University of Szeged

Faculty of Pharmacy

Department of Pharmacodynamics and Biopharmacy

The role of the aquaporin 5 in the function of late pregnant rat uterus: pharmacological studies

Ph.D. Thesis Summary

Adrienn Csányi

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University of Szeged
Faculty of Pharmacy

Doctoral School of Pharmaceutical Sciences
Program director: Dr. István Zupkó Ph.D.

Department of Pharmacodynamics and Biopharmacy
Supervisor: Dr. Eszter Ducza Ph.D.

Adrienn Csányi

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Final examination committee:

Head: Dr. István Zupkó Ph.D.
Members: Dr. Gabriella Kékesi Ph.D.
Dr. Éva Szőke Ph.D.

Reviewer committee:

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Introduction

The Aquaporin (AQP) water channels have an important role in the regulation of the water homeostasis in living organisms. Their function is essential not only in organs such as blood vessels, lungs, kidney, but also in the reproductive tissues. They are responsible for vaginal lubrication and they can be found also in the ovaries where they show different expression patterns during various stages of ovulation. The AQPs are present in the uterus and cervix; they enable the cervical ripening, the embryo implantation, and endometrial development.

There are evidences that AQP5 isoform is expressed in the uterus and it has different distribution during the peri-implantation period. In our earlier study we detected the presence of AQP1, 2, 3, 5, 8, and 9 in the late-pregnancy rat uterus, with a prevalent accumulation of the AQP5 subtype which was dramatically down-regulated on the last day of pregnancy. The AQP5 expression is regulated by oxytocin in the pregnant rat uterus, and it may play a role in the regulation of delivery.

Studies suggest that this water channel is modulated by different sexual hormones. The AQP5 expression changes according to the hormonal changes during menstrual cycle and it showed an elevated expression in the peri-implantation period.

Infections are a common factor which causes preterm birth and increase the prevalence of the prenatal morbidity and mortality. So antibiotics are widely used to avoid the infection caused preterm labor. Fosfomycin and amoxicillin are also widely used antibiotic drugs for treating bacteriuria and bacterial vaginosis during pregnancy and their effects on the AQP expression is unknown.

Transient receptor vanilloid 4 (TRPV4) is voltage-gated Ca^{2+} channel which is activated by physical (heat, cell swelling) and chemical impacts. It can be found among others in the cardiovascular, nervous, pulmonary and reproductive system. It is supported by some evidences that the TRPV4 and AQP5 channels are in functional relationship. In salivary gland cells, the cooperation of TRPV4 and AQP5 is essential for the regulatory volume decrease. This cation channel is detected in myometrial smooth muscle cells in rats as well.

Aims

The dynamic change of AQP5 expression was proved during pregnancy so we investigated the hormonally effects on the AQP5 expression. The first aim of our study was (**1**) to identify the effects of estrogen and progesterone receptor agonists pretreatment on the mRNA and protein expressions of AQP5 in the rat uterus during the terminal phase of gestation. To attain

this goal we have (**2**) determined the changes of AQP5 expression in the hormonally-induced preterm birth model also.

The usage of some antibiotics is allowed during pregnancy but limited information is available about their effect on AQP5 expression and thereby on uterine contraction. Therefore our next aim was to (**3**) investigate the changes of the AQP5 expressions and uterine contraction after antibiotic (amoxicillin, fosfomycin, doxycycline) pretreatment to determine the effect of these prophylactic antibiotics on the preterm birth.

We hypothesized an osmotic pathway - through AQP5 - might have influence on the changes of TRPV4 function and uterus contraction. To prove this we (**4**) investigated the co-expression and cooperation of AQP5 and TRPV4 in the pregnant uterus and (**5**) their mutual regulatory effect on myometrial contraction.

Materials and methods

Housing and handling of the animals

Experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (permission numbers: IV/198/2013 and IV/3796/2015.).

The mature Sprague-Dawley rats (females 180-200g and males 240-260g) were mated in a special mating cage, in the early morning hours. Swabs were taken from the vagina of the female rats. In case of positive examination, the female animals were regarded and separated as first-day pregnant rats.

In vivo female sexual hormone and antibiotic treatments of the rats

The pretreatment of pregnant animals with 17β-estradiol (E), tamoxifen (T), and clomiphene (C) was started on day 14 and day 18 of gestation. The active agents were suspended in olive oil and injected subcutaneously in a dose of 1 µg/0.1 ml of E, 5 mg/0.1 ml of T and 1 mg/0.1 ml of C once a day for four days.

The pregnant rats were treated with progesterone (P), levonorgestrel (L), and medroxyprogesterone acetate (MPA) from day 11 and 15 of gestation. The compounds were suspended in olive oil. The animals were injected subcutaneously every day with 0.5 mg/0.1 ml of P and L, and 5 mg/0.1ml of MPA for seven days. On day 18 and 22, the uterine samples were collected and molecular studies were carried out.

We treated the preterm birth (PB) group as reported by Elovitz et al.. The pregnant rats received a mifepristone, dissolved in olive oil, subcutaneous injection in a dosage of 3 mg/0.1 ml on day 19 of pregnancy in the morning. The intravaginal prostaglandin E2 was

given at a dose of 0.5 mg/ml on the same day in the afternoon. Next day appear preterm delivery on day 20 of gestation. After the beginning of the PB, uterine samples were collected and molecular studies were carried out.

The pregnant animals received amoxicillin (A) from the day 16 of gestation. A was given orally in a dose of 40 mg/kg of body weight by oral gavage once a day for seven days. We prepared the suspension with purified water.

The fosfomycin (F) treatment of the pregnant rats was started on day 21 of gestation. The solution was prepared with purified water. The animals were treated orally with F in a dose of 40 mg/kg of body weight once.

The pregnant rats got the doxycycline (D) treatment from the day 16 of gestation. They received D orally, in a dose of 30 mg/kg of body weight once a day for seven days. The suspension was prepared with methyl cellulose. On the last day of pregnancy uterine samples were collected and molecular studies were carried out.

RT-PCR Studies

RT-PCR studies were carried out with uterine tissues (n=6 in each experiment). Total cellular RNA was isolated by extraction with guanidiniumthiocyanate-acid-phenol-chloroform according to the procedure of Chomzynski and Sacchi. Reverse transcription and amplification of the PCR products were performed by using the TaqMan RNA-to-C_T-Step One Kit and an ABI StepOne Real-Time cycler. β-actin served as an internal control. All samples were run in triplicate. The fluorescence intensities of the probes were plotted against PCR cycle number. The amplification cycle displaying the first significant increase of the fluorescence signal was defined as the threshold cycle (C_T).

Western Blot Analysis

Twenty-five micrograms of sample protein per well was subjected to electrophoresis on 4%-12% NuPAGE Bis-Tris Gel in XCellSureLock Mini-Cell Units. Proteins were transferred from gels to nitrocellulose membranes using the iBlot Gel Transfer System. Ponceau S was used to check the standard running and transfer conditions. The blots were incubated overnight on a shaker with AQP5, TRPV4 and β-actin polyclonal antibodies in blocking buffer. Antibody binding was detected with the Western Breeze® Chromogenic immunodetection kit.

Images were captured with the EDAS290 imaging system, and the optical density of each immunoreactive band was determined with Kodak 1D Images analysis software. The β-actin was used for protein normalization for this semi-quantitative method.

Immunohistochemistry

After preparing the uterus sections for the immunohistochemistry, they were placed on blocking solution, treated with rabbit polyclonal anti-TRPV4 and AQP5 primary antibodies. Incubation was performed with the Histo-Labeling system anti-rabbit secondary antibody conjugated with peroxidase and the reaction was visualized using 3,3-diaminobenzidine tetrachloride. Histological counterstaining was performed with haematoxylin. For double immunofluorescence analysis, the Tyramide Signal Amplification Kit was used with fluorescent-labeled tyramide to detect color red and directly labeled secondary antibody to detect color green. Micrographs were generated using an Olympus Fluoview-1000 system. The scale bar represents 50 µm. The counting of TRPV4 and AQP positive myometrial cells was performed in 3 different standardized areas from each slide, using ImageJ software.

***In Vitro* Contractility Studies**

Uterus tissues were removed from the 18 or 22 day pregnant rats. 5-mm-long muscle rings were sliced from both horns of the uterus. The initial tension of the preparation was set to about 1.5 g and the tension dropped to about 0.5 g by the end of the equilibration period. The tension of the myometrial rings was measured with a gauge transducer and recorded with a SPEL Advanced ISOSYS Data Acquisition System. Spontaneous contractions were recorded for 4 minutes and cumulative oxytocin concentration-response curves (10^{-12}–10^{-8}M) were constructed in each experiment.

For the investigation of the effects of TRPV4 agonist and antagonist compounds, KCl solution was used to induce rhythmic contractions. The effects of a TRPV4 antagonist (RN1734) and a TRPV4 agonist (RM1747) were tested on the uterine contractions in the concentration range of $3x10^{-8}$-10^{-5}M in a cumulative mode. Recording was performed at each concentration of the examined agents for 5 minutes. The effects of RN1734 and RN1747 were expressed as the percentage of the area under curve (AUC) of KCl induced contractions. The dose-response curves were fitted and the statistical analysis of EC_{50} and E_{max} values was performed.

Results

The effects of estrogen analog compounds on AQP5 expression in pregnant rat uterus

We found that E pretreatment did not result any significant changes in the AQP5 mRNA levels, it was inefficient both on day 18 and day 22 of gestation **(Fig. 1A)**. The protein expression of AQP5 showed a significantly increase both on day 18 and day 22 of pregnancy, compared to the control **(Fig. 1B)**.

In case of T pretreatment we did not observe any changes on the AQP5 mRNA expression **(Fig. 2A)**. However, the level of AQP5 protein elevated on day 18, but did not change on the last day of gestation, compared to the control **(Fig. 2B)**.

Either the AQP5 mRNA **(Fig 3A)** or the protein **(Fig 3B)** levels did not alter significantly as a result of C pretreatment on the investigated days of pregnancy.

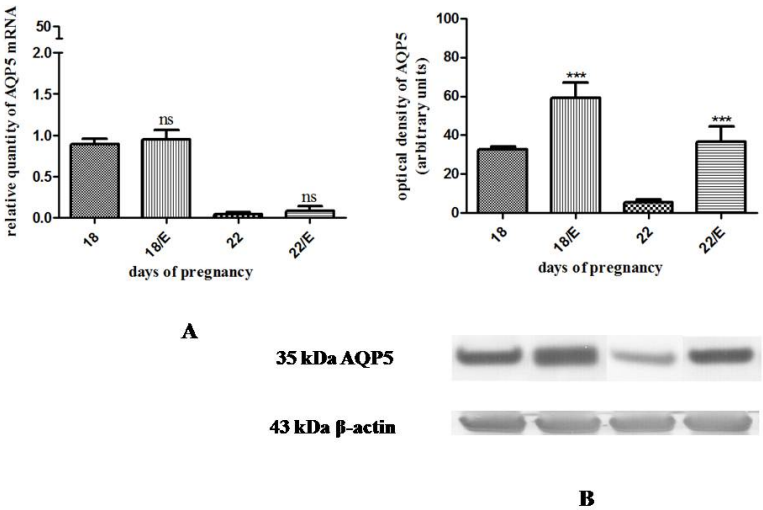

Figure 1. Results of RT-PCR and Western immunoblotting analysis after E treatment. The changes of mRNA and protein expression of AQP5 after E pretreatment in pregnant rat uterus on days 18 and 22.ns > 0.05, *** $p < 0.001$; E: 17β-estradiol treated group

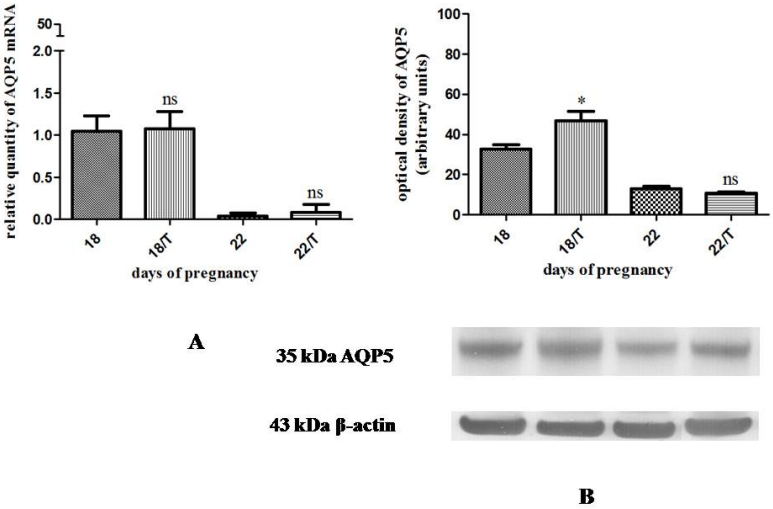

Figure 2. Results of RT-PCR and Western immunoblotting analysis after T treatment. The changes of mRNA and protein expression of AQP5 after T pretreatment in pregnant rat uterus on days 18 and 22.ns > 0.05, *$p < 0.05$; T: tamoxifen treated group

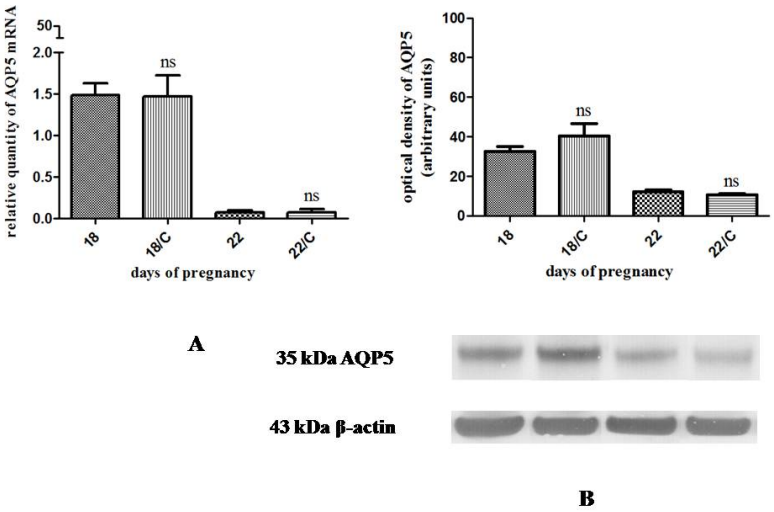

Figure 3. Results of RT-PCR and Western immunoblotting analysis after C treatment. The changes of mRNA and protein expression of AQP5 after C pretreatment in pregnant rat uterus on days 18 and 22.ns > 0.05; C: clomiphene treated group

The effects of gestagen analog compounds on AQP5 expression in pregnant rat uterus

All of the gestagen-related compounds: P, L and MPA pretreatment induced a significant raise both in AQP5 mRNA and protein levels on day 18 and day 22 of gestation **(Fig. 4-6)**.

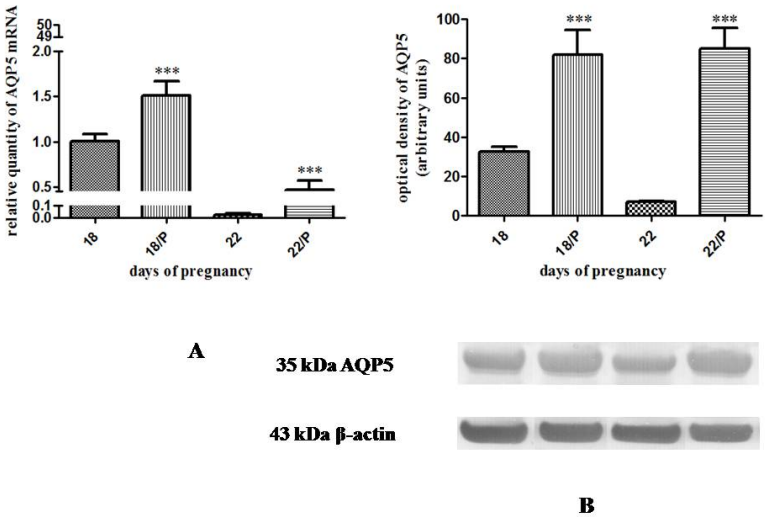

Figure 4. Results of RT-PCR and Western immunoblotting analysis after P treatment. The changes of mRNA and protein expression of AQP5 after P pretreatment in pregnant rat uterus on days 18 and 22. *** $p < 0.001$; P: progesterone treated group

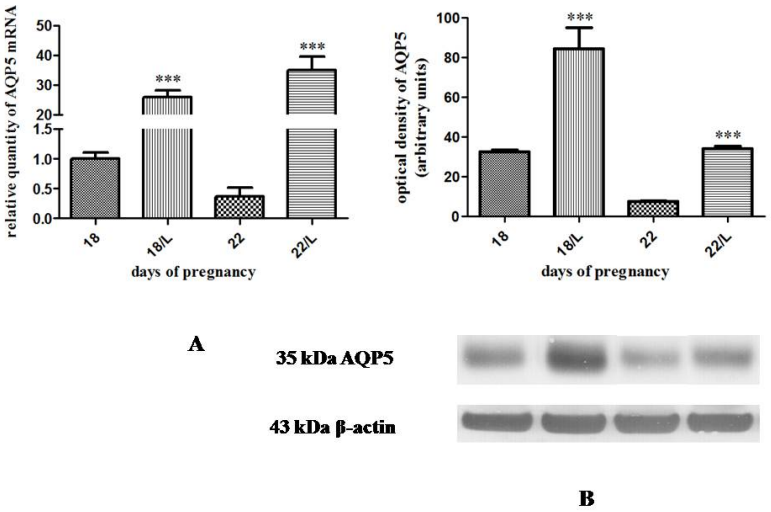

Figure 5. Results of RT-PCR and Western immunoblotting analysis after L treatment. The changes of mRNA and protein expression of AQP5 after L pretreatment in pregnant rat uterus on days 18 and 22. *** $p < 0.001$; L: levonorgestrel treated group

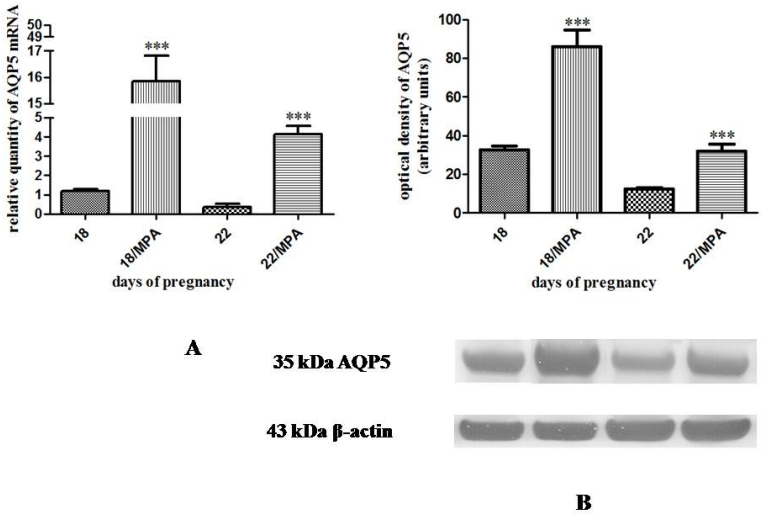

Figure 6. Results of RT-PCR and Western immunoblotting analysis after MPA treatment. The changes of mRNA and protein expression of AQP5 after MPA pretreatment in pregnant rat uterus on days 18 and 22. *** $p < 0.001$; MPA: medroxyprogesterone-acetate treated group

The effect of hormonally-induced preterm delivery on AQP5 expression in pregnant rat uterus

We found a significant reduction of AQP5 mRNA and protein levels on day 20 of pregnancy, compared to the non-treated rats on pregnancy day 20. Interestingly this drop was similar to the physiological last day of gestation **(Fig. 7)**

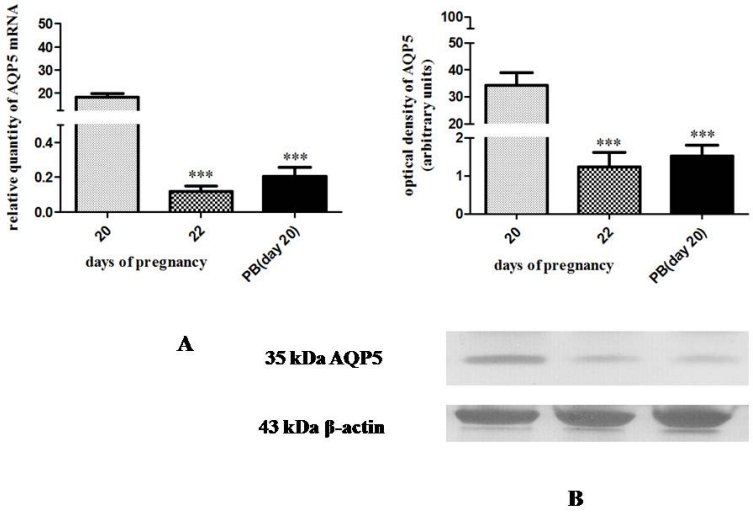

Figure 7. Results of RT-PCR and Western immunoblotting analysis after the hormonally-induced PB. The changes of mRNA and protein expression of AQP5 after the hormonally-induced PB in pregnant rat uterus. *** $p < 0.001$; PB: preterm birth group

The effects of different antibiotic drugs on AQP5 expression in pregnant rat uterus

The AQP5 mRNA **(Fig. 8A)** and protein **(Fig. 8B)** levels showed a significant drop on day 22 of pregnancy after the 7 days A pretreatment, compared to the non-treated rat uterus on the last day of gestation.

A single dose of F significantly increased the AQP5 mRNA levels on the last day of pregnancy, compared to non-treated animals **(Fig. 9A)**. In opposition to the mRNA level, the protein level of AQP5 showed reduction on day 22 of gestation **(Fig. 9B)**.

In case of 7 days D pretreatment we did not observe any changes either in the AQP5 mRNA or the protein levels on the last day of pregnancy **(Fig. 10)**.

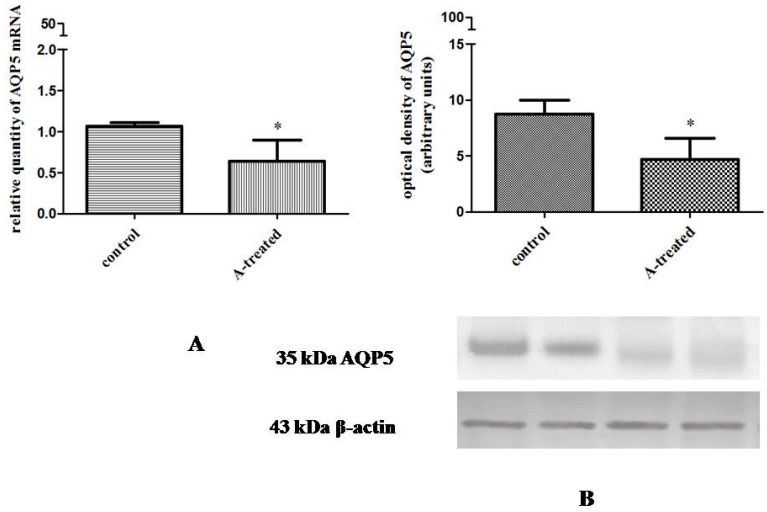

Figure 8. Results of RT-PCR and Western immunoblotting analysis after A treatment. Changes in mRNA (**A**) and protein (**B**) expression of AQP5 after 7 days of A pretreatment in rat uterus on the last day of pregnancy. * $p < 0.05$; A: amoxicillin treated group

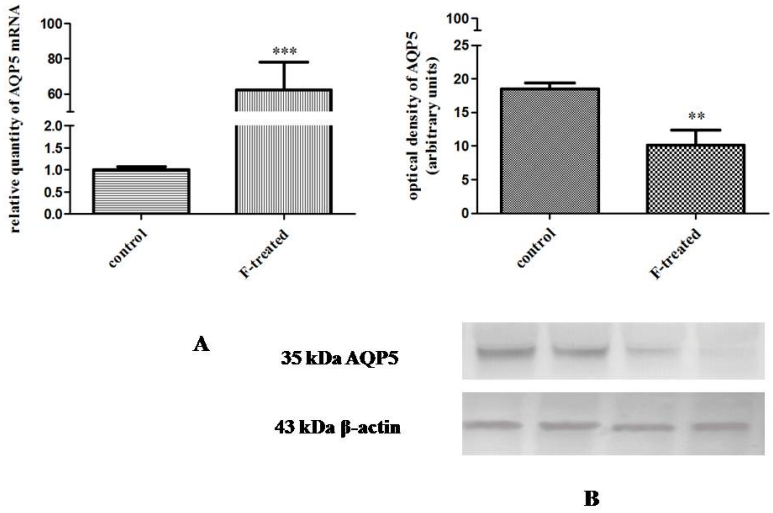

Figure 9. Results of RT-PCR and Western immunoblotting analysis after F treatment. Changes in mRNA (**A**) and protein (**B**) expression of AQP5 after F pretreatment in pregnant rat uterus on gestation day 22. ** $p < 0.01$, *** $p < 0.001$; F: fosfomycin treated group

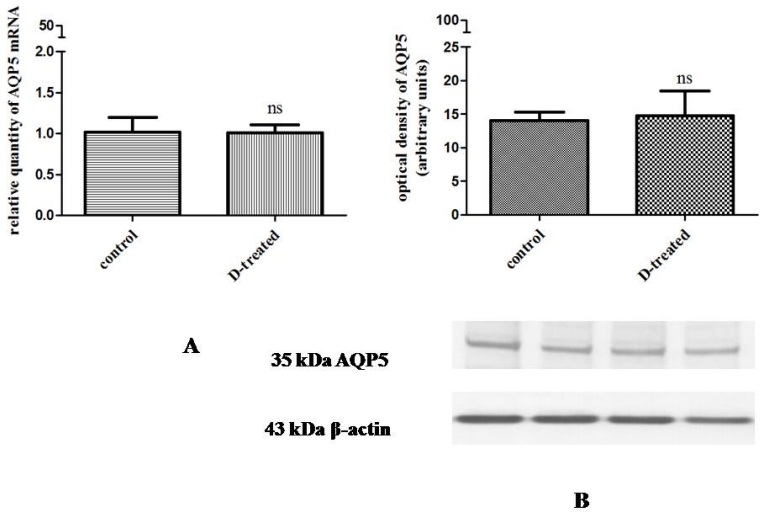

Figure 10. Results of RT-PCR and Western immunoblotting analysis after D treatment. Changes in mRNA (**A**) and protein (**B**) expression of AQP5 after D pretreatment in pregnant rat uterus on gestation day 22.ns $p > 0.05$; D: doxycycline treated group

The effect of antibiotic pretreatment on the oxytocin evoked myometrial contractions *in vitro*

As seen on **Figure 11**, oxytocin (10^{-12}-10^{-8} M) enhanced the uterus contractions in the 22 day pregnant uterus. A or F pretreatments significantly increased the uterine contracting effects of oxytocin. In contrast, D pretreatment did not influence the oxytocin-induced contractions **(Fig. 11)**.

As for the spontaneous myometrial contractions, it was decreased significantly by F and A pretreatment on the 22 day pregnant uterus. In opposition to D treated uterus, there was no change in the spontaneous contractility **(Fig.12)**.

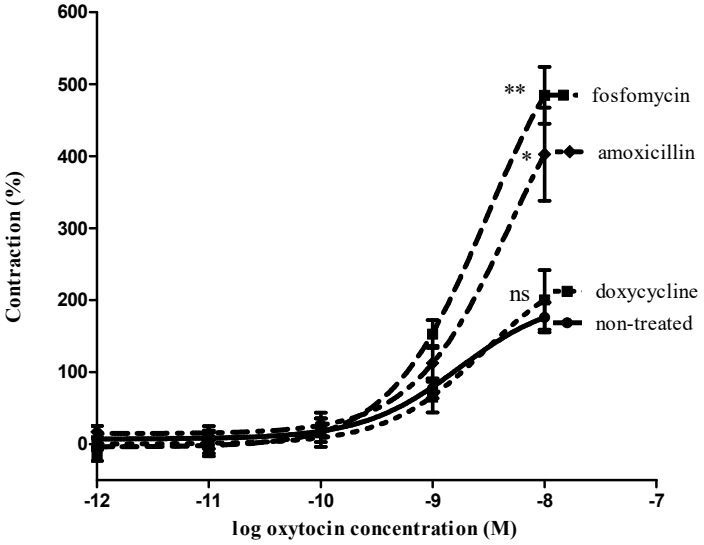

Figure 11. Effects of F, A and D pretreatment on the oxytocin-induced contractions in the 22-day-pregnant rat uteri.; ns $p > 0.05$; * $p < 0.05$; ** $p < 0.01$.

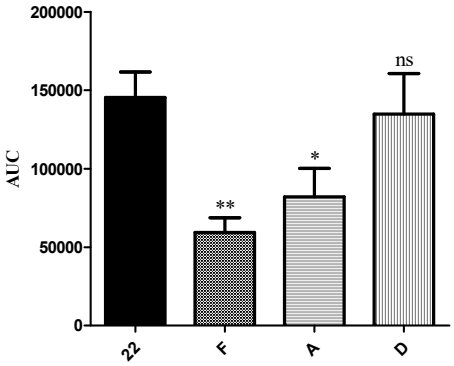

Figure 12. Spontaneous myometrial contractility in the 22 day pregnant rats after F, A and D pretreatments. ns $p > 0.05$; * $p < 0.05$; ** $p < 0.01$. 22: control 22-day pregnant rat uterus; F: fosfomycin; A: amoxicillin; D: doxycycline treated 22-day pregnant rat uterus

The TRPV4 and AQP5 co-expression in rat uterus

We determined the TRPV4 mRNA and protein expression in the non-pregnant and pregnant rat uterus **(Fig. 13)**. There is a correlation between the changes of the mRNA and protein levels on the investigated days. On day 18 of gestation, the mRNA and protein expression was the lowest, and it elevated continuously until the day of labor. From day 18 to 22 of pregnancy, the AQP5 and TRPV4 mRNA expression showed a strong correlation (r^2=0.9577) **(Fig. 13C)**. Between AQP5 and TRPV4 protein expression, we determined a moderate correlation (r^2=0.6452) **(Fig. 13D)**.

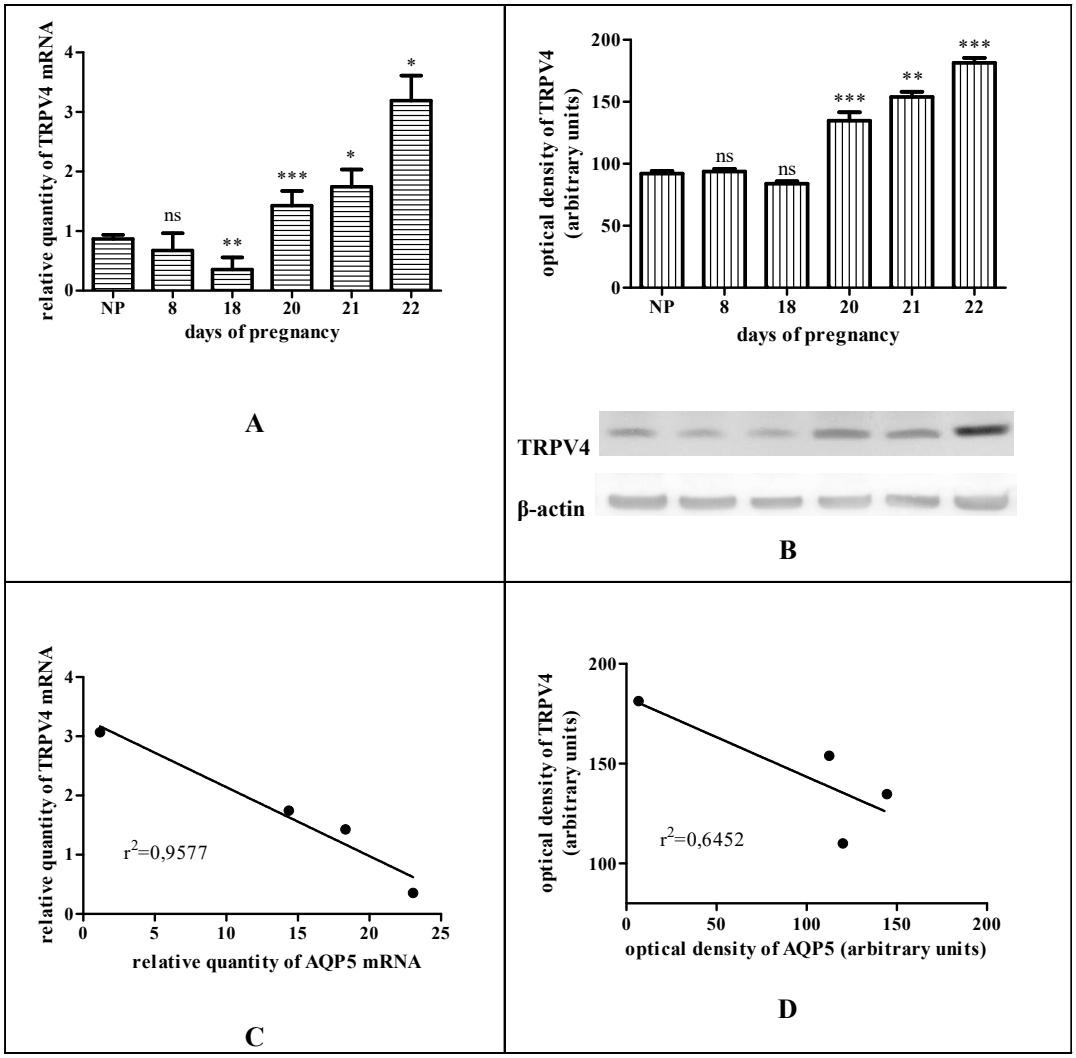

Figure 13. The changes in mRNA (**A**) and protein expression (**B**) of TRPV4 in the non-pregnant uterus (estrus phase) and on different gestational days in pregnant rat uterus. Correlation between TRPV4 and AQP5 mRNA (**C**) and protein (**D**) expression from pregnancy day 18 to day 22 in uterus. NP: non-pregnant, ns >0.05, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

On day 18 and 22 of gestation, the number of the AQP5 and TRPV4 channels did not show any significant changes **(Fig. 14D and E)** in the myometrium.

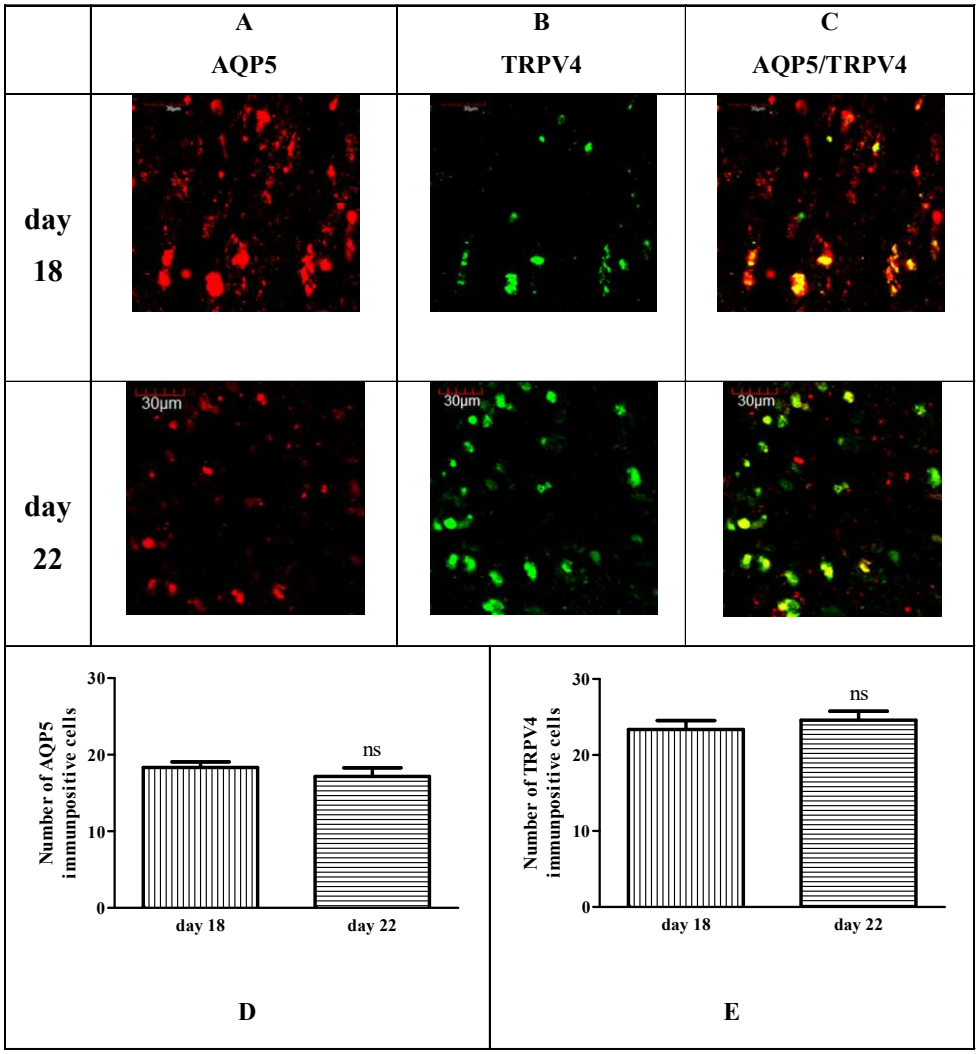

Figure 14. Representative pictures showing expressions of the AQP5 (**A**) and TRPV4 (**B**) ; these co-expressions (**C**) and the number of AQP5 (**D**) / TRPV4 (**E**) immunopositive cells in the myometrium on days 18 and 22 of pregnancy. ns >0.05.

On the last day of gestation, the numbers of AQP5 immunpositive cells were significantly decreased **(Fig. 15D)**. On the other hand, the numbers of TRPV4 immunpositive cells were significantly elevated on day 22 **(Fig. 15E)**.

It was proven, that the AQP5 and TRPV4 channels are co-expressed in the endometrium and myometrium, on both investigated days of late gestation **(Fig. 14C and 15C)**.

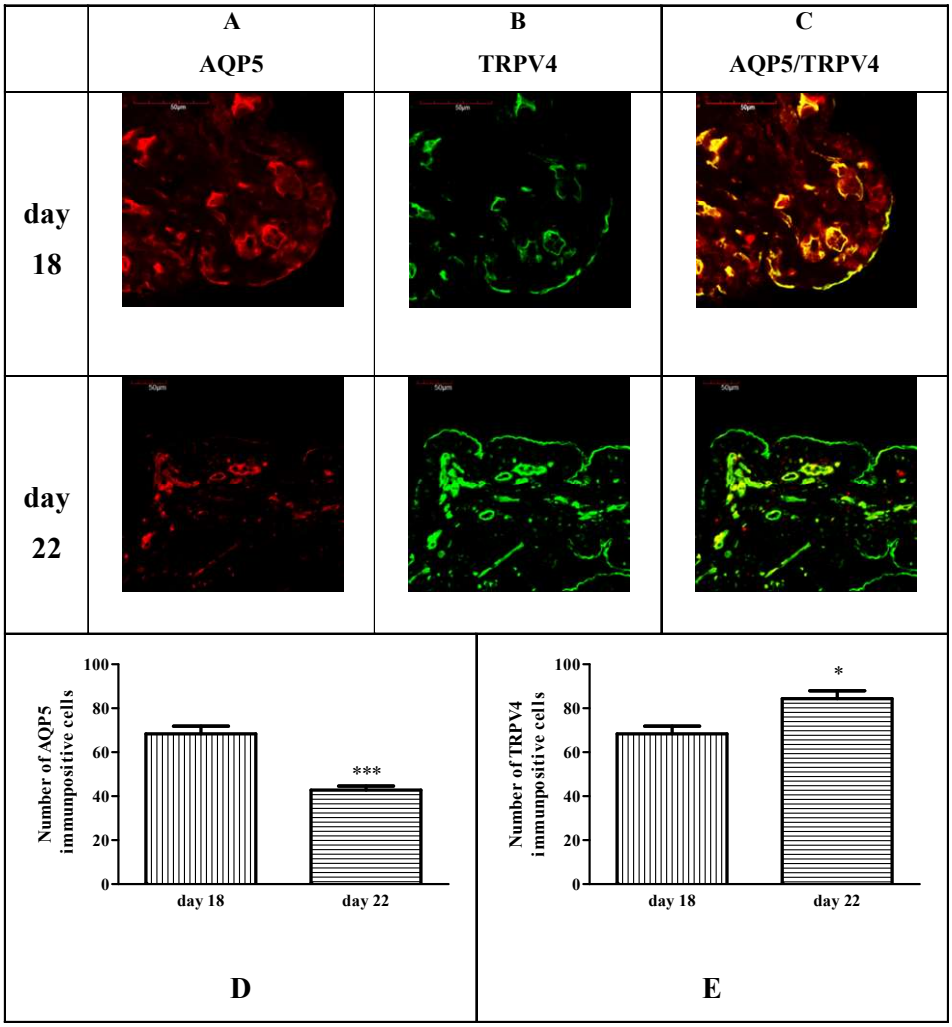

Figure 15. Representative pictures showing expressions of the AQP5 (**A**) and TRPV4 (**B**); these co-expressions (**C**) and the number of AQP5 (**D**) / TRPV4 (**E**) immunopositive cells in the endometrium on days 18 and 22 of pregnancy. * $p<0.05$, *** $p<0.001$.

It was also investigated, how the TRPV4 agonist and antagonist influence the uterus contraction in an isolated organ bath system **(Fig. 16)**. The agonist (RN1747) displayed a minor relaxing effect on day 18 (9.93 %). The antagonist (RN1734) showed a pronounced relaxing effect (30.33 %) on the same day **(Fig. 16A)**.

On the last day of pregnancy, the agonist did not have any effect on relaxation (-16.87 %), but it induced uterus contraction. The antagonist had a remarkable relaxing effect (44.52 %) on day 22 of gestation **(Fig. 16B)**.

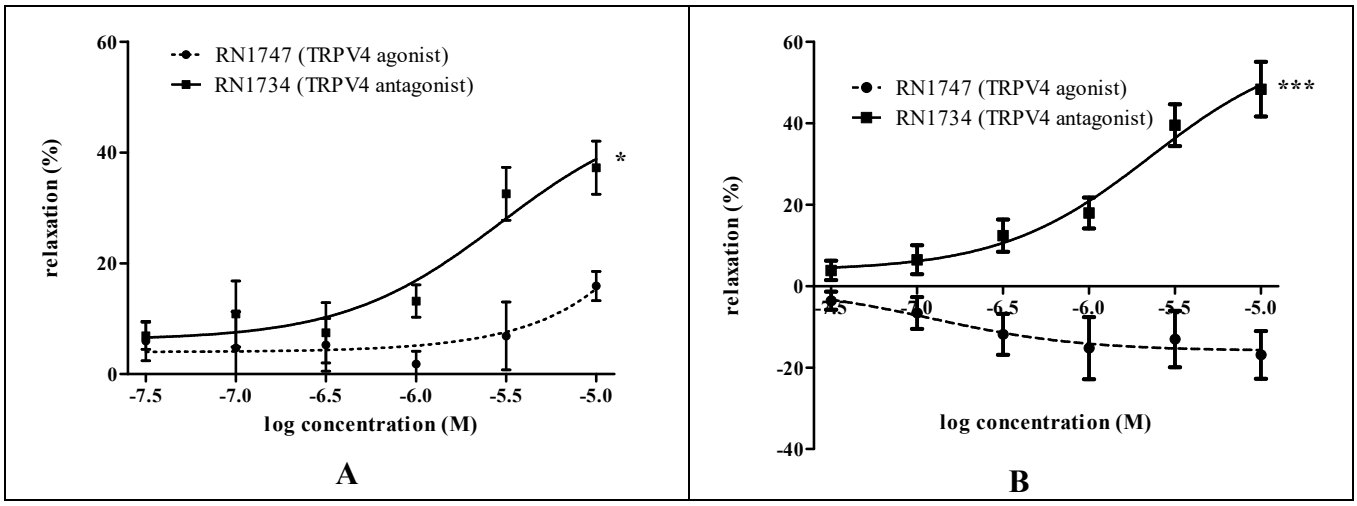

Figure 16. Effect of selective TRPV4 agonist (RN1747) and antagonist (RN1734) on KCl-evoked control contraction of rat uteri on days 18 (**A**) and 22 (**B**) of gestation. *p < 0.05; ***p < 0.001

Discussion

Our earlier studies revealed that AQP5 water channel is the one subtype of the AQP family which undergoes major changes in the course of gestation in pregnant rat uterus. There are numerous comprehensive studies which investigated AQP5 water channel in different species and tissues but to date, we cannot found any experiment that have focused on the expression of this channel in late pregnant rat uterus after the pretreatment of female sexual hormones or antibiotics. Besides, no data is available on the effect of antibiotics on the uterine contractility and the connection between AQP5 and TRPV4 channel in the pregnant rat uterus.

AQP5 channel, female sexual hormones and preterm birth

Evidences show that sexual hormones can influence AQP water channels. According to our findings, P, L and MPA pretreatment results a significant elevation on the AQP5 expression. This was supported by another investigation where it was proven that AQP5 was particularly expressed in response to high levels of P in the uterus of dogs.

In our experiment E pretreatment increased just the amount of AQP5 protein and T and C treatment did not change the AQP5 expression. We hypothesize that the four-day long pretreatment is not enough for influencing the hypothalamic-pituitary axis.

We wanted to prove that P has notable effect on AQP5 expression, so we used hormonally-induced preterm birth (PB) model. It was induced by antigestagen mifepristone and prostaglandin E2. The AQP5 expression decreased significantly on day 20 of pregnancy when the PB begins. This change in the AQP5 expression was similar to the last day of gestation which occurs on day 22. This phenomenon could be explained by the process, during which

the level of P dropped significantly at the time of the PB, which was followed by the decrease of AQP5 expression.

AQP5 channel and antibiotic treatment

The antibiotic treatment can reduce the incidence of preterm birth, which often leads to long-term neurological impairments and respiratory and gastrointestinal complications in children. Amoxicillin (A) pretreatment significantly reduced the AQP5 level in the pregnant rat uterus on the last day of gestation. The uterine tissues showed enhanced contractility responses to oxytocin as a result of A pretreatment. Fosfomycin (F) treatment increased the sensitivity of the myometrium to oxytocin stimulation. These synergetic effects of A and F on the uterus contraction must be considered in pharmacotherapy during pregnancy.

Cooperation between the AQP5 channel and TRPV4 receptor

Based on our earlier study, AQP5 channel is selectively down-regulated by oxytocin, which is responsible for uterus contractions. The AQP5 expression is up-regulated by the myometrial relaxing progestogens. Based on these findings, we suppose an inverse correlation between the AQP5 level and uterus contraction. We hypothesize an osmotic pathway to control the uterus smooth muscle cells.

TRPV4 channel is a Ca^{2+} channel and it is activated by osmotic stimuli and it can be found in different smooth muscles. Based on literature data it is known that the AQP5 water channel is an interacting partner of the TRPV4 channel in the smooth muscle cells.

The changes of TRPV4 expression in the rat uterus could be the effect of hormonal changes. TRPV4 expression was decreased by P in human tracheal and mammary gland ductal epithelial cell lines. This statement supports our results, considering that the level of P drops at the end of pregnancy which increases TRPV4 expression.

Based on our studies we found an inverse correlation between the AQP5 and TRPV4 expression in the late pregnant uterus. The co-expression and putative cooperation between AQP5 and TRPV4 was revealed by our immunohistochemical findings.

TRPV4 might have a role in contractility and Ca^{2+} signaling. In our *in vitro* experiments, the TRPV4 antagonist resulted relaxing effect on the pregnant rat uterus.

According to our findings, we suppose the decreased AQP5 expression triggers an osmotic stress, which activates TRPV4 and increases uterus contraction on the day of labor.

Conclusion

In light of our results; (**1**) the pretreatment of P and P-related compounds resulted a significant elevation in the AQP5 mRNA and protein expression. We assumed that AQP5 expression is

directly proportional to P level and both of them are essential for support gestation. We proved that (**2**) PB also caused a drop in the AQP5 expression on day 20 of pregnancy. The explanation could be that the level of P decreased such as at the time of delivery. We identified (**3**) that AQP5 expression could be modulated by antibiotics what sharpen the contractions on oxytocin evoked uterus. We suppose that A and F may sensitize the uterus to oxytocin by reducing the AQP5 expression. Based on our results, (**4**) the expression of AQP5 and TRPV4 channels showed an inverse proportion and our immunohistochemical findings uncovered the co-expression of AQP5 and TRPV4. The uterus relaxant effect of TRPV4 antagonist was proved (**5**) on the day of labor as a new target for the tocolysis.

List of publications

1. Publication related to the Ph.D. thesis

I. Csányi A, Bóta J, Falkay G, Gáspár R, Ducza E: The effects of female sexual hormones on the expression of AQP 5 in the late-pregnant rat uterus.
International Journal of Molecular Sciences 17: (8) Paper: 1300, 13p. (2016) ***[IF: 3.226; Q1 in Medicine (miscellaneous) (2016)]***

II. Ducza E, **Csányi A**, Gáspár R: AQPs during Pregnancy: Their Function and Significance.
International Journal of Molecular Sciences 18: (12) p.E2593 (2017) ***[IF: 3.687); Q1 in Medicine (miscellaneous) (2017)]***

III. Csányi A, Hajagos-Tóth J, Kothencz A, Gáspár R, Ducza E: Effects of different antibiotics on the uterine contraction and the expression of AQP 5 in term pregnant rat.
Reproductive Toxicology 81 pp. 64-70., 7 p. (2018) ***[IF: 2.580; Q2 in Toxicology (2017)]***

IV. Submitted for publication: Ducza E, **Csányi A**, Szőke É, Pohóczky K, Hajagos-Tóth J, Kothencz A, Tiszai Z, Gáspár R: Significance of transient receptor potential vanilloid 4 and AQP 5 co-expression in the rat uterus at term.
Heliyon ***[IF- : ; Q1 in Multidisciplinary]***

2. Presentations related to the Ph.D. thesis

I. Ducza E, **Csányi A**, Gáspár R.
Pharmacological influence of myometrial AQP 5 expression in pregnant rat

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