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

Adrienn Csányi

Supervisor:

Eszter Ducza Ph.D.

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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.
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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.
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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.
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2. Presentations related to the Ph.D. thesis

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Pharmacological influence of myometrial AQP 5 expression in pregnant rat
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II. Ducza E, **Csányi A**, Gáspár R.
Az AQP 5 expressziójának változása hormonális hatásokra vemhes patkány uteruszban
FAMÉ, Magyar farmakológiai, anatómus, mikrocirkulációs és élettani társaságok közös tudományos konferenciája, Pécs, Magyarország, (2016) (Poster presentation)

III. Csányi A, Hajagos-Tóth J, Gáspár R, Ducza E.
Antibiotikumok hatása az AQP 5 expressziójára vemhes patkány uteruszban

Tavaszi Szél Konferencia, Nemzetközi Multidiszciplináris Konferencia, Miskolc, Magyarország, Doktoranduszok Országos Szövetsége, (2017) (Oral presentation)

IV. Csányi A, Hajagos-Tóth J, Gáspár R, Ducza E.
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3. Other publication unrelated to this thesis:

I. Hajagos-Tóth J, Bóta J, Ducza E, **Csányi A**, Tiszai Z, Borsodi A, Samavati R, Benyhe S, Gáspár R: The effects of estrogen on the α2- adrenergic receptor subtypes in rat uterine function in late pregnancy in vitro.
Croatian Medical Journal 57 : 2 pp. 100-109. , 10 p. (2016) ***[IF: 1.619; Q2 in Medicine (miscellaneous) (2016)]***

II. Kothencz A, Hajagos-Tóth J, **Csányi A**, Gáspár R: Alpha-tocopherol succinate increases cyclooxygenase-2 activity: Tissue-specific action in pregnant rat uterus in vitro.
Life Sciences 192 pp. 199-204. , 6 p. (2018) ***[IF: 3.234; Q1 in Medicine (miscellaneous) (2017)]***

List of abbreviations

A: amoxicillin

AQP: aquaporin

C: clomiphene citrate

C_T: threshold cycle

D: doxycycline

E: 17β-estradiol

EDHF: endothelium-derived hyperpolarizing factor

F: fosfomycin

L: levonorgestrel

MPA: medroxyprogesterone acetate

NP: non-pregnant

NPA motif: asparagine-proline-alanine motif

P: progesterone

PB: hormonally-induced preterm birth

PROM: premature rupture of the membranes

RT-PCR: real-time reverse transcription polymerase chain reaction

SEM: standard error of the mean

SD: standard deviation

T: tamoxifen citrate

TRP: transient receptor potential

TRPV4: transient receptor potential vanilloid 4

WB: western blot

1   Introduction

Aquaporin (AQP) water channels are small hydrophobic integral membrane proteins which enable the passive rapid movement of water across the cell membrane. (Denker et al., 1988) There are three subfamilies of this water channel, according to their permeability. Classical AQPs are AQP0, 1, 2, 4, 5, 6, and 8, which selectively permeable to water. (Ishibashi et al., 2011) While aquaglyceroporins allow not only the transport of water but also non-polar solutes, such as glycerol, urea and gases, like carbon dioxide and ammonia. This subfamily contains AQP3, 7, 9, and 10. (Hara-Chikuma and Verkman, 2006; Madeira et al., 2015) Unorthodox AQPs include AQP11 and 12; the function of them is under investigation. (Itoh et al., 2005; Yakata et al., 2007)

Structural studies revealed that the monomer units of this water channel consist of six transmembrane α-helices and five connecting loops. (Eriksson et al., 2013) Loops B and E contain the conserved Asn-Pro-Ala (NPA) motifs, which fold as seventh pseudo transmembrane helix insert from the opposite sides of the membrane. These motifs located near the center of the water pore and part of the hydrophilic surface of natural dipole pore. The selectivity of these channels for water is due to the backbone α-carbonyl groups and the NPA motifs containing aromatic and arginine residues (**Fig. 1**). They act as hydrogen-bond donors and acceptors that responsible for the coordination of water transport through the channel. (Törnroth-Horsefield et al., 2010)

AQPs are widely expressed in living organisms and their proper function is elemental for different physiological processes. In the human body AQP1 is expressed in the central nervous system, corneal endothelium, erythrocytes, kidney and in all vascular endothelial cells. AQP2 can be found in the kidney, in the luminal side of collecting duct principal cells. AQP3 is located in the eyes, lung, kidney, macrophages, T cells, erythrocytes, epidermal cells and in the epithelial cells of the intestine. AQP4 is expressed in the astrocytes end-feet, lacrimal gland, inner ear, olfactory epithelial cells, stomach, airway cells and in the kidney. AQP5 is located in the salivary glands, lacrimal gland, alveolar type I cells and in the uterus. AQP7 is expressed in the S3 segment of the proximal tubule and in the adipocytes. AQP8 is found in the epithelial cells of the intestine. AQP9 is expressed in the erythrocytes and AQP0 is expressed in the lens. (Verkman et al., 2014)
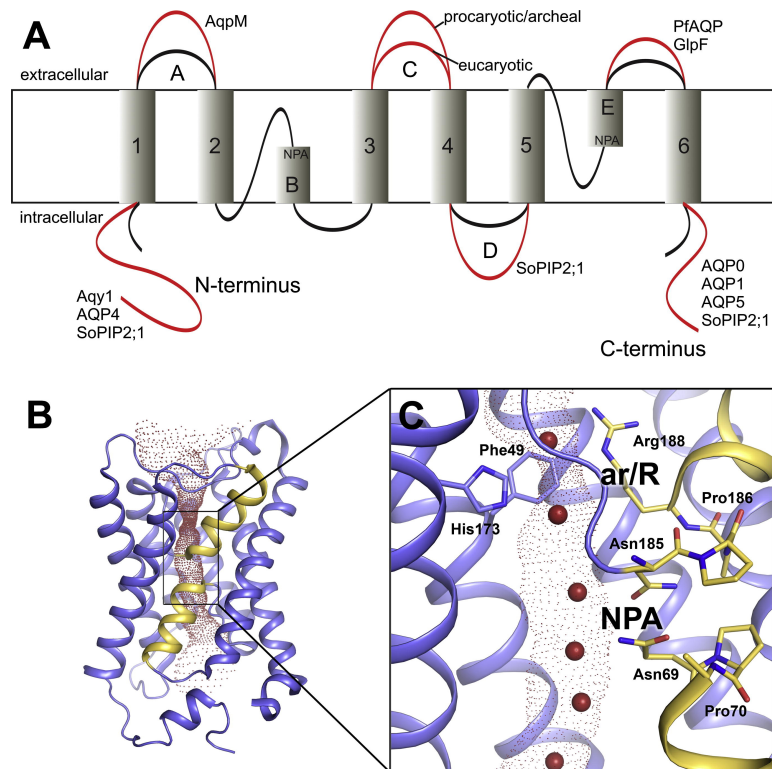
Figure 1. The structure of AQP water channels. (A) The topologic map of the elemental constructive fold of AQP highlighting the unusually long regions.(B) Comprehensive structure of AQPs showing the six trans-membrane helices (blue) and the two half-helices (yellow) which constitute the pseudo seventh transmembrane helix. (C) The conformation of the NPA and ar/R motifs in the AQP channel with the red water molecules. (B) and (C) were obtained from the X-ray structure of human AQP5. (Törnroth-Horsefield et al., 2010)

1.1 The role of the AQP water channels in the female reproductive system

AQPs can be found in the female reproductive system: in the vagina, cervix, uterus, oviduct, ovary, oocyte, and also in the placenta, fetal membrane and in the embryo. (Zhang et al., 2012) Their importance during the gestation is unquestionable (**Table 1**).

AQP1 is primarily expressed in the capillaries and venules of the human vagina and AQP2 in the cytoplasm of the vaginal epithelium. AQP3 can be found mainly in the plasma membrane of vaginal epithelium. In the cytoplasm of the vaginal epithelium AQP5 and AQP6 are located. The location of AQP1, 2, and 3 are very similar in rats to humans. (Park et al., 2008) They are responsible for sexual arousal like genital swelling and increased vaginal lubrication. (Berman et al., 2000)

In the human ovary, AQP1 can be found in ovarian microvascular and small vessel epithelial cells. (Yang et al., 2005) AQP1, 2, 3, and 4 can be found in both theca and granulose cells of human follicles and they show different expression patterns during various stages of ovulation. (Thoroddsen et al., 2011) In the oviduct they enable gamete transport and embryo

development in the early stage. (Leese et al., 2001) During ovarian folliculogenesis, the transcellular water transport is mediated by AQP7, 8, and 9. (McConnell et al., 2002) AQP1, 3, 8, and 9 are expressed in mouse oocytes and they may affect the oocyte maturation. (Edashige et al., 2000)

In the human uterus and cervix, AQP1 is expressed in the endothelium of small blood vessels. AQP2 is predominantly present in luminal and glandular epithelial cells of the endometrium. (Feng et al., 2008) AQP3 is localized in basolateral membranes of human uterine endometrium, and AQP9 is present in the cytoplasm of epithelial cells of the human oviduct. (Ji et al., 2013; Mobasheri et al., 2005) AQP1, 3, and 8 are constitutively expressed in non-pregnant mice. (Jablonski et al., 2003)

Richard et al. studied the expression of AQP0 to 9 in mice uterus on days 1 to 8 of gestation. They found that, the expression of AQP5 and 9 increased in the glandular epithelium of the uterus at the time of implantation. (Richard et al., 2003) Overall, AQP1, 2, 3, 4, 5, 7, 8, and 9 have been detected in the rat uterus. They take part in endometrial development, cell migration, embryo implantation and maybe in myometrial contractions. AQP5 is mainly involved in embryo implantation and it is a major regulator during pregnancy. (Ducza et al., 2014; Lindsay and Murphy, 2007; Zou et al., 2011) The cervix undergoes significant changes through pregnancy and labor. Anderson et al. revealed that AQP3, 5 and 8 may responsible for the complicated regulations of this tissue. (Anderson et al., 2006)

The amniotic fluid regulation occurs through the placenta and the fetal membrane; the normal volume of amniotic fluid is crucial for maintaining the fetal development. AQP3, 4, 8, and 9 are located in human syncytiotrophoblasts and AQP1 can be found in placental vessels. AQP1, 3, 8, and 9 are located in the human amnion and chorion. (Zhang et al., 2012)

	AQP1	AQP2	AQP3	AQP4	AQP5	AQP6	AQP7	AQP8	AQP9	AQP10	AQP11	AQP12
Blastocyts (implantation)	h-R	h-R	h-R	h-R	h-R	h-R	h-R	m-P	h-R m-P		h-R	h-R
Uterus (implantation)	r-mm-P m-mm-P+R p-P	h-em-P+R	h-em-R	m-mm-P+R c-em-R	m-mm-P+R r-mm-P p-P			m-mm-P	r-P+R p-P			
Amnionic membrane	h-P+R o-P+R m-fc-P		h- P+R o-P+R m-ec,fc-P					h-P+R o-P+R m-ec-P	h-P+R o-P+R m-aec-P		h-P+R o-P+R	
Placenta	h-pv-R r-bz,lz-P m-vec-P+R o-vec-P+R		h-pv-R r-R m-tec-P+R o-tec-P+R	h-pv-R h-st,ec-P	h-pv-R			h-pv-R r-bz, lz-P m-P+R o-tec-R	h-pv-R r-cp, bz, lz-P m-R		h-pv-R	
Uterus (late pregnant)	r-P+R	r-P+R	r-P+R		r-P+R			r-P+R	r-P+R			
Cervix (late pregnant)			m-bcl-P+R	m-acl P+R	m-acl P+R			m-acl P+R				

Table 1. The expression of AQP isotypes in different species during gestation.

species: h: human; r: rat; m: mouse; c: cattle; o: ovine; p: pig; P: protein, R: mRNA, fc: fibroblast cells; ec. epithelial cells; aec: apoptotic epithelial cells; vec: vascular epithelial cells; tec: trophoblast epithelial cells; st: syncytiotrophoblast cells; pv: placenta villi; cp: complete placenta; mm: myometrium; em: endometrium, bcl: basal cell layers of cervical epithelium, acl: apical cell layers of cervical epithelium, bz: bazal zone, lz: labyrinth zone (Ducza et al., 2017)

1.2   AQP5 in the pregnant rat uterus

According to the studies of Lindsay et al. it is clear that some AQP isoforms are expressed in rat and mouse uterus and the distribution and expression of these channels are regulated during the peri-implantation period. (Lindsay and Murphy, 2007, 2006, 2004) There was a redistribution of AQP5 from the cytoplasm to the apical plasma membrane at the time of the blastocyst implantation. It suggests that AQP5 may allow locating the blastocyst and has a crucial role in the reduction of uterine luminal fluid during the implantation. (Lindsay and Murphy, 2004) They found elevated AQP5 protein expression in the apical plasma membrane of uterine epithelial cells in the same conditions as implantation. (Lindsay and Murphy, 2006) In our earlier study we investigated the expression of AQP isoforms in the pregnant rat uterus. The mRNAs of AQP1, 2, 3, 5, 8, and 9 isoforms were detectable in the myometrium of non-pregnant and late pregnant (day 18, 20, 21, 22) rats. Among the six water channel subtypes, AQP5 mRNA and protein levels showed the most remarkable changes during pregnancy. AQP5 mRNA was the highest on days 18-21 of gestation and dramatically dropped on the last day (day 22) of pregnancy. The amount of AQP5 mRNA peaked on day 18, while the AQP5 protein level was the highest on day 20 of gestation, but also decreased on the last day (**Fig. 2**). In an *in vitro* study we examined the effect of oxytocin on the expression of AQP5 on day 18 of pregnancy, when the mRNA level is the highest. We found that AQP5 mRNA level significantly decreased within 5 minutes after oxytocin administration. This effect occurred via the oxytocin receptor, because the oxytocin receptor antagonist atosiban inhibited the effects of oxytocin on the expression of AQP5 mRNA. According these results, we suppose that AQP5 is regulated by oxytocin at the end of gestation and may play important role in the initiation of delivery. (Ducza et al., 2014)

There is another study which also revealed that AQP5 was dramatically down regulated during parturition. They used microarray analysis to detect the changes of different gene expressions in the rat myometrium during late pregnancy and during labor. Helguera et al. reported that both AQP5 and AQP8 are upregulated until the first 20 days of gestation. On the other hand the protein expression of both water channels showed significant decrease from the day 20 to the last day of gestation. (Helguera et al., 2009)
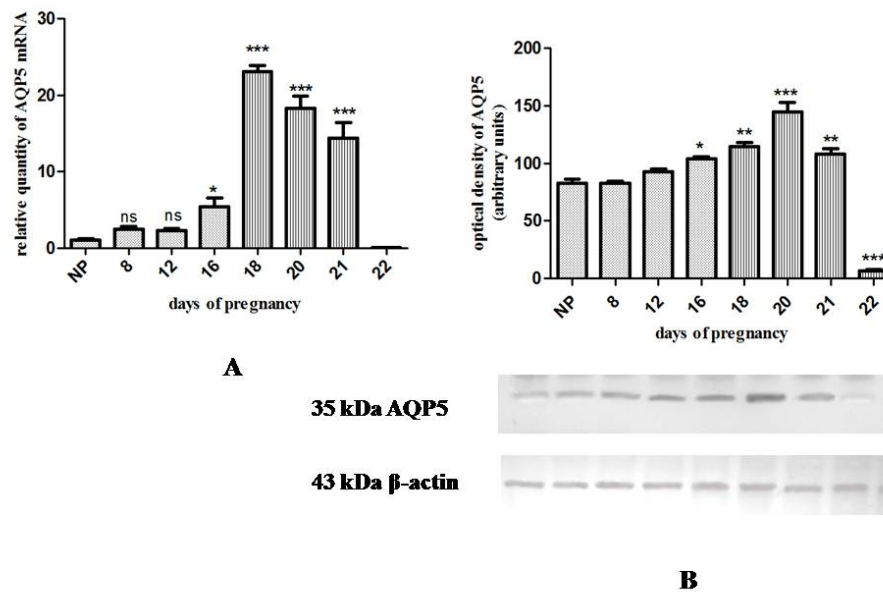
Figure 2. Changes in mRNA (A) and protein (B) expression of AQP5 on different days of pregnancy in rat uterus and in non-pregnant rat uterus (NP). Amount of AQP5 mRNA **(A)** and protein **(B)** were significantly reduced on the last day of gestation. ns > 0.05, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the previous day. Each bar denotes the mean ± S. D. (Ducza et al., 2014)

1.3 The effects of sexual hormones on the AQP5 expression

There are evidences that the expression of water channels is influenced by sexual hormones. This is supported by the fact that the expression of AQPs changes according to the menstrual cycle. Aralla et al. investigated the water channel regulation in the uterus of cycling bitches, and they found that AQP5 was particularly expressed in response to high levels of progesterone. (Aralla et al., 2009) Another study revealed that AQP2 expression correlates with serum 17β-estradiol and progesterone levels in human endometrium. (He et al., 2006) The explanation of these phenomena is that there is an estrogen response element in the promoter region of the AQP5 and 2 genes, which provides the immediate regulation of these water channels by estrogen. (Kobayashi et al., 2006; Zou et al., 2011) An *in vitro* study investigated the effects of progesterone and estradiol treatments on the AQP5 expression in the porcine uterus. They observed the down-regulation of AQP5 gene expression after the progesterone and estrogen treatments during the mid-luteal phase, while in the time of luteolysis, it was increased by estrogen. (Skowronska et al., 2015) Ovariectomized rats were treated with testosterone, estrogen, or combination of them. It was determined that testosterone enhanced the expression of AQP5 in the uterus and this effect was abated by a consecutive estrogen treatment. (Salleh et al., 2015) It was also observed that AQP5 protein

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expression increased due to progesterone treatment alone or in combination with estrogen in ovariectomized rat uterus. (Lindsay and Murphy, 2006)

Richard et al. confirmed that AQP1, 4, and 5 are extremely expressed in the peri-implantation period in mouse uterus, and AQP5 expression depends on the estrogen stimulation of the progesterone-primed uterus. (Richard et al., 2003) According to a study, the location of the AQP5 channel changed from the cytoplasm to the apical plasma membrane at the time of implantation in the rat uterus and it may enable the proper positioning of the blastocyst. (Lindsay and Murphy, 2004)

1.4 Preterm birth, antibiotic treatment and AQP

Preterm labor has multiple pathophysiogical background, like infection, allergic phenomena, abnormal allograft reaction, ischemia, cervical disease, endocrine disorder or uterine over-distension. (Romero et al., 2006) It is well known that preterm birth increases prenatal morbidity and mortality, which often leads to long-term neurological impairments gastrointestinal and respiratory complications in children. (Owen et al., 1993) Genital tract infection is a common factor which raises the frequency of preterm delivery. (Joergensen et al., 2014) Antibiotics are widely used to diminish the hazard of infection caused preterm labor, principally in case of the premature rupture of the membranes (PROM). (Kenyon et al., 2013) There is a meta-analysis about the effect of prophylactic antibiotic treatment after PROM which indicates that, for example amoxicillin could alleviate the prevalence of sepsis and intraventricular hemorrhage among newborns. (Egarter et al., 1996) Another meta-analysis described the importance of antibiotics for asymptomatic bacteriuria in gestation, which has been associated with low birth weight and preterm delivery. Antibiotic treatment was effective in decreasing the frequency of acute pyelonephritis in the mother. Beside this it reduced the incidence of low birth weight babies and preterm birth. It means that these children more likely avoid the neonatal complications. (Smaill and Vazquez, 2015) As a study revealed, urinary tract infection is successfully treated with a single-dose fosfomycin during pregnancy and it was well tolerated. (Krcmery et al., 2001) Beside fosfomycin, amoxicillin is also frequently used antibiotic drug for treating bacteriuria and bacterial vaginosis through pregnancy. (Duff et al., 1991; Estebanez et al., 2009) These two antibiotics are generally used in the treatment of pregnant women but doxycycline is contraindicated. (Bookstaver et al., 2015) However, it was proven that doxycycline induces AQP5 expression in the lungs of transgenic mice; therefore it is the only one known AQP5-influencing antibiotic. (Tichelaar, 2000)

## 1.5   Transient receptor potential vanilloid 4 and AQP5 channel

Up to the present day, six subfamilies of transient receptor potential (TRP) channels have been identified in mammals. (Rosasco and Gordon, 2017) These six subfamilies are TRPV (vanilloid), TRPC (canonical), TRPM (melastatin), TRPA (ankyrin), TRPML (mucolipin), and TRPP (polycystin). The seventh subfamily of TRP channels is TRPN (*Drosophila* NOMPC), which only found in invertebrates and fish. (Björkgren and Lishko, 2017) They can be activated by diverse stimuli that include voltage; heat; mechanical force; for example osmotic differences, pressure, shear stress and chemicals; such as capsaicin, menthol, Ca^{2+}, calmodulin, pH. (Zheng, 2013)

TRPV4 is activated by different physical (heat, cell swelling, mechanical stimuli) and chemical (endocannabinoids, arachidonic acid) impacts. (Vennekens et al., 2008) These channels are members of the voltage-gated superfamily of ion channels within this they belong to voltage gated K^+, Na^+, and Ca^{2+} channels. The TRP channels have tetramer form and they can appear as homo and also as heterotetramers. All of the subunits contain six membrane-spanning helices with a reentrant pore loop, which is located between the fifth and sixth transmembrane helices. We can found also an intracellular amino- and carboxy-terminal. Four transmembrane portions (**Fig. 3**) construct the voltage-sensing or voltage-sensing-like region. The other two transmembrane part of the channel build up the ion-conducting pore. (Rosasco and Gordon, 2017)
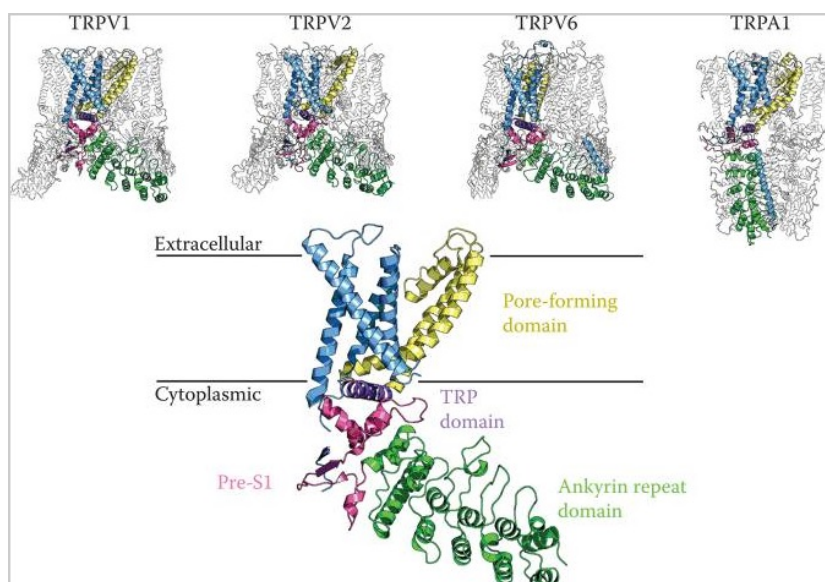
Figure 3. The structure of the TRP channels. The upper line shows the structures of the TRP channels. Different colors indicate the building subunits. On the bottom of the picture we can see the enlarged subunit of TRPV1. Parts of this unit are the voltage sensor-like domain (blue), pore domain (yellow), the pre-S1 domain (pink), and the ankyrin repeat domain (green). (Rosasco and Gordon, 2017)

The distribution in the tissues and the function of TRP channels are very diverse (**Fig. 4**). In the human body they play role in the brain and nervous system, the following channels expressed here: TRPC1; TRPV2, -4, -5, -6; TRPM2, -4, -5, -6, -7; TRPP1, -2, -3; TRPML1, -2 and TRPA1. Some of the TRP channels can be found in the pulmonary, cardiovascular, renal, digestive, integumentary system and in the liver. These channels take place also in the reproductive system, TRPC1, -3, -6; TRPV2, -4, -5, -6; TRPM2, -4, -5, -6, -7; TRPP1, -2, -3; TRPML1, -2; TRPA1. (Rosasco and Gordon, 2017)
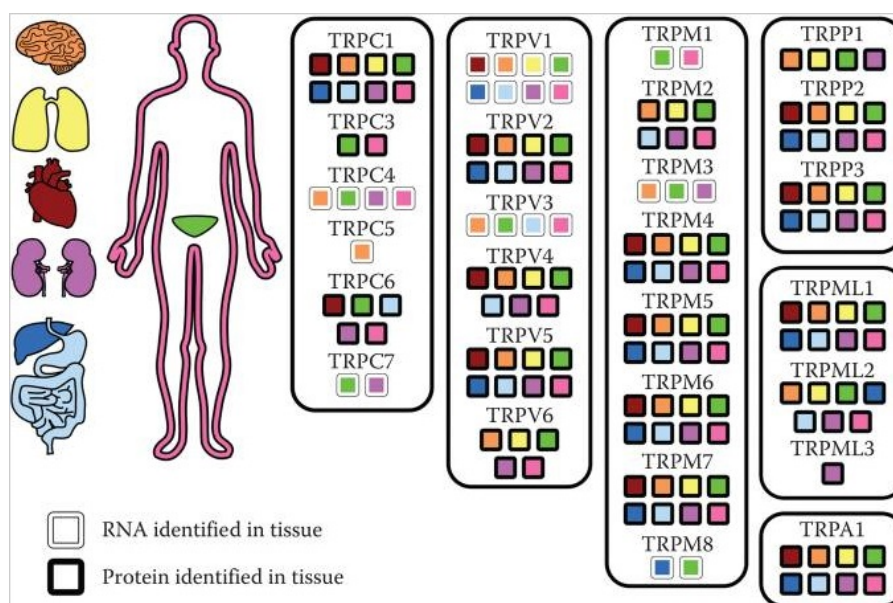
Figure 4. The distribution of TRP channels in the human body. Different TRP channels are listed on the right side of the picture. The colored squares mean where these channels can be found. Square with a white border indicate the presence of RNA expression, and the square with a black border demonstrate the existence of protein expression of the mentioned TRP channel. The meaning of the colors: orange: brain/nervous system; yellow: pulmonary system; red: cardiovascular system; purple: renal system; dark blue: liver; light blue: digestive system; pink: integumentary system; and green: reproductive system. (Rosasco and Gordon, 2017)

As seen on the figure, TRPV channels are expressed in the male and female reproductive tissues and play crucial regulatory task in various physiological actions. They are responsible for oocyte maturation and activation and for the fertilization. (Björkgren and Lishko, 2017) Expression of TRPV4 mRNA was observed in rat prostatic tissue. (Guibert et al., 2011) TRPV1 is present in human placenta and the deregulation in TRPV1 expression was found in preeclampsia. (Martínez et al., 2016)

It is supported by numerous evidences that AQP5 water channel is an interacting partner of TRPV4 channel. A study indicates that hypotonic reduction of AQP5 expression requires

TRPV4. (Liu et al., 2006) Based on this article, the cooperation of TRPV4 and AQP5 is essential for the mechanism of regulatory volume decrease (RVD) in salivary gland cells. It is proved that AQP5 necessary for salivary fluid secretion and this channel regulates the volume of acinar cells. So the functional connection was recognized between AQP5 and TRPV4 in these cells. (Aure et al., 2010)

TRPV4 is the osmosensor by hypotonicity-activated Ca^{2+} entry pathway. In this process AQP5 regulates the water permeability and the cell volume.

A study revealed the decreased expression of AQP5 depends on the TRPV4 channel in hypo-osmotic conditions. Plenty of AQP5 is coordinated by extracellular osmolalities and it can be regulated in hypotonic circumstances by TRPV4 activation. (Sidhaye et al., 2006)

The activation of TRPV4 channel causes nitric oxide and endothelium-derived hyperpolarizing factor (EDHF)-mediated endothelium-dependent relaxation in the rat pulmonary artery. (Sukumaran et al., 2013) TRPV4 channel mediated EDHF-type activation implicates the gestation-induced remodeling and vasodilatation in uterine radial artery of pregnant rats. (Senadheera et al., 2013) In the myometrial smooth muscle cells, the external Ca^{2+} entry is essential for the uterine contraction. Among other voltage-gated calcium channels, the TRPV4 channel is a potential candidate concerned in Ca^{2+} entry generating myometrial contractions. (Matthew et al., 2004; Ying et al., 2015)

Ying et al. found that TRPV4 gene and protein expression elevated during pregnancy in murine model. The TRPV4-mediated Ca^{2+} entry and contractility were raised in myometrial smooth muscle cells of pregnant mice compared to the non-pregnant. They proved also the direct pharmacological stimulation of TRPV4 escalate uterine contraction. (Ying et al., 2015)

2 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 expression and uterine contraction after antibiotic (amoxicillin, fosfomycin, doxycycline) pretreatment to determine the effect of these prophylactic antibiotics on 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.

3 Materials and methods

3.1 Housing and handling of the animals

The animals were treated in accordance with the European Communities Council Directives (2010/63/EU) and the Hungarian Act for the Protection of Animals in Research (Article 32 of Act XXVIII). 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 experimental animals were purchased from INNOVO Ltd. (Gödöllő, Hungary) and were kept at a controlled temperature (20-23 °C), in relative humidity of 40-60% and under a 12 h light/dark cycle. The experimental animals were fed a standard rodent pellet diet (INNOVO Ltd., Gödöllő, Hungary) and tap water was available *ad libitum.*

3.2 Mating of the animals

The mature Sprague-Dawley rats (females 180-200g and males 240-260g) were mated in a special mating cage, which has a time-controlled mobile metal door. The male and female animals are separated until the gate opened in the early morning hours, because rats are active nocturnally. Four or five hours after the possible sexual intercourse, swabs were taken from the vagina of the female rats. In case of the existence of sperms in the native vaginal smear or the presence of copulation plug the female animals were regarded and separated as first-day pregnant rats.

3.3 Treatments of the rats

3.3.1 *In vivo* female sexual hormone treatments of the rats

The pretreatment of pregnant animals with 17β-estradiol valerate, tamoxifen citrate, and clomiphene citrate (Sigma-Aldrich, Budapest, Hungary) 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 17β-estradiol (Bóta et al., 2015; Hódi et al., 2014); 5 mg/0.1 ml of tamoxifen citrate (Cyr, 2000); and 1 mg/0.1 ml of clomiphene citrate (Bharti et al., 2013; Nutu et al., 2010) once a day for four days. On day 18 and the last day of pregnancy, day 22, the uterine samples were collected and molecular studies were carried out.

The pregnant rats were treated with progesterone, levonorgestrel, and medroxyprogesterone acetate (Sigma-Aldrich, Budapest, Hungary) 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 progesterone and levonorgestrel, and 5 mg/0.1ml of medroxyprogesterone acetate for seven days.(Bóta et al., 2015; Brandon and Raval, 1979; Graham and Milad, 2013; Hódi et al., 2014) On day 18 and 22, the uterine samples were collected and molecular studies were carried out. We chose these durations for the hormonal treatments according to our earlier investigations.

We treated the preterm birth group as reported by Elovitz et al..(Elovitz and Mrinalini, 2004) The pregnant rats received mifepristone (Sigma-Aldrich, Budapest, Hungary), dissolved in olive oil. It was injected subcutaneously in a dosage of 3 mg/0.1 ml on day 19 of pregnancy at 9 A.M.. The intravaginal prostaglandin E2 was given at a dose of 0.5 mg/ml on the same day at 4 P.M.. Next day appear preterm delivery on day 20 of gestation. After the beginning of the preterm birth, uterine samples were collected and molecular studies were carried out.

3.3.2 *In vivo* antibiotic treatments of the rats

The pregnant animals received amoxicillin from the day 16 of gestation. The Ospamox granulates for suspension (Sandoz Ltd. Kundl, Austria) was given orally in a dose of 40 mg/kg of body weight by oral gavage once a day for seven days. (Berney and Francioli, 1990; Moreillon et al., 1986) We prepared the suspension with purified water. On the last day of pregnancy (day 22) uterine samples were collected and molecular studies were carried out.

The fosfomycin treatment of the pregnant rats was started on day 21 of gestation. The solution was prepared with purified water. The animals were treated orally by oral gavage with Monural granulates (Zambon Ltd. Bresso, Italy) in a dose of 40 mg/kg of body weight once. (Lingscheid et al., 2015; Ozok et al., 2012) On the last day of pregnancy (day 22) uterine samples were collected and molecular studies were carried out.

The pregnant rats got the doxycycline treatment from the day 16 of gestation. They received Doxycylin AL (AliudPharma Ltd. Laichingen, Germany) (100 mg hard capsule) in a dose of 30 mg/kg of body weight once a day for seven days, orally by oral gavage. (Mata et al., 2015; Wang et al., 2014) The suspension was prepared by opening the capsules and grinding the powder with methyl cellulose. On the last day of pregnancy (day 22) uterine samples were collected and molecular studies were carried out.

The durations of the therapies were determined based on human therapeutic protocols. (Bader et al., 2017; Coker and Dierfeldt, 2016; Dijkmans et al., 2017)

3.4 RT-PCR Studies

3.4.1 Tissue Isolation

The experimental animals (250-300 g) were sacrificed by CO_2 inhalation and newborn rats were sacrificed by immediate cervical dislocation. Uterine tissues, tissue between two implantation sites, from pregnant animals (n=6 in each experiment) were rapidly removed and placed into RNAlater Solution (Sigma-Aldrich, Budapest, Hungary). The tissues were frozen in liquid nitrogen and stored at -75°C until the extraction of total RNA.

3.4.2 Total RNA Preparation

Total cellular RNA was isolated by extraction with guanidinium thiocyanate-acid-phenol-chloroform according to the procedure of Chomzynski and Sacchi (Chomzynski, 1987). After precipitation with isopropanol, the RNA was washed with 75% ethanol and then resuspended in diethyl pyrocarbonate-treated water. RNA purity was controlled at an optical density of 260/280 nm with BioSpec-nano (Shimadzu, Kyoto, Japan); all samples exhibited an absorbance ratio in the range of 1.6-2.0. RNA quality and integrity were assessed by agarose gel electrophoresis.

3.4.3 Real-Time Quantitative Reverse-Transcriptase PCR

Reverse transcription and amplification of the PCR products were performed by using the TaqMan RNA-to-C_T-Step One Kit (Thermo Fisher Scientific, Budapest, Hungary) and an ABI StepOne Real-Time cycler. Reverse-transcriptase PCR amplifications were performed as follows: 48°C for 15 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The generation of specific PCR products was confirmed by melting curve analysis. **Table 2** contains the assay IDs for the primers used and the reaction parameters. 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).

TaqMan Assays	Assay ID (ThermoFisher Scientific)	Accession Number	Assay Location	Amplicon Length	Annealing Temp. (C°)	Reaction Volume (µl)
AQP5	Rn00562837_m1	NM_012779.1	473	69	60	20
TRPV4	Rn00576745_m1	NM_023970.1	950	59	60	20
β-actin	Rn00667869_m1	NM_031144.3	881	91	60	20

Table 2. **Parameters of the applied primers and PCR reactions**. In our studies the parameters of inventoried TaqMan assays were defined by Life Technologies (Thermo Fisher Scientific, Budapest, Hungary).

3.5 Western Blot Analysis

The uterine tissues from pregnant rats (tissue between two implantation sites) were homogenized using a Micro-Dismembrator (Sartorius AG, Goettingen, Germany) and centrifuged at 5,000×g for 15 min at 4°C in RIPA Lysis Buffer System (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), which contains phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate and protease inhibitor cocktail. Total protein amounts from supernatant were determined by spectrophotometer (BioSpec-nano, Shimadzu, Japan).

Twenty-five micrograms of sample protein per well was subjected to electrophoresis on 4%-12% NuPAGE Bis-Tris Gel in XCell SureLock Mini-Cell Units (Thermo Fisher Scientific, Budapest, Hungary). Proteins were transferred from gels to nitrocellulose membranes using the iBlot Gel Transfer System (Thermo Fisher Scientific, Budapest, Hungary). Ponceau S (Sigma-Aldrich, Budapest, Hungary) was used to check the standard running and transfer conditions. The blots were incubated overnight on a shaker with AQP5 (35 kDa), TRPV4 (98 kDa) and β-actin (43 kDa) polyclonal antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, USA, diluted 1:200, host: rabbit, specificity: mouse, rat and human) in blocking buffer. Antibody binding was detected with the Western Breeze® Chromogenic immunodetection kit (Thermo Fisher Scientific, Budapest, Hungary).

Images were captured with the EDAS290 imaging system (Csertex Ltd., Budapest, Hungary), 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. Optical densities were calculated as arbitrary units after local area background subtraction.

3.6 Immunohistochemistry

The localization of TRPV4 and AQP5 in the uterus was examined by immunohistochemistry. Late pregnant (pregnancy days 18 and 22) uteri were fixed in paraformaldehyde and then embedded in paraffin, sectioned (5-μm-thick tissue sections) deparaffinized, rehydrated and incubated in acidic citrate buffer (pH6) in microwave for antigen recovery, then treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. After washing, sections were placed on normal blocking solution, treated with rabbit polyclonal anti-TRPV4 (Proteintech, UK) and AQP5 (Thermo Fischer Scientific, Hungary) primary antibodies in a dilution of 1:200 for 1 h at room temperature. Incubation was performed with the Histo-Labeling system anti-rabbit secondary antibody conjugated with peroxidase (Histols Reagent, Hungary) and the reaction was visualized using 3,3-diaminobenzidine tetrachloride (Histols

DAB, Histols Reagent, Hungary). Histological counterstaining was performed with haematoxylin. For double immunofluorescence analysis, the Tyramide Signal Amplification Kit (Molecular Probes/Thermo Fischer Scientific, Hungary) was used with fluorescent-labeled tyramide (Alexa Fluor 594-labeled, cat. no. T20925, Invitrogen, 1:100) to detect color red and directly labeled secondary antibody (Alexa Fluor 488 goat anti-rabbit, Invitrogen, 1:200) to detect color green. Micrographs were generated using an Olympus Fluoview-1000 system on an Olympus IX81 microscope stage equipped with an Olympus DP70 digital camera and through an Olympus UPlan FL N, Phase2 objective. 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.

3.7 *In Vitro* Contractility Studies

Uterus tissues were removed from the 18 or 22-day pregnant rats (n=6 in each experiment). 5 mm long muscle rings were sliced from both horns of the uterus (2-2 rings were chosen from the centre of each horn) they were cleaned up of fat, the foeto-placental units were removed and mounted vertically in an organ bath containing 10 ml de Jongh solution (composition: 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM NaH_2PO_4, 6 mM glucose, pH = 7.4). The temperature of the organ bath was maintained at 37 °C, and carbogen (95% O_2 + 5% CO_2) was perfused continuously through the bath. After mounting, the rings were allowed to equilibrate for approximately 60 min before experiments were started, with a buffer change every 15 min. 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 (SG-02; Experimetria Ltd., Budapest, Hungary) and recorded with a SPEL Advanced ISOSYS Data Acquisition System (Experimetria Ltd., Budapest, Hungary). In the following step, spontaneous contractions were recorded for 4 minutes and cumulative oxytocin concentration–response curves (10^{-12}–10^{-8} M) were constructed in each experiment. Following the addition of each concentration of oxytocin, recording was performed for 4 minutes.

For the investigation of the effects of TRPV4 agonist and antagonist compounds, 25 mM KCl solution was used to induce rhythmic contractions in the *in vitro* contractility studies. Without washing out the contractile agent, the effects of a TRPV4 antagonist (RN1734, Sigma-Aldrich, Hungary) and a TRPV4 agonist (RN1747, Sigma-Aldrich, Hungary) were tested on the uterine contractions in the concentration range of $3x10^{-8}$-10^{-5} M in a cumulative mode.

Control uteri were treated with the solvent of the compounds. Recording was performed at each concentration of the examined agents for 5 minutes. The tension of the myometrial rings was measured with a strain gauge transducer (SG-02; MDE Ltd., Budapest, Hungary) and contractions were recorded and later analyzed with the SPEL Advanced ISOSYS Data Acquisition System (MDE Ltd., Budapest, Hungary). 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.

3.8 Statistical Analysis

All experiments were carried out on six animals and the *molecular biology studies* were repeated three times. Statistical analyses were performed using the Prism 5.0 software (Graph Pad Software, Inc., San Diego, CA, USA). ANOVA Dunnett's test or two-tailed unpaired *t* test were used.

The results of the *isolated organ studies* were analyzed with Dunnett's multiple comparison tests.

D'Agostino-Pearson omnibus test was performed to determine the normal distribution of the data. One-way ANOVA followed by Bonferroni's post hoc test was used for statistical *analysis of the immunochemistry*. The value of $p < 0.05$ was considered statistically significant.
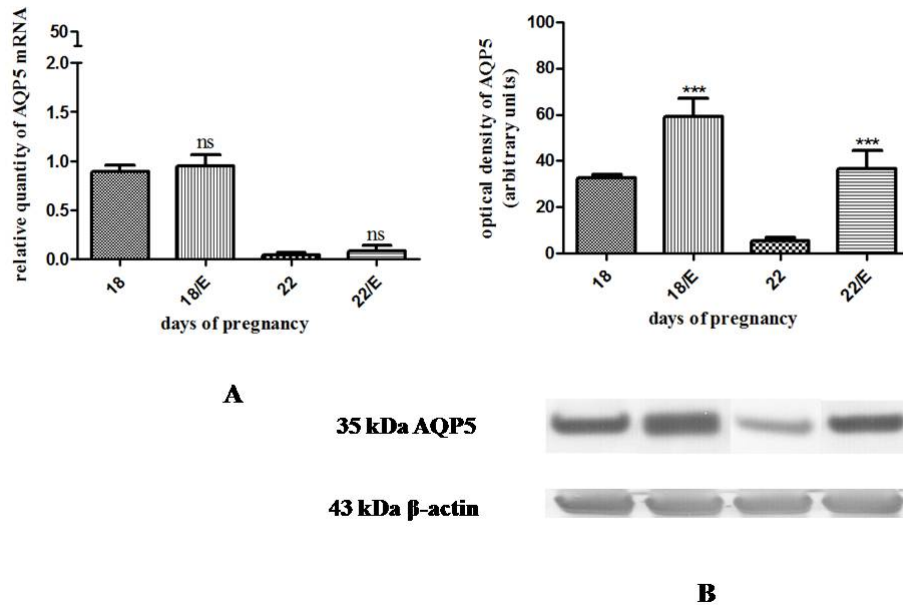
4 Results

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

We found that 17β-estradiol pretreatment did not result any significant changes in the AQP5 mRNA levels, it was inefficient both on day 18 and day 22 of gestation compared to the non-treated control uterus **(Fig. 5A)**. The protein expression of AQP5 showed a significantly increase both on day 18 and day 22 of pregnancy, compared to the control **(Fig. 5B)**.

In case of tamoxifen citrate pretreatment we did not observe any changes on the AQP5 mRNA expression **(Fig. 6A)**. 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. 6B)**.

Either the AQP5 mRNA **(Fig 7A)** or the protein **(Fig 7B)** levels did not alter significantly as a result of clomiphene citrate pretreatment on the investigated days of pregnancy.
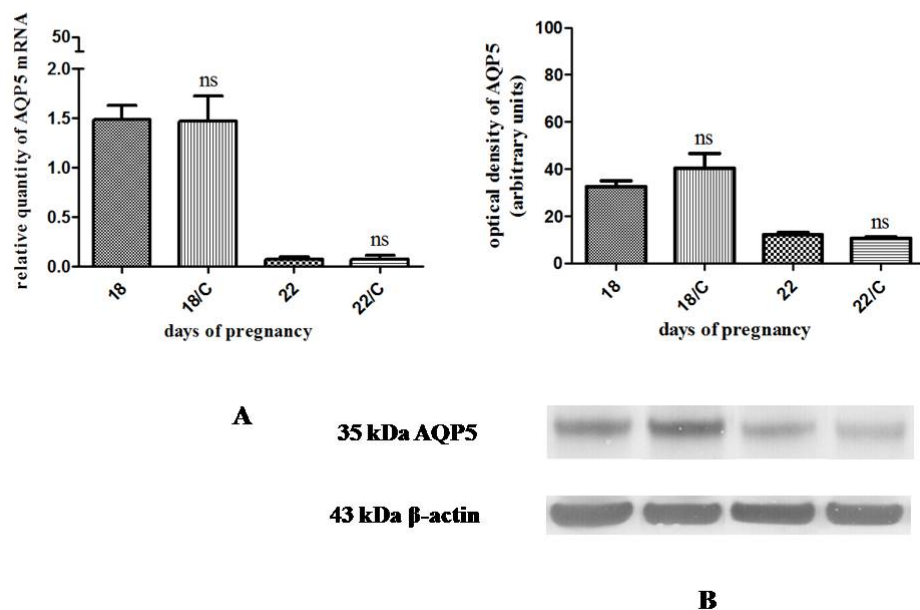

Figure 5. Results of RT-PCR and Western immunoblotting analysis after 17β-estradiol treatment. The changes of mRNA and protein expression of AQP5 after 17β-estradiol (E) pretreatment in pregnant rat uterus on days 18 and 22. ns > 0.05, *** $p < 0.001$ as compared to the data of non-treated control. Each bar denotes the mean ± S. D.
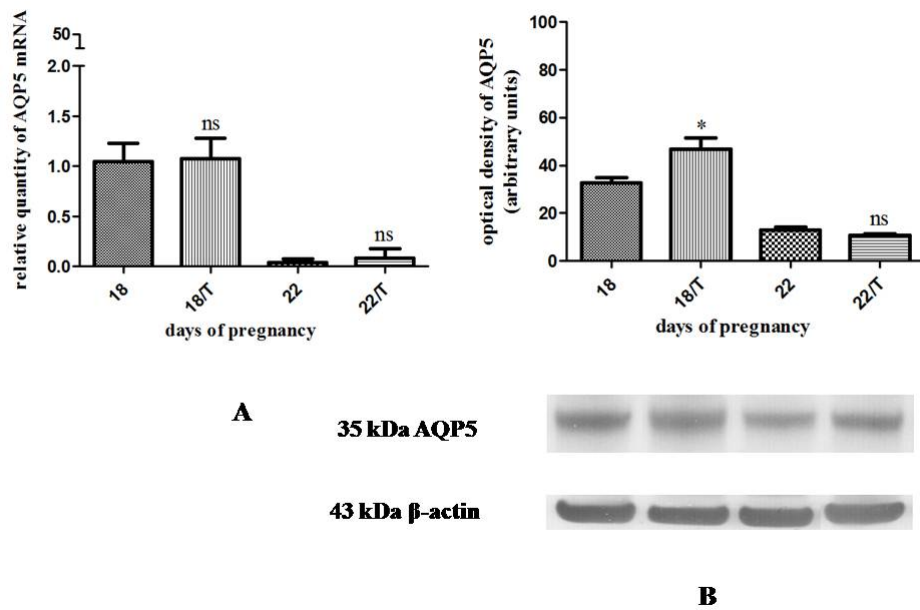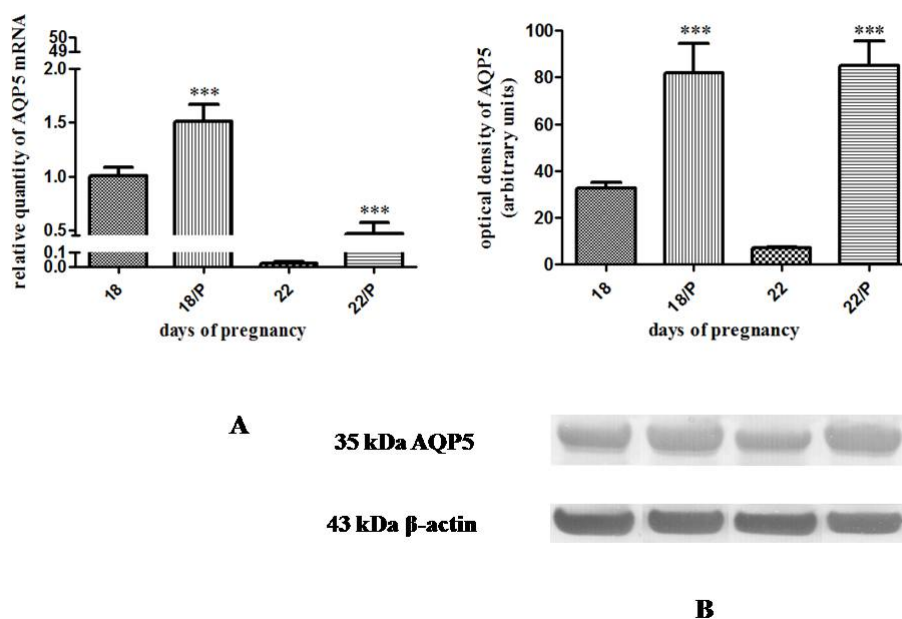
Figure 6. Results of RT-PCR and Western immunoblotting analysis after tamoxifen citrate treatment. The changes of mRNA and protein expression of AQP5 after tamoxifen citrate (T) pretreatment in pregnant rat uterus on days 18 and 22. ns > 0.05, $*p < 0.05$ as compared to the data of non-treated control. Each bar denotes the mean ± S. D.
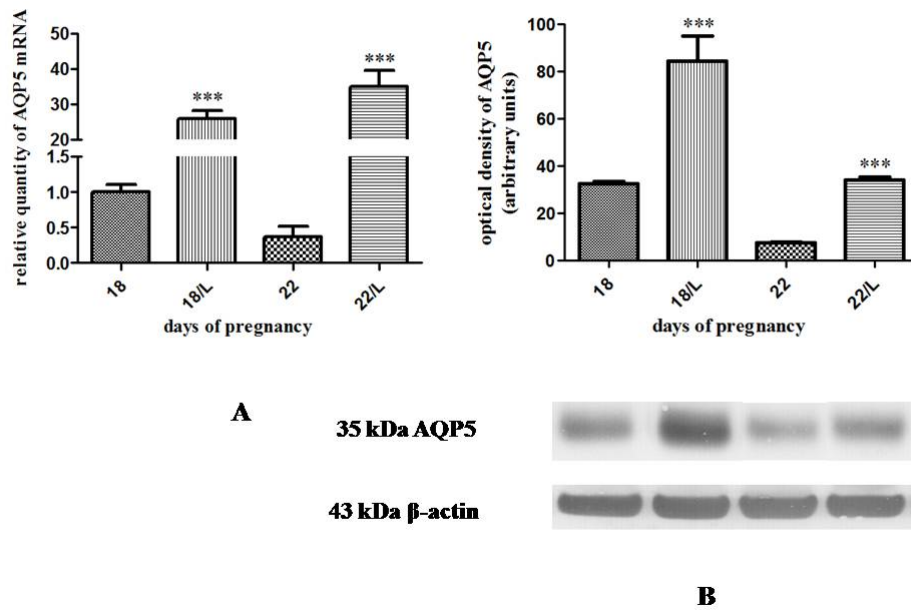

Figure 7. Results of RT-PCR and Western immunoblotting analysis after clomiphene citrate treatment. The changes of mRNA and protein expression of AQP5 after clomiphene citrate (C) pretreatment in pregnant rat uterus on days 18 and 22. ns > 0.05 as compared to the data of non-treated control. Each bar denotes the mean ± S. D.

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

All of the gestagen-related compounds: progesterone, levonorgestrel and medroxyprogesterone acetate pretreatment induced a significant raise both in AQP5 mRNA and protein levels on day 18 and day 22 of gestation **(Fig. 8-10)**.

Figure 8. Results of RT-PCR and Western immunoblotting analysis after progesterone treatment. The changes of mRNA and protein expression of AQP5 after progesterone (P) pretreatment in pregnant rat uterus on days 18 and 22. *** $p < 0.001$ as compared to the data of non-treated control. Each bar denotes the mean ± S. D.

Figure 9. Results of RT-PCR and Western immunoblotting analysis after levonorgestrel treatment. The changes of mRNA and protein expression of AQP5 after levonorgestrel (L) pretreatment in pregnant rat uterus on days 18 and 22. *** $p < 0.001$ as compared to the data of non-treated control. Each bar denotes the mean ± S. D.
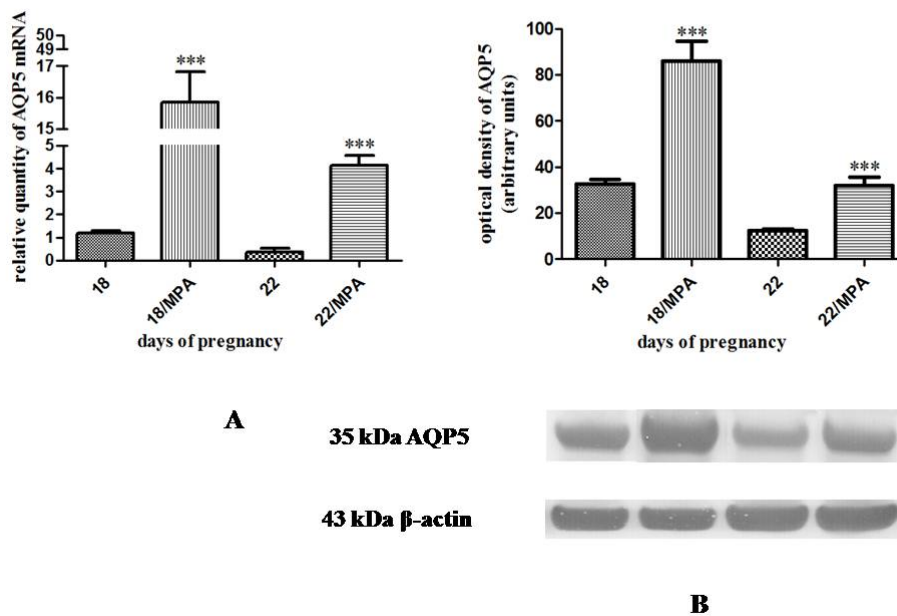
Figure 10. Results of RT-PCR and Western immunoblotting analysis after medroxyprogesterone acetate treatment. The changes of mRNA and protein expression of AQP5 after medroxyprogesterone acetate (MPA) pretreatment in pregnant rat uterus on days 18 and 22. *** $p < 0.001$ as compared to the data of non-treated control. Each bar denotes the mean ± S. D.

4.3 The effect of hormonally-induced preterm delivery on AQP5 expression in pregnant rat uterus
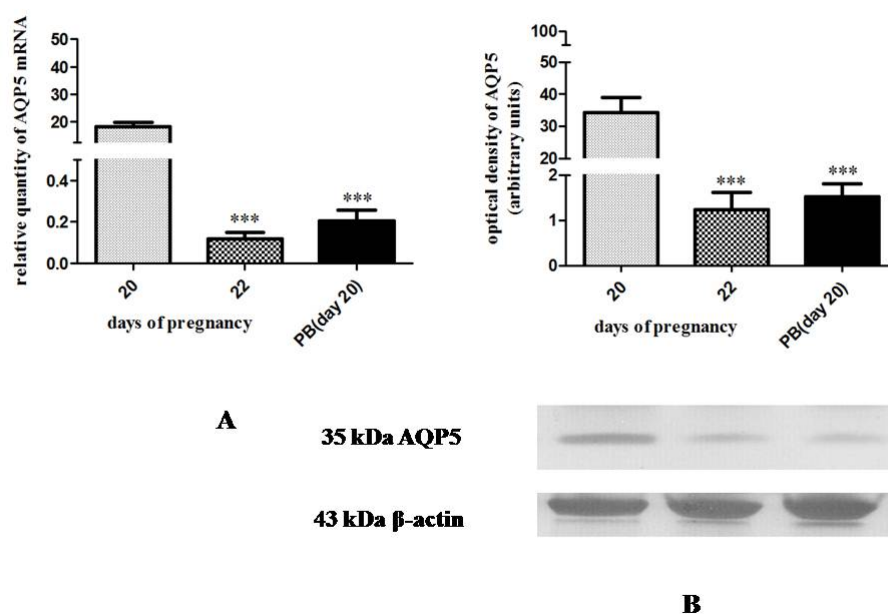
We found a significant reduction of AQP5 mRNA and protein levels on day 20 of pregnancy, compared to the non-treated rats on pregnancy day 20. Interestingly this drop was similar to the physiological last day of gestation **(Fig. 11)**
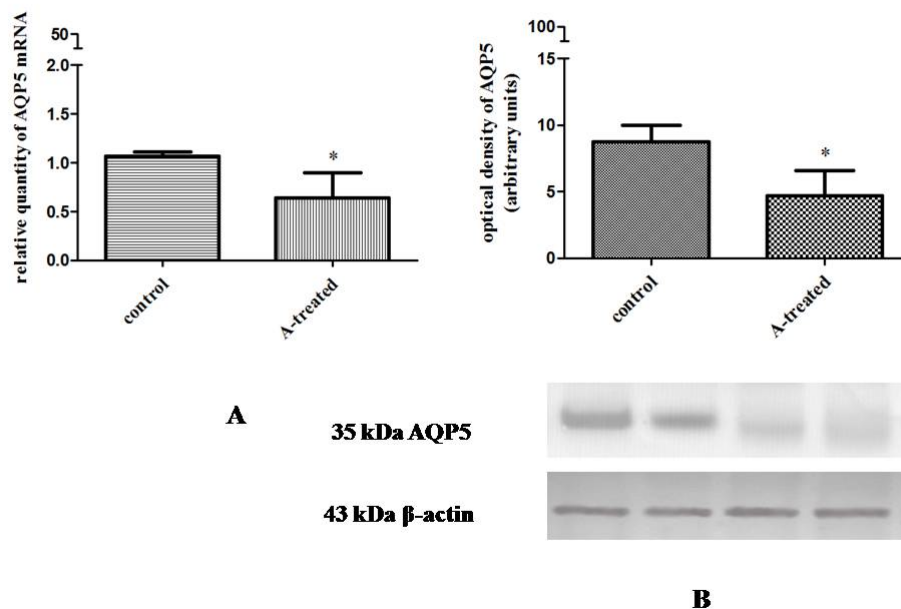

Figure 11. Results of RT-PCR and Western immunoblotting analysis after the hormonally-induced preterm delivery. The changes of mRNA and protein expression of AQP5 after hormonally-induced preterm birth (PB) in pregnant rat uterus. *** $p < 0.001$ as compared to the data of non-treated pregnancy day 20. Each bar denotes the mean ± S. D.

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

The AQP5 mRNA **(Fig. 12A)** and protein **(Fig. 12B)** levels showed a significant drop on day 22 of pregnancy after the 7 days amoxicillin pretreatment, compared to the non-treated rat uterus on the last day of gestation.

A single dose of fosfomycin significantly increased the AQP5 mRNA levels on the last day of pregnancy, compared to non-treated animals **(Fig. 13A)**. In opposition to the mRNA level, the protein level of AQP5 showed reduction on day 22 of gestation **(Fig. 13B)**.

In case of 7 days doxycycline pretreatment we did not observe any changes either in the AQP5 mRNA or the protein levels on the last day of pregnancy **(Fig. 14)**.

Figure 12. Results of RT-PCR and Western immunoblotting analysis after amoxicillin treatment. Changes in mRNA (**A**) and protein (**B**) expression of AQP5 after 7 days of amoxicillin (A) pretreatment in rat uterus on the last day of pregnancy. $*$ $p < 0.05$ as compared to the non-treated uterus. Each bar denotes the mean $\pm$ S.D.
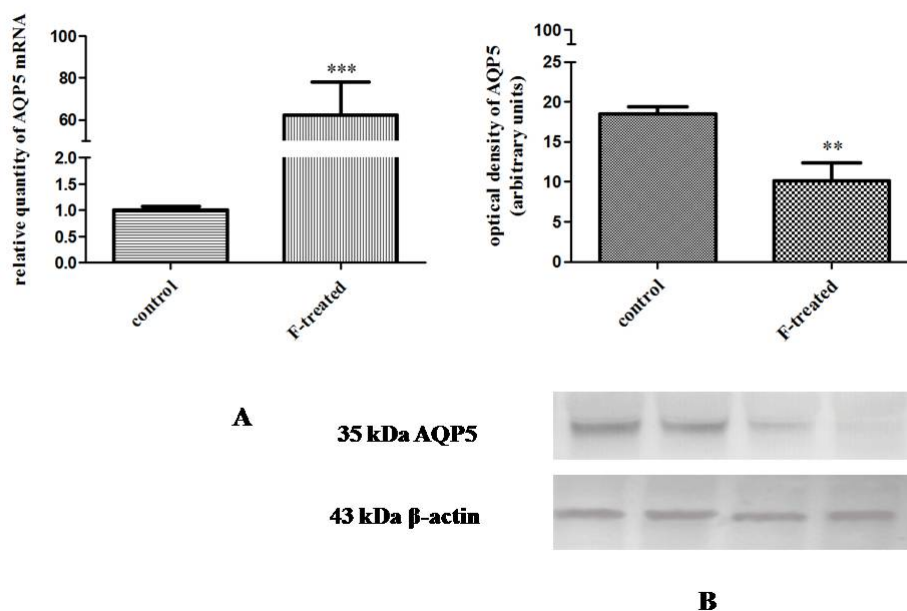
Figure 13. Results of RT-PCR and Western immunoblotting analysis after fosfomycin treatment. Changes in mRNA (**A**) and protein (**B**) expression of AQP5 after fosfomycin (F) pretreatment in rat uterus on the last day

of pregnancy. ** $p < 0.01$, *** $p < 0.001$ as compared to the non-treated uterus. Each bar denotes the mean ± S.D.
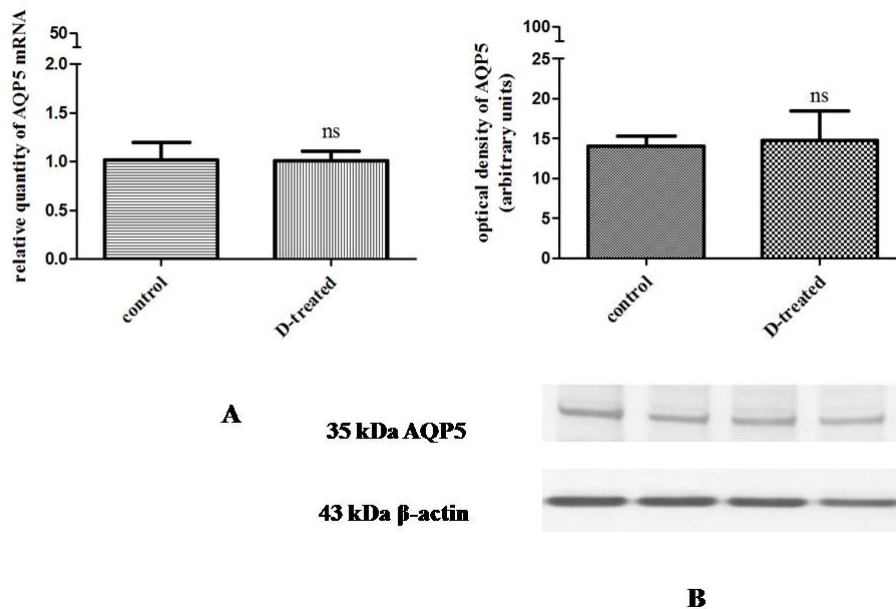

Figure 14. Results of RT-PCR and Western immunoblotting analysis after doxycycline treatment. Changes in mRNA (**A**) and protein (**B**) expression of AQP5 after doxycycline (D) pretreatment in rat uterus on the last day of pregnancy. ns $p > 0.05$ as compared to non-treated uterus. ns: non-significant. Each bar denotes the mean ± S.D.

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

As seen on **Figure 15**, oxytocin (10^{-12}-10^{-8} M) enhanced the uterus contractions on day 22 of pregnancy. Amoxicillin or fosfomycin pretreatments significantly increased the uterine contracting effects of oxytocin. In contrast, doxycycline pretreatment did not influence the oxytocin-induced contractions **(Fig. 15)**.
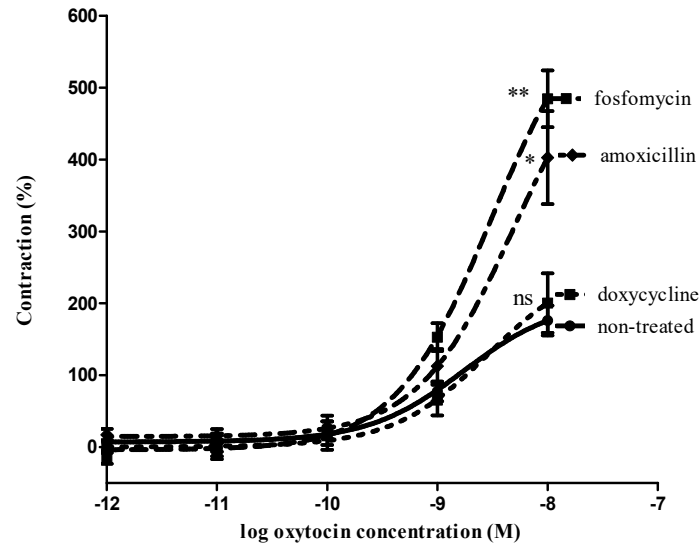
Figure 15. Effects of fosfomycin, amoxicillin and doxycycline pretreatment on the oxytocin-induced contractions in the 22-day pregnant rat uteri. The change in contraction was calculated by area under the curve alteration and expressed in % ± SD as compared to the area under the curve of control contractions. ns: non-significant; ns $p > 0.05$; * $p < 0.05$; ** $p < 0.01$.
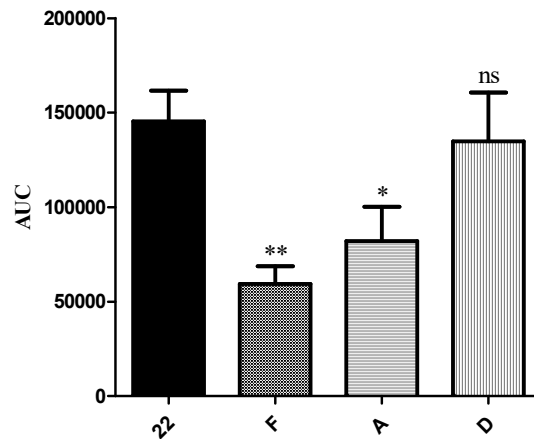
As for the spontaneous uterus contractions, it was decreased significantly by fosfomycin and amoxicillin pretreatment on the 22-day pregnant uterus. In opposition to doxycycline treated uterus, there was no change in the spontaneous contractility **(Fig.16)**.

Figure 16. Spontaneous uterine contractility in the 22-day pregnant rats after fosfomycin, amoxicillin and doxycycline pretreatments. The changes of the area under the curve of spontaneous uterus contractions after the pretreatment with antibiotics compared to the non-treated uterus. Each value denotes the mean ± S.E.M.. ns: non-significant; 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

4.6 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. 17**). 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 (**Fig. 17A and B**). From day 18 to 22 of pregnancy, the AQP5 (**Fig. 2**) and TRPV4 mRNA expression (**Fig. 17A**) showed a strong correlation ($r^2=0.9577$) (**Fig. 17C**). Between AQP5 and TRPV4 protein expression, we determined a moderate correlation ($r^2=0.6452$) (**Fig. 17D**).
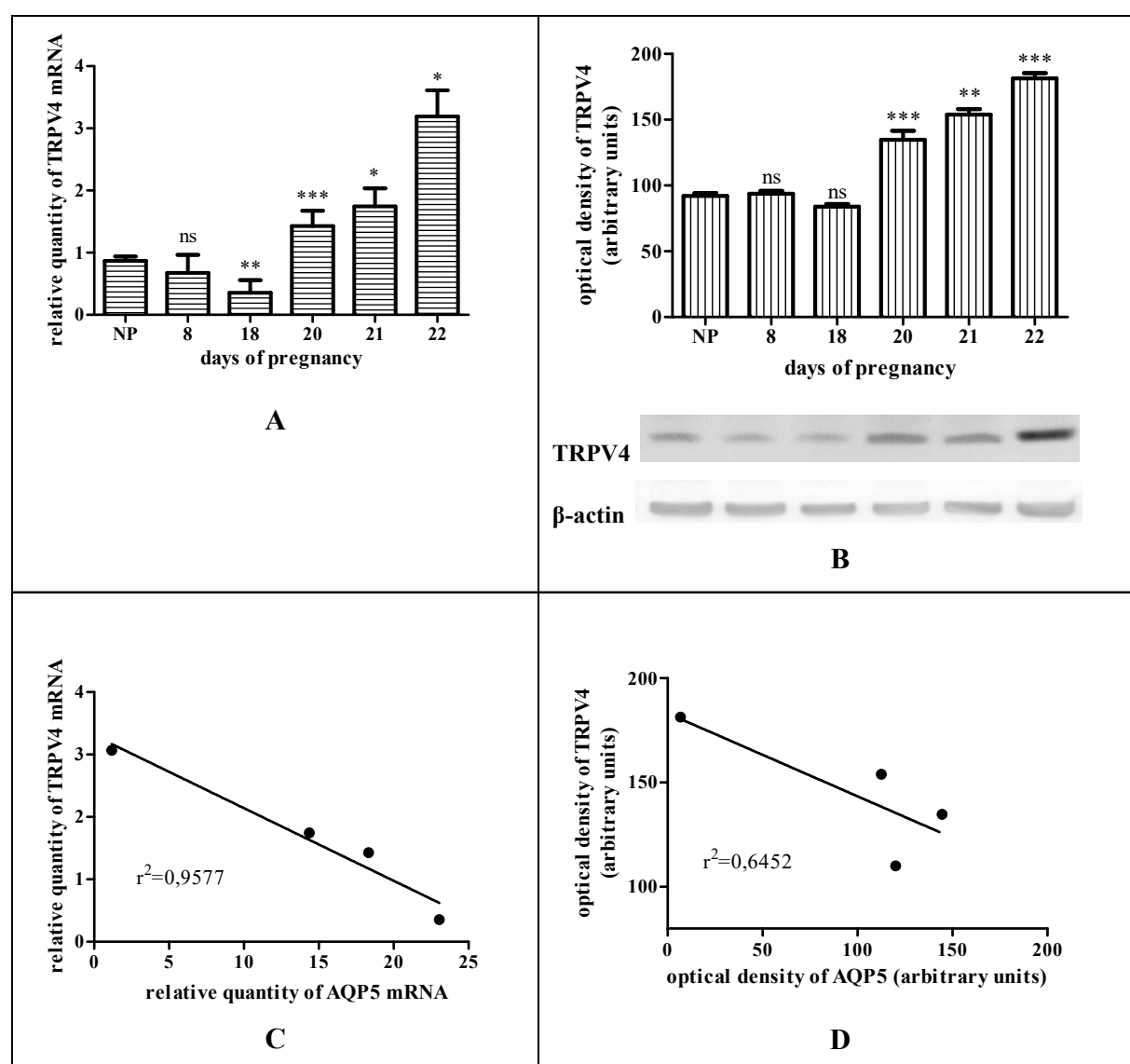
Figure 17. The changes in mRNA (A) and protein expression (B) of TRPV4 in the non-pregnant uterus (estrus phase) and on different gestational days in pregnant rat uterus. Correlation between TRPV4 and AQP5 mRNA (C) and protein (D) expression from pregnancy day 18 to day 22 in uterus. NP: non-pregnant, ns >0.05, * $p<0.05$, ** $p<0.01$, *** $p<0.001$; compared to the previous day.

On day 18 and 22 of gestation, the number of the AQP5 and TRPV4 channels show inverse expression but it was not significant change (**Fig. 18D and E**) in the myometrium, according to the immunohistochemical studies.
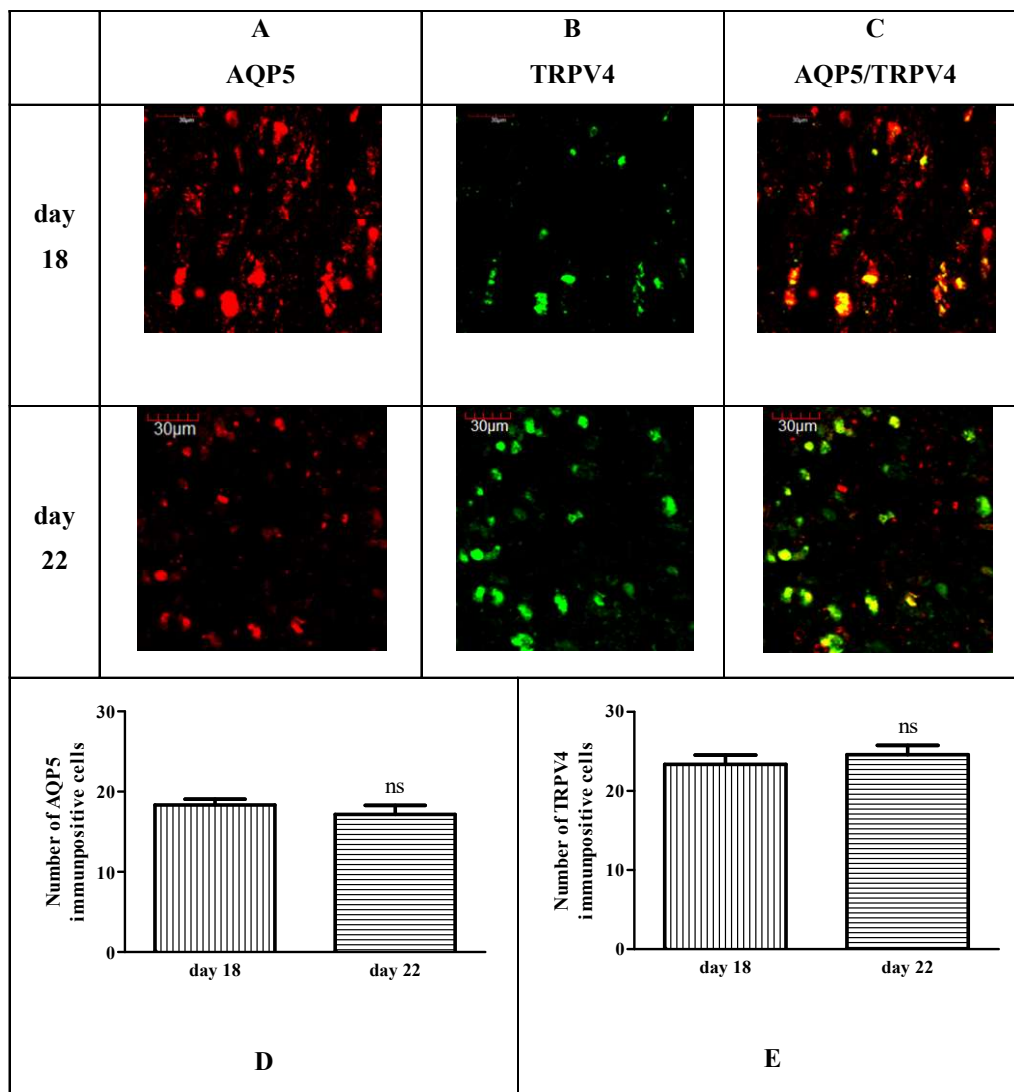
Figure 18. Representative pictures showing expressions of the AQP5 (A) and TRPV4 (B) ; these co-expressions (C) and the number of AQP5 (D)/ TRPV4 (E) immunopositive cells in the myometrium on days 18 and 22 of pregnancy. ns >0.05, compared to day 18 of pregnancy.

On the last day of gestation, the numbers of AQP5 immunpositive cells were significantly decreased (**Fig. 19D**). On the other hand, the numbers of TRPV4 immunpositive cells were significantly elevated on day 22 (**Fig. 19E**).

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. 18C and 19C**).
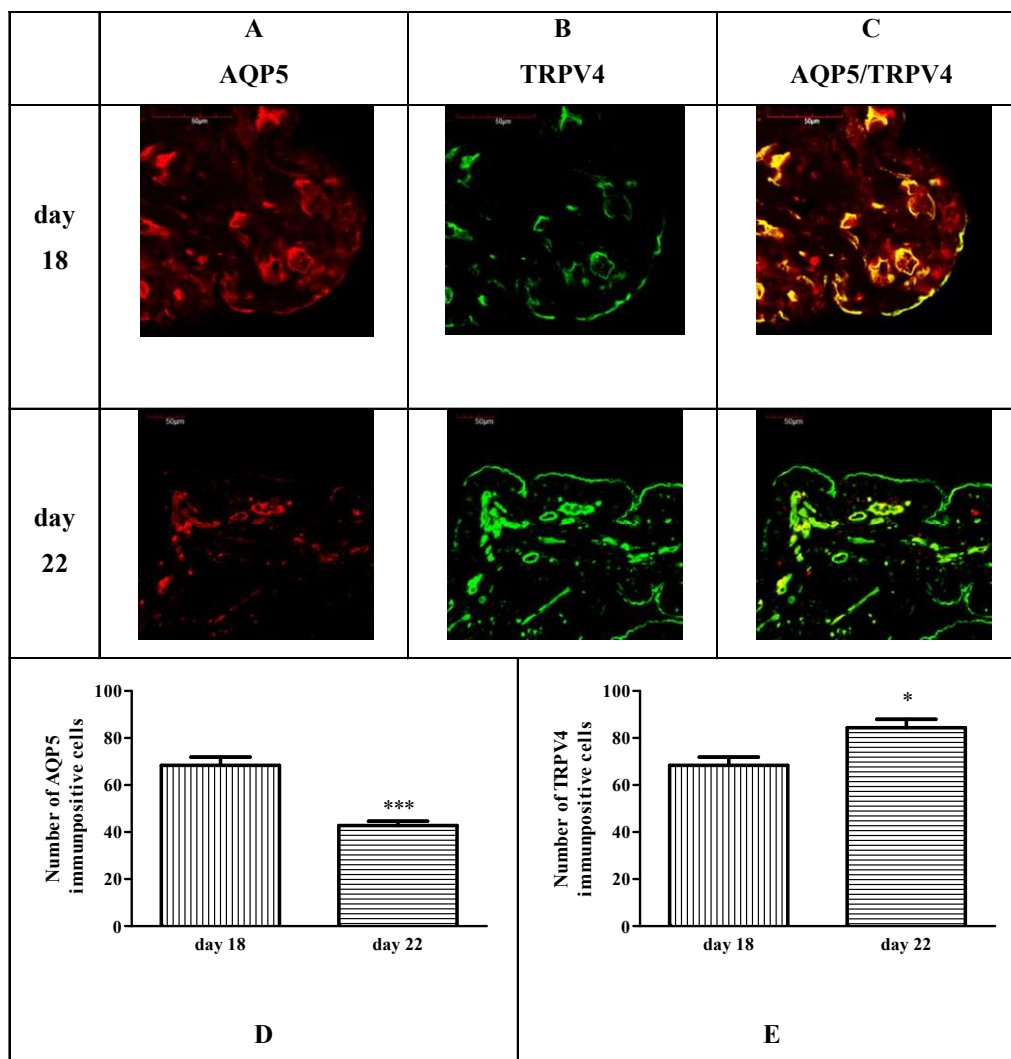
Figure 19. Representative pictures showing expressions of the AQP5 (A) and TRPV4 (B); these co-expressions (C) and the number of AQP5 (D)/ TRPV4 (E) immunopositive cells in the endometrium on days 18 and 22 of pregnancy. * $p<0.05$, *** $p<0.001$ compared to day 18 of pregnancy.

It was also investigated, how the TRPV4 agonist and antagonist influence the uterus contraction in an isolated organ bath system (**Fig. 20**). The agonist (RN1747) displayed a minor relaxing effect on day 18 (9.93 %) in 10^{-5} M concentration. The antagonist (RN1734) showed a pronounced relaxing effect (30.33 %) on the same day and in the same concentration (**Fig. 20A**).

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. 20B**).
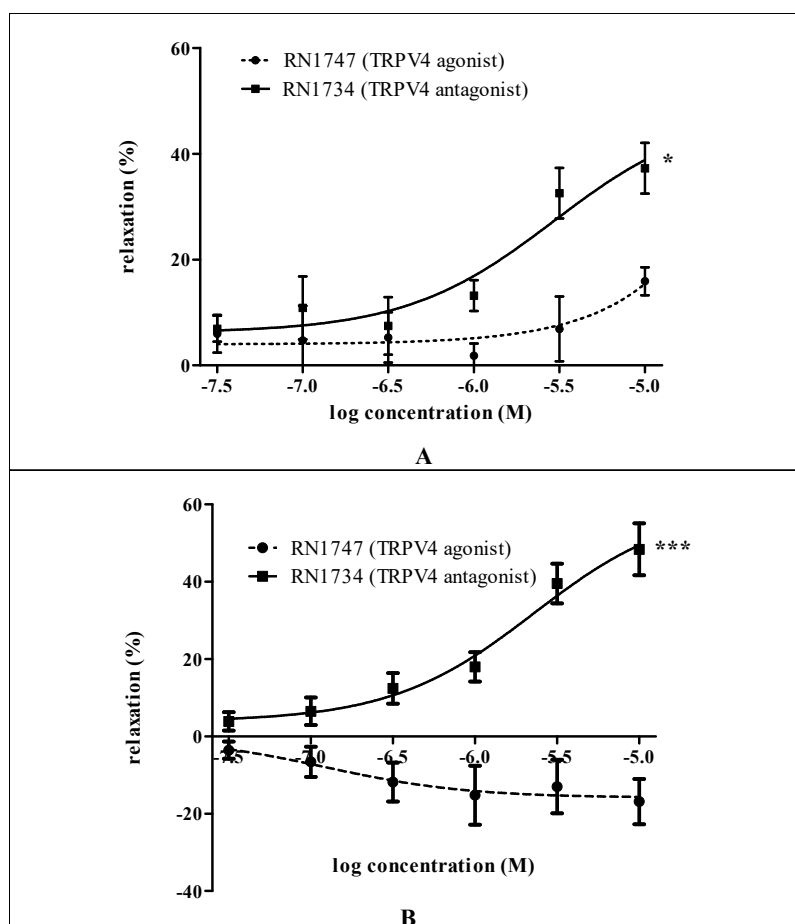
Figure 20. Effect of selective TRPV4 agonist (RN1747) and antagonist (RN1734) on KCl-evoked control contraction of rat uteri on days 18 (A) and 22 (B) of gestation. The statistical analyses were carried out with the two-tailed unpaired t-test. Each value denotes the mean ± S.E.M, *$p<0.05$; ***$p<0.001$.

5   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 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. There is no data about the effect of antibiotics on the uterine contractility and the connection between AQP5 and TRPV4 channel in the pregnant rat uterus.

5.1   AQP5 channel, female sexual hormones and preterm birth

At the time of gestation several hormonal changes occur. In case of human, the two dominant female sexual hormones, estrogen and progesterone show a continually growing level during pregnancy. The serum progesterone level decreased slightly at the end of gestation, although the amount of estrogen in the blood stays elevated. (Boroditsky et al., 1978; Tulchinsky et al., 1972) In pregnant rats, the progesterone level rises constantly at the time of gestation until day 19 of pregnancy, after that it drops dramatically. (Morishige et al., 1973) The serum estradiol level is consistent in rats, but it doubles from day 18 until day 21 of pregnancy. The amount of estradiol drops slightly on the last day of gestation. (Shaikh, 1971)

There are evidences that the AQP water channels can be influenced by sexual hormones. For example, the expression of AQP1 channel is moderately regulated by estrogen in the non-pregnant mouse uterus. The AQP2 level shows elevated expression as a result of estrogen. (Jablonski et al., 2003) Richard et al. revealed that the expression of AQP1, 4, and 5 elevated extremely at the time of implantation in the mouse myometrium. As a result of estrogen stimulation, the expression of AQP5 showed elevation on progesterone-primed uterus. (Richard et al., 2003) It is clear that AQP5 has an important role in the fluid regulation of uterus during the implantation period. The hormonal changes which regulate implantation are responsible for the positioning of AQP5 water channel in the uterus. (Lindsay and Murphy, 2006)

According to recent studies there is no information about the effects of female sexual hormones on the AQP5 expression in late pregnant rat uterus. In our study we found that progesterone pretreatment significantly increased the AQP5 expression. In case of progesterone, the AQP5 mRNA level was higher than the AQP5 protein level. In contrast, the protein expression increased similarly after levonorgestrel and medroxyprogesterone acetate

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treatment, as compared to progesterone treatment. According to the basic perception of molecular biology, the information streams from DNA by the help of mRNA to proteins. There are variable factors which could impact this process that is why we cannot always find a solid correlation between mRNA and protein expression. (Maier et al., 2009)

17β-estradiol pretreatment caused a significant increase in the amount of AQP5 protein, but did not result any changes in the AQP5 mRNA expression, both on day 18 and day 22 of gestation. In the light of our result, we presume that estrogen may protect the stability the AQP5 protein and this procedure could lead to the elevation in protein expression without modification of mRNA expression.

Tamoxifen is a nonsteroidal triphenylethylene derivative with a selective estrogen receptor modulator effect. (Dutertre and Smith, 2000) The tamoxifen citrate pretreatment significantly increased the level of AQP5 protein on day 18 of gestation.

Clomiphene citrate also has selective estrogen receptor modulator effect and it has nonsteroidal conformation too. (Hughes et al., 2000) Clomiphene citrate did not result any difference either in the AQP5 mRNA expression or in the AQP5 protein level. We presume that the four-day long pretreatment is not enough for influencing the hypothalamic-pituitary axis and evoke the hormonal changes.

Preterm delivery could have different pathophysiological backgrounds, such as endocrine disorders, allergic phenomena, abnormal allograft reaction, cervical disease, uterine over-distension, ischemia and infection.(Romero et al., 2006) This is the reason why we have various types of animal models for preterm birth. We wanted to prove that progesterone has notable effect on AQP5 expression, so we used hormonally-induced preterm birth model. Preterm delivery was induced by antigestagen mifepristone and intravaginal prostaglandin E2. The preterm birth was initiated by mifepristone which blocks the progesterone receptors. (Telleria and Deis, 1996) Prostaglandin E2 enables the induction of labor and the cervical ripening. (Thomas et al., 2014) The preterm birth started on day 20 of gestation in this model. The AQP5 mRNA and protein expression decreased significantly on day 20 of pregnancy when the preterm delivery 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 progesterone dropped significantly at the time of the preterm delivery, which was followed by the decrease of AQP5 expression.

In the light of our results we can conclude that both estrogen and gestagen-related compounds can influence the expression of AQP5 water channel. Progesterone and the other gestagen hormones have more pronounced effect on the expression in the late pregnant rat uterus. The

hormonally-induced preterm birth model study suggests that the lack of progesterone leads to reduced AQP5 expression and may contribute to the initiation of preterm labor.

5.2   AQP5 channel and antibiotic treatment

Among the several risk factors which can lead to preterm birth, the most common are intrauterine inflammation and infection. (Epstein et al., 2000; Gonçalves et al., 2002) As the results show, the antimicrobial therapy seems to be the appropriate solution for prevent the preterm delivery and the complications of it. There is evidence about the usage of antibiotics in pregnancy with premature rupture of the membranes: it can prolong the period between the rupture of membranes and the onset of labor. (Ananth et al., 1996) The antibiotic treatment also can reduce the incidence of problems which are caused by preterm birth, for example: intraventricular hemorrhage, chorioamnionitis and neonatal infection. (Kenyon et al., 2013) Two randomized controlled studies examined the penicillin family of antibiotic drugs in case of preterm birth. These investigations did not found any positive effect as a result of antibiotic prophylaxis irrespective of the use of tocolysis or steroid treatment. (Kenyon et al., 2004, 2001)

We have information about the teratogenic effect and other risk factors about antibiotic drugs during gestation; however, no data is available regarding their response on the pregnant uterine contractility. Effect of antibiotics on uterine contractions can be an important question especially in a case, when a mother obtains antibiotic treatment.

We investigated three different antibiotic drugs regarding their influence on the uterine contractions and additionally on the AQP5 expression. Our earlier results supported that the uterine contractility is inversely proportional to AQP5 expression. (Csányi et al., 2016; Ducza et al., 2014) Amoxicillin 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 amoxicillin pretreatment, in the *in vitro* isolated organ experiment. (Csányi et al., 2018) This phenomenon might explain the ineffectiveness amoxicillin and other beta-lactam antibiotics in the prevention of preterm delivery. (Lee et al., 2016)

AQP5 mRNA level was significantly elevated while the protein level declined as a result of fosfomycin pretreatment. The AQP5 protein expression was reduced by fosfomycin pretreatment and this treatment increased the sensitivity of the uterus to oxytocin stimulation in the concentration of 10^{-8}M.

Doxycycline pretreatment did not have any effect on the AQP5 expression or on the uterine contracting effect of oxytocin.

Some studies investigated the effect of antibiotic treatment on the contractility of smooth muscles and they found that it can modulate directly the contractility. (Granovsky-Grisaru et al., 1998; Paradelis et al., 1982; Tagaya et al., 1995) Piccino et al. reported that three antibiotics (amoxicillin, enrofloxacin and rifaximin) can modulate the contractility of the non-pregnant bovine uterine tissue. They revealed that amoxicillin has concentration-dependent relaxing effect on the basal contractility in the lutheal and follicular phases of the cycle. (Piccinno et al., 2016)

Based on these results, we have found more evidence for the cross-talk between AQP5 and oxytocin in the control of uterine contraction. Even though the correct mechanism of this phenomenon is not clear, we presume that the changes in the cell volume by AQP5 were induced by antibiotics that influence the intracellular calcium concentration. It was earlier proved that high intracellular calcium concentration resulted elevated AQP5 expression in kidney cell lines, in which strongly expressed human AQP5 and also in eccrine sweat glands. (Concepcion et al., 2016; Inoue, 2016) The canonical pathway of the uterus stimulation of oxytocin is the calcium-dependent activation of calmodulin and myosin light chain kinase. It is a known fact that the oxytocin receptor density elevates at the onset of labor, it is enable the increased sensitivity of the uterine tissue to oxytocin which is essential for the normal process of delivery at term.

We can conclude that amoxicillin and fosfomycin therapies may sensitize the uterus to oxytocin via the reduction of AQP5 expression. This synergetic effect must be considered in pharmacotherapy during pregnancy.

5.3   Cooperation between the AQP5 channel and TRPV4 receptor

The exact mechanisms of preterm delivery are largely unknown. The management of preterm birth involves the identification of the factors which induced the untimely uterine contraction. (Marzano et al., 2008) In our study we looked for new mechanisms through which uterine contraction is adjustable.

It is well known, that AQPs are present during the gestation and they regulate somehow the uterus contractions and the cervical ripening during labor. (Ducza et al., 2017) The AQP5 expression represented an increasing trend to the day 18 of pregnancy and this expression dramatically decreased on the last day of gestation in rat. It was also elucidated that this water channel is selectively down-regulated by oxytocin, which is responsible for uterus

contractions. (Ducza et al., 2014) The AQP5 expression was up-regulated by the uterus relaxing progesterone and progesterone-related compounds. (Csányi et al., 2016) According to these findings we confirmed an inverse correlation between AQP5 expression and myometrial contraction. We hypothesize that a low AQP5 level may induce or increase the contractility of late pregnant uterine tissue. The key question is the clarification of the mechanism of this phenomenon, especially at the time of delivery. We hypothesize an osmotic pathway to control the smooth muscle cells in the uterus. The main investigated days were the day 18 and 22 of pregnancy, because on these days were the most pronounced changes in the AQP5 and TRPV4 expression in rat uterus.

The TRPV channels are present in the female and male reproductive tissues and they are responsible for different physiological function. For example, in rat prostatic tissue Guibert et al. observed TRPV4 expression. (Guibert et al., 2011) These channels enable the oocyte maturation and activation and also fertilization. (Björkgren and Lishko, 2017) Another study revealed TRPV1 expression in the human placenta and their abnormal function in preeclampsia. (Martínez et al., 2016) The TRPV6 is expressed in the bovine uterine endometrium and placenta during the gestation. (Sprekeler et al., 2012)

The TRPV4 channel is a Ca^{2+} channel and it is activated by osmotic stimuli and it can be found in different smooth muscles. (Rosasco and Gordon, 2017) Based on current evidence, the calcium homeostasis is regulated in the uterine smooth muscle near term to facilitate the uterine contractility. The entry of extracellular Ca^{2+} enables the maintenance of spontaneous rhythmic contractions in the human uterus. (Parkington et al., 1999)

It is well known that the AQP5 water channel is an interacting partner of the TRPV4 channel in the smooth muscles of the airway system and in the gastrointestinal tract. (Aure et al., 2010; Sidhaye et al., 2006) There is a functional connection between AQP5 and TRPV4 in salivary glands. (Aure et al., 2010) According to other studies, the hypotonic reduction of AQP5 expression needs the TRPV4 action; moreover it is necessary for the mechanism of regulatory volume decrease in salivary gland cells. (Hosoi, 2016; Liu et al., 2006)

In our study we determined the changes of TRPV4 expression in rat uterus during gestation. This alteration of TRPV4 expression in the uterus could be the effect of hormonal changes. TRPV4 expression was decreased by progesterone in human tracheal and mammary gland ductal epithelial cell lines. Decreased TRPV4 expression and promoter activity were also observed in the presence of progesterone in human aortic vascular smooth muscle cells (Jung et al., 2009) This statement supports our results, considering that the level of progesterone drops at the end of pregnancy (Boroditsky et al., 1978), which increases TRPV4 expression. It

is known from our previous results that AQP5 expression is up-regulated by progesterone. (Csányi et al., 2016) We suppose a potential hormone regulated cooperation between the AQP5 and TRPV4 expression.

Based on our results we found an inverse correlation between the AQP5 and TRPV4 mRNA and protein expression. The putative cooperation between AQP5 and TRPV4 was revealed by our immunohistochemical findings. We proved the changes in the AQP5 and TRPV4 expression and the co-expression of these proteins in the late pregnant rat uterus.

In the volume homeostasis of brain there are a similar co-expression and functional relationship of TRPV4 and AQP4 channels. (Benfenati et al., 2011)

Up to this day, different attempts were to modulate the entry of extracellular Ca^{2+} into the smooth muscle cells, and thereby regulate uterine contractility, but they are not efficient enough to prevent or delay the preterm birth. (Muglia and Katz, 2010) Current evidence suggests that uterine smooth muscle cell Ca^{2+} homeostasis is modulated near term to promote uterine contractility. TRPV4 might have a role in uterine contractility and Ca^{2+} signaling (Ying et al., 2015) We determined the effect of the TRPV4 channel in the regulation of late pregnant rat uterus contraction with TRPV4 agonist and antagonist. The TRPV4 agonist had a poor relaxing effect on the uterus contraction on day 18 in the highest concentration. In contrast, a contraction-inducing effect was measured on the last day of pregnancy.

The TRPV4 antagonist (RN1734) completely inhibits both ligand-induced and hypotonicity-induced activation of TRPV4, without modulating the function of other TRP channels. (White et al., 2016) We determined the relaxing effect of RN1734 on both investigated days; with the major effect on the last day of pregnancy. We presume it can be explained by the increased expression of TRPV4 and the decreased expression of AQP5. Singh et al. proved that the other type of TRPV4 antagonist (HC067047) inhibited the PGF2α induced contraction in non-pregnant and pregnant mouse uterus. (Singh et al., 2015) There is no opportunity to examine the physiological role of AQP5 in the uterus contractions because of the lack of non-toxic tissue- and subtype-selective agonists or antagonists.

Based on our findings we presume the decreased AQP5 expression triggers an osmotic stress, which activates TRPV4 and increases uterus contraction on the day of labor. This phenomenon can confirm the future role of TRPV4 antagonist in the tocolytic therapy.

6 Conclusion

In the light of our results, we can conclude that (**1**) AQP5 expression is influenced by both female hormones with progesterone predominance in the late pregnant rat uterus.

We found (**2**) a marked decrease of AQP5 levels in hormonally induced preterm model and this change in AQP5 expression was similar to the day of normal birth. The explanation for this phenomenon could be that in the preterm delivery, the progesterone level decreases significantly, followed by the reduction of AQP5 expression. These changes suggest that the lack of progesterone effect leads to reduced AQP5 expression and may contribute to the initiation of preterm labor. These results may confirm an inverse correlation between the AQP5 expression and uterus contraction.

Antibiotics are used in pregnancy-associated urinary tract infections and to reduce the risk of infection-related preterm birth, especially in case of the premature rupture of the membranes. Some studies have reported the direct contractility modulating effect of antibiotics on smooth muscles. Based on our finding, (**3**) the therapy with amoxicillin and fosfomycin requires increased attention because they may sensitize the uterus to oxytocin via the reduction of AQP5 expression. This synergetic effect must be considered in pharmacotherