterus (Carson *et al.* 2000).

We previously described that AQP1, 5 and 9 protein expression in the porcine uterus is influenced by the estrous cycle and pregnancy (Skowronski, 2010). In the present study, E_2 treatment resulted in a rapid (by 3 hours) decrease in *AQP1* and *AQP5* gene expression, although longer (24 hours) treatment elevated *AQP5* and *AQP1* gene expression but only to the control level. Similarly, P_4 did not change *AQP1* or *AQP5* mRNA expression at the time of the study. However, the content of AQP1 and 5 proteins was up-regulated in the presence of steroid hormones after 3 and 24 h exposure. These results indicate that despite low *AQP1* and *5* mRNA transcript abundance, AQP1 and 5 proteins was stable expressed in uterine explants displaying a low turnover rate. In general, our results are consistent with other authors and elucidated that E_2 and P_4 are important regulators of AQP1 and 5 expression in the porcine uterus and have an important role in the implantation process. Taking into account our immunohistochemistry data, we suggest that the finding of AQP5 in glandular epithelial cells in control explants and the presence of AQP5 in both the apical and basolateral plasma membranes of glandular epithelium in uterine explants in response to P_4 and E_2, strongly corroborates the hypothesis of a transcellular route for the movement of water across in the cells. Furthermore, since AQP5 is hormonally translocated to the basolateral plasma membranes of glandular epithelium, this may provide a functional mechanism for both absorption across the glandular epithelium and reabsorption of glandular contents. Hence, our finding may reveal the importance of steroid hormone regulation of AQP5. Very recently, Zhang and co-workers (2015) demonstrated upon E_2 treatment that AQP5 knockout mice showed significantly decreased luminal fluid volume and number of implantation sites compare with wild-type mice, suggesting that AQP5 are responsible for E_2-induced abnormal fluid accumulation. In turn, Zou *et al.* (2011) demonstrated that E_2 may regulate AQP2 expression via an estrogen-response element (ERE) in the AQP2 promoter. One of the prominent effects of estrogen is water imbibition and for the regulation of water transportation, AQP5 play a critical role (Kobayashi *et al*. 2006). Lindsay and Murphy (2006) showed that up-regulation of AQP1 and AQP5 in the rat uterus was dependent on progesterone alone or in combination with estrogen. Furthermore, in the mouse uterus

exogenous estrogen strongly up-regulated the expression of AQP2, without any effect on AQP5 (Jablonski *et al*. 2003).

Our findings agreed with several previous observations in other species by Li *et al.* (1997) and Kobayashi *et al.* (2006) who studied this steroid action on uterine expression of AQP1 in rats and AQP5 in mice, respectively. Our data differ partially from the results obtained by Richard et al. (2003), who found increased *AQP1* mRNA in mice myometrium in response to estrogen, but did not evaluate the expression of AQP1 at the protein level, and AQP5 expression was induced by estrogen only in progesterone-primed animals. Thus, it seems that steroid hormones may regulate AQPs gene expression both in a positive and negative regulative manner, depending on the physiological status of animal and tissue types and function. As observed in the present study, the lack of a full relationship between the concentration of gene transcripts and respective proteins may also result from: 1. differentiated stability of mRNAs or proteins, as well as the regulation of transcription, post-transcriptional processes or translation, 2. functional feedback, i.e. a high protein product may suppress gene transcript into mRNA, and a higher mRNA level may diminish the post-transcriptional process. Here, we can hypothesize that regulation of AQPs expression seemed to occur at the post-transcriptional level as protein expression upon Western-blot was pronounced. Further, those inverse relations are not accidental, they also happened in other studies performed by Klein *et al*. (2013) and Ducza *et al.* (2014). Therefore, it is not clear how tightly transcription and translation are correlated during such development processes. One limitation of the analysis of uterine explants is the complex and dynamics tissue composition. There are luminal and glandular epithelial cells, stromal cells, endothelial cells and various immune cells. Implantation and embryo development are the critical moments in reproduction, it is a complex process involving numerous genes (regulating uterine receptivity and blastocyst implantation), mRNAs, proteins in the endometrium and synchronism of maternal uterus and embryo and the intricate maternal-fetal dialogue (Liu *et al*. 2014). Pregnant gilts were used in our experiment and their endometrium would have already been exposed to the embryo signal and resulted in altered expression of target genes of interest.

The present experiments showed that OT increased *AQP1* mRNA expression after 24-h and decreased *AQP5* mRNA after 3 and 24 hours incubation, but had no effect on AQP1 or AQP5 protein content. These results partially are in agreement with the data obtained very recently by Ducza *et al.* (2014). They indicated that OT treatment increased *AQP2* mRNA levels but also specifically reduced the expression of *AQP5* in the rat uterus on Day 18 of

pregnancy. In our recent published paper, oxytocin has been shown to decreased *AQP1/5* mRNA expression, without visible changes in protein content *in vitro* (Skowronska *et al.* 2015). However, our unpublished data (Skowronska *et al.* unpublished) revealed that the myometrial slices are responsive to OT leading to an increase in both AQP isoforms. The changes in AQPs expression were more pronounced in myometrial slices compare to endometrial slices. We speculate that the endometrial/myometrial responsiveness to oxytocin increases because of the changes in the steroid environment, and perhaps due to an inhibitory action of OT on AQPs uterine expression is abolished after separation of the uterine endometrium and myometrium.

The present study results showed that AQP1 and AQP5 mRNA and protein were regulated by arachidonic acid in the porcine uterus during implantation. Prostaglandin E_2 (PGE_2) is the major cyclooxygenase product of arachidonate metabolism in the porcine uterus (Hertelendy *et al.* 2004). Arachidonic acid decreased *AQP1* mRNA expression after 3-h incubation, while longer (24h) exposure to AA increased both *AQP1* mRNA and protein expression. Although the *AQP5* mRNA expression was down-regulated after a 3-h AA treatment, the AQP5 protein concentration increased, so an inverse relation was found between expression both in gene and protein levels. These results indirectly indicate that AA-prostaglandins (PG) are involved in the regulation of AQP1 and AQP5 protein expression in uterine tissue during implantation, because elevated AQP1 and 5 protein concentrations in the porcine uterus. Additionally, immunohistochemistry results confirmed the changes in cellular localization of AQP5 in response to AA. After 3-h treatment with AA, prominent AQP5 labeling was seen in both the apical and basolateral plasma membranes of the glandular epithelial cells. Previously, other research groups (Geisert *et al*. 1986) demonstrated that pharmacological inhibition of PG synthesis does not affect trophoblast elongation in pigs. However, inhibition of PG synthesis during the period of trophoblast attachment (Days 13 to 18) results in embryonic mortality (Kraeling *et al.* 1985). Prostaglandins produced by the uterus play an essential role in regulation of the estrous cycle and during maternal recognition of pregnancy in many species, including the pig (Kennedy *et al*. 2007) and are involved in implantation, control of cytokine release, cell growth and differentiation and vascular responses (Kelly *et al*. 2001, Bazer *et al*. 2010). Based on those literature reports and the present results, we propose that AA is a regulator of AQP 1 and 5 expression at the time of implantation because it may regulate both AQP1 and AQP5 gene and protein content in the porcine uterus. In line with those reports, we suggest that AQP1 and 5 amplified by AA may

play an important role in balancing uterine fluid, stromal edema and embryo-maternal communication. There is limited data describing the effect of PGs on AQPs expression. The effect of PGE_2 on the actions of arginine vasopressin (AVP) and AQP2 phosphorylation and distribution was examined in the rat renal medulla, and it was found that PGE_2 stimulated the retrieval of AQP2 from the plasma membrane in AVP-stimulated tissue (Zelenina *et al*. 2000).

The present study showed that FSK and cAMP differ in the regulation of the expression of AQP1 and AQP5 gene and protein. FSK and cAMP increased AQP1 mRNA and protein expression after 24 hours of incubation but had no effect after 3 hours. However, FSK had no effect on *AQP5* mRNA expression after 3 and 24 hours, but increased AQP5 protein concentration. Furthermore, cAMP up-regulated *AQP5* mRNA expression after 24-h incubation but, as well as FSK, increased AQP5 protein concentration after 3 and 24 hours. These results showed that the stimulatory effect of FSK and cAMP on AQP1, but not AQP5 protein expression could depend on the incubation period. The above results strongly suggest that expression of both AQPs are regulated by cAMP and FSK but specially the AQP5, because AQP5 protein content was elevated after 3 as well as 24 hours in the porcine uterine explants during implantation. These results are generally consistent with the data obtained previously with the use of different cells/tissues (Wang *et al.* 2006, Wang *et al.* 2007, Yang *et al.* 2003) or cell lines (Wang *et al*. 2003, Belkacemi *et al.* 2008).

In this study, we revealed by immunohistochemistry the changes in cellular localization of AQP5 in response to the studied factors in the uterine tissue explants (Figure 6). AQP1 localization was mainly associated with the apical and basal membranes of the uterine endothelium cell, but AQP5 was mainly associated, in the control explants, with the apical membranes of epithelial cells, the secretion side of the uterine glandular epithelium, It is noteworthy that P_4, E_2, AA, FSK and cAMP caused an emergence of AQP5 in the basolateral membrane of the epithelial cells. It might be hypothesized that these changes are connected with potentially bidirectional transcellular water movement through uterine epithelial cells at the time of implantation in the pig. The changes in cellular localization of AQPs in the uterus during the implantation period have been already observed in rodents (Richard *et al.* 2003, Lindsay and Murphy, 2004, 2006, 2007). These results suggest that specific AQPs localization provides a mechanism for transcellular fluid transport. Furthermore, Garcia *et al*. (2001) indicated that cAMP may induce translocation and insertion of AQP8 within intracellular vesicular structures to plasma membranes in the rat hepatocytes.

Our recent studies (Skowronska *et al.* 2015) performed on the uterine explants from cyclic gilts demonstrated that steroid hormones, AA, FSK and cAMP cause translocation of AQP5 from the apical to basolateral plasma membrane of the glandular epithelial cells.

With reference to our recent published research (Skowronska *et al.* 2015), the uterine explants from non-pregnant pigs collected on Days 14-16 of the cycle differed from those collected on Days 14-16 of pregnancy. The pregnant gilts (present data) expressed 4.5-fold higher abundance of *AQP1* mRNA compare to the corresponding stage of the estrous cycle (Days 14-16). Whereas, cyclic gilts expressed 3-fold higher abundance of *AQP5* mRNA than the pregnant gilts. Moreover, an inverse relationship was found in regulation of *AQP1* and *AQP5* gene expression during the luteolysis and early pregnancy. The third difference observed between the pregnant and cyclic uterine explants was in the protein expression, responses of AQP1 to P_4, E_2, AA, FSK and cAMP treatment after short incubation appeared to be higher during luteolysis versus implantation. These results indicate that the presence of conceptus may influence *AQPs* expression in the uterine explants

In conclusion, the present study is the first *in vitro* demonstration that P_4, E_2, AA, FSK and cAMP up-regulated AQP1 and AQP5 expression in the porcine uterus during implantation period. The changes in AOPs expression appeared to be dependent on duration of the tissue exposure to experimental factors. Moreover, treatment with P_4, E_2, AA, FSK and cAMP caused the expression of AQP5 also in the basolateral plasma membrane of the epithelial cells, suggesting transcellular (through the epithelial cells) water movement between the uterine lumen and blood vessels. Overall, these results indicate the important role of AQP1 and AQP5 in keeping local fluid balance within the pig uterus and embryo-maternal communication during implantation.

Conflict of Interest

The authors have declared that no competing interests exist.

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Figure legends

Figure 1

AQP1 mRNA expression in porcine uterine explants determined by real-time PCR. The expression of *AQP1* mRNA in the pig uterine tissue harvested on Days 14-16 (n = 5) of pregnancy after treatment with progesterone (P_4; 10^{-5} M), estradiol (E_2; 10^{-9} M), oxytocin (OT; 10^{-7} M), arachidonic acid (AA; 10^{-5} M), forskolin (FSK; 10 µg/mL), and cyclic-AMP (cAMP; 200 µM). The uterine explants (~400 mg) were pre-incubated (18 h; 37°C, 95% O_2 + 5% CO_2) and then incubated without (C) or with the addition of experimental factors for 3 and 24 h. The data are presented as the mean values ± standard error of the mean relative to the mean ref. expression of *GAPDH* and *18 sRNA*. Different letters (a,b,c,d) indicate significant differences ($p<0.05$) between each treatment and respective control for 3- (a,b) or 24-h (c,d) incubations.

Figure 2

AQP5 mRNA expression in porcine uterine explants determined by real-time PCR. The expression of *AQP5* mRNA in the pig uterine tissue harvested on Days 14-16 (n = 5) of pregnancy after treatment with progesterone (P_4; 10^{-5} M), estradiol (E_2; 10^{-9} M), oxytocin (OT; 10^{-7} M), arachidonic acid (AA; 10^{-5} M), forskolin (FSK; 10 µg/mL), and cyclic-AMP (cAMP; 200 µM). The uterine explants (~400 mg) were pre-incubated (18 h; 37°C, 95% O_2 + 5% CO_2) and then incubated without (C) or with addition of experimental factors for 3 and 24 h. Data are presented as the mean values ± standard error of the mean relative to the mean ref. expression of *GAPDH* and *18 sRNA*. Different letters (a,b,c,d) indicate significant differences ($p<0.05$) between each treatment and respective control for 3- (a,b) or 24-h (c,d) incubations.

Figure 3

Protein content of AQP1 in uterine explants. Semi-quantitative Western blot analysis of AQP1 in homogenates of the pig uterine explants from Days 14-16 of pregnancy (each lane represents a sample from 1 pig), after treatment with progesterone (P_4; 10^{-5} M), estradiol (E_2; 10^{-9} M), oxytocin (OT; 10^{-7} M), arachidonic acid (AA; 10^{-5} M), forskolin (FSK; 10 µg/mL), and cyclic-AMP (cAMP; 200 µM). The uterine explants (~400 mg) were pre-incubated (18 h; 37°C, 95% O_2 + 5% CO_2) and then incubated without (C) or with addition of experimental factors for 3 and 24 h. Densitometric analysis of AQP1 protein levels was performed and normalized against β-actin (42 kDa) and different letters (a, b, c, d) indicate significant differences ($p<0.05$) between each treatment and respective control for 3- (a,b) or 24-h (c,d) incubations.

Figure 4

Protein content of AQP5 in uterine explants. Semi-quantitative Western blot analysis of AQP5 in homogenates of the pig uterine explants from Days 14-16 of pregnancy (each lane represents a sample from 1 pig), after treatment with progesterone (P_4; 10^{-5} M), estradiol (E_2; 10^{-9} M), oxytocin (OT; 10^{-7} M), arachidonic acid (AA; 10^{-5} M), forskolin (FSK; 10 µg/mL), and cyclic-AMP (cAMP; 200 µM). The uterine explants (~400 mg) were pre-incubated (18 h; 37°C, 95% O_2 + 5% CO_2) and then incubated without (C) or with addition of experimental factors for 3 and 24 h. Densitometric analysis of AQP5 was performed and normalized against β-actin (42 kDa) and different letters (a, b, c, d) indicate significant differences ($p<0.05$) between each treatment and respective control for 3- (a,b) or 24-h (c,d) incubations.

Figure 5

Immunohistochemical localization of AQP1 in uterine tissue explants. Immunoperoxidase staining of AQP1 in paraffin-embedded sections of the uterine explants from pigs on Days 14-16 of pregnancy. Anti-AQP1 antibody labels endothelial cells of the uterine explants (arrows). Both apical and basal plasma membranes exhibited stable AQP1 labeling in control (a) and P_4-treated uterine explants for 3-h (b). Negative control for AQP1 was performed by using non-immune IgG, and no specific immunostaining was observed (c). Immunoperoxidase labeling of AQP1 in the pig kidney cortex (d/ positive control). The labeling is seen in both of the apical and basolateral plasma membrane in proximal tubule cells. L – lumen. Bar = 50 µm.

Figure 6

Immunohistochemical localization of AQP5 in uterine tissue explants. Immunoperoxidase staining of AQP5 in paraffin-embedded sections of the uterine explants from pigs on Days 14-16 of pregnancy. AQP5 antibody stains epithelial cells of the uterine explants (arrows). The labeling is seen only in the apical plasma membranes of the epithelial cells in control on Days 14–16 of pregnancy (a). Continuously, 3- and 24-h treatments on Days 14–16 of pregnancy of the tissue explants with P_4 (b and c), E_2 (d and e), FSK (f and g) and cAMP (h and i), respectively, and 3- h with AA (j) prominent AQP5 labeling is seen in both the apical and basolateral plasma membranes of the epithelial cells. AQP5 antibody also stains smooth muscle cells in control (k and l) and P_4-treated uterine explants for 3-h (m and n). No staining was observed with using non-immune immunoglobulins (o and p/ negative control). The anti-AQP5 labels apical membrane of type I pulmonary epithelial cells of the pig (q and r/ positive control). L – lumen. Bar = 50 µm.

Table 1

Primer pairs used in the study

PCR product	Sequence
AQP1	Forward: 5′-CCAGCGAGTTCAAGAAGAAG-3′ Reverse: 5′-GCGACACCTTCACGTTATC-3′
AQP5	Forward: 5′-CTATGAGTCCGAGGAGGATT-3′ Reverse: 5′-GCTTCGCTGTCATCTGTT-3′
18SrRNA	Forward: 5′-GGCTACCACATCCAAGGAAG-3′ Reverse: 5′-TCCAATGGATCCTCGCGGAA-3′
GADPH	Forward: 5′-GACCTCCACTACATGGTCTA-3′ Reverse: 5′-AAGATGGTGATGGCCTTTC-3′

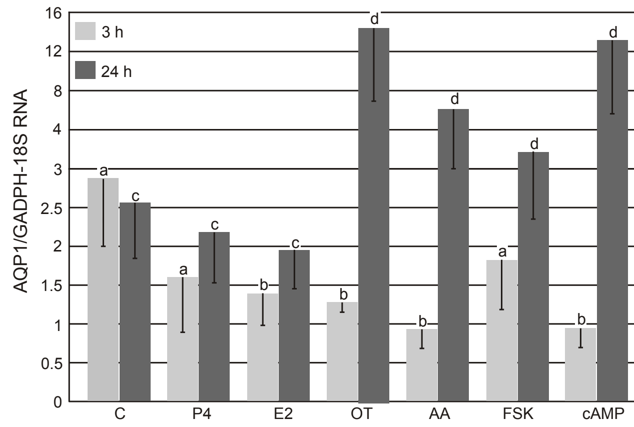

Figure 1

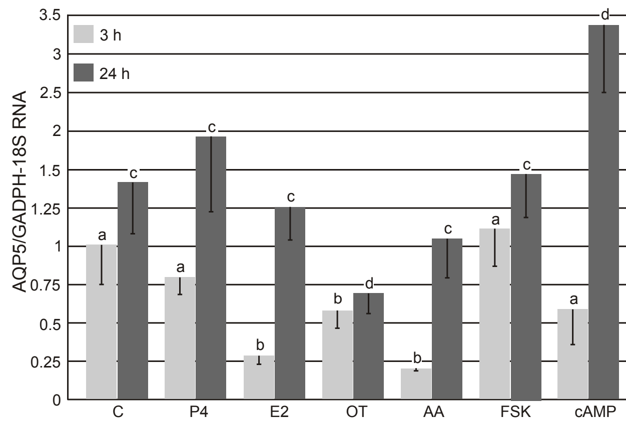

Figure 2

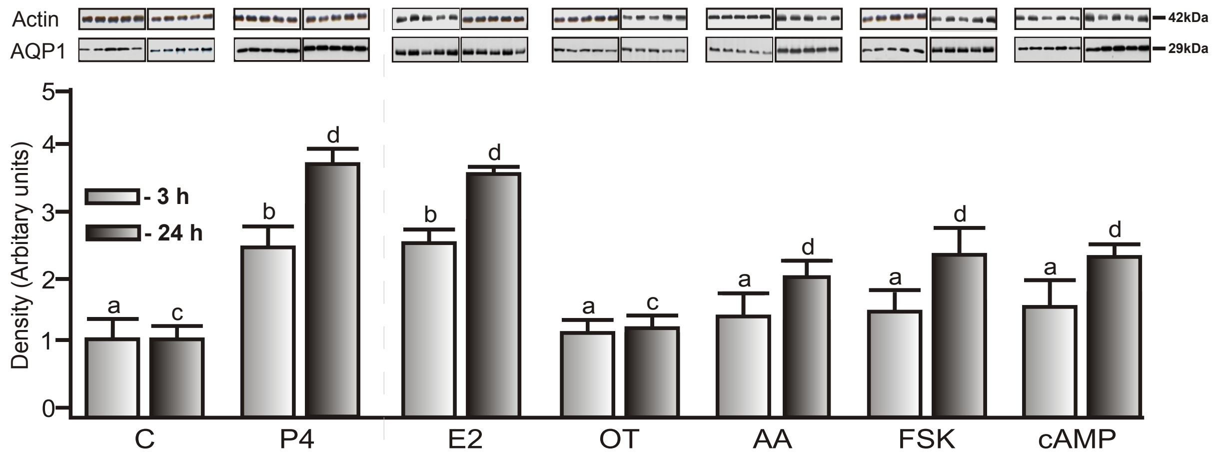

Figure 3

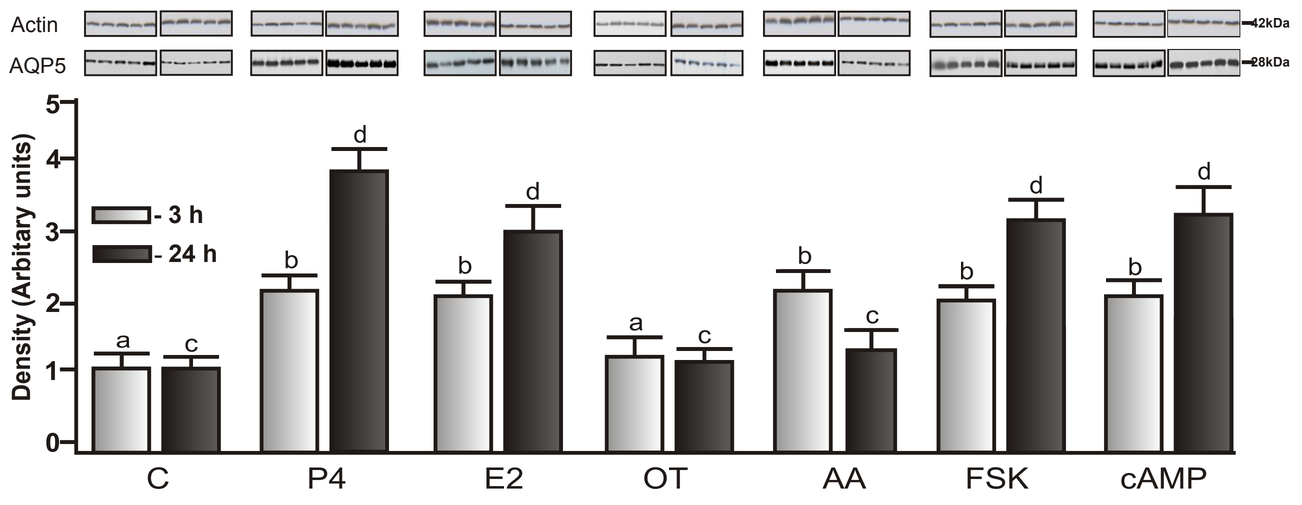

Figure 4

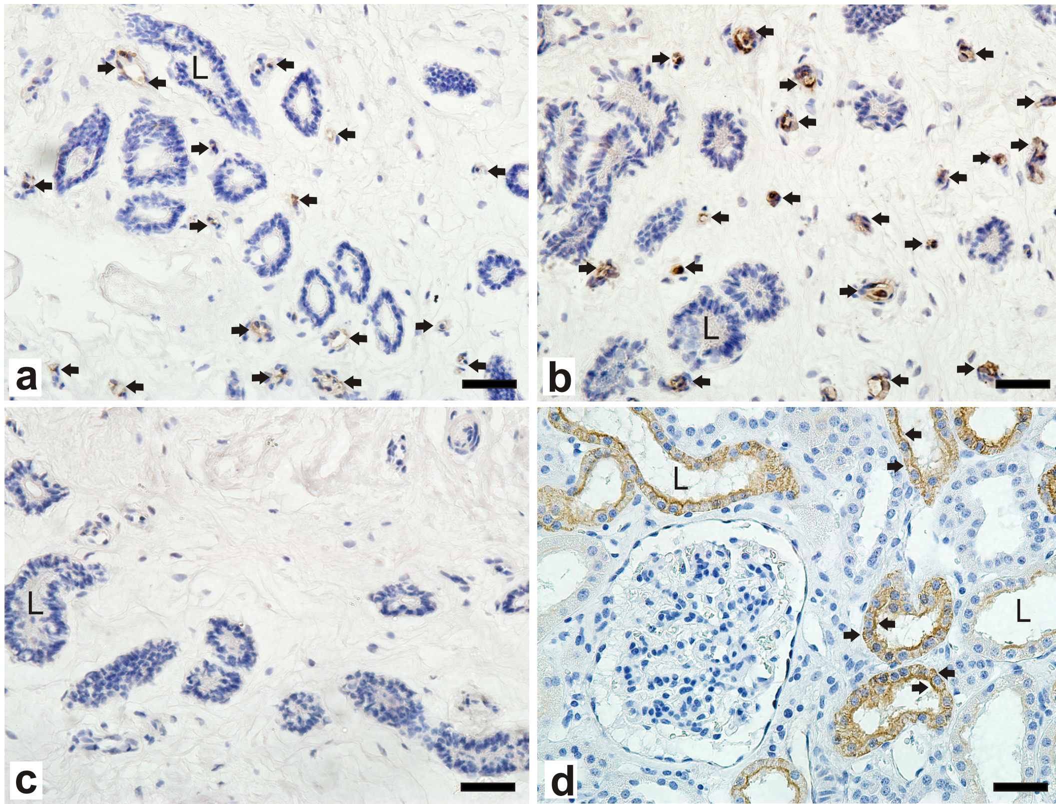

Figure 5

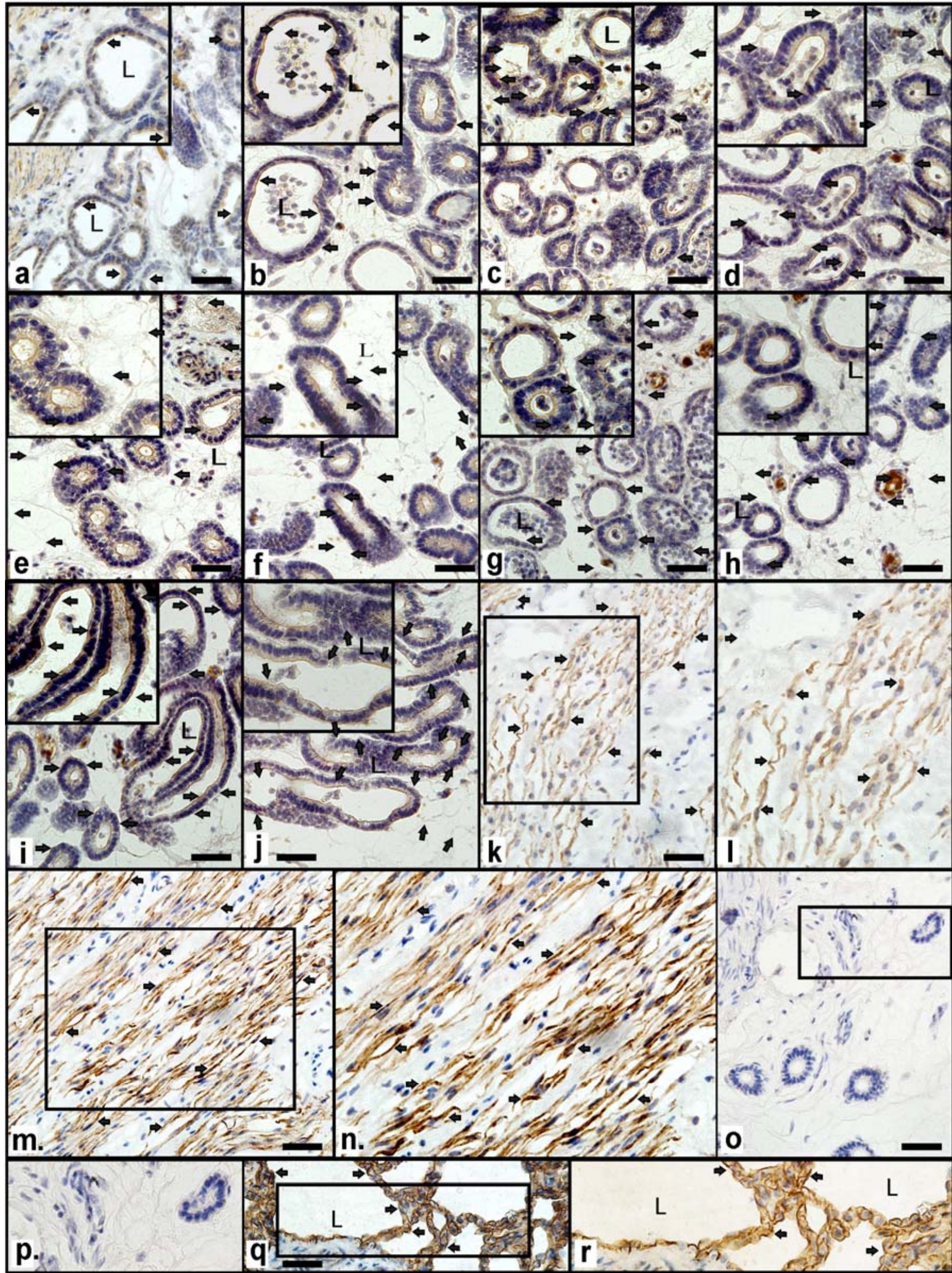

Figure 6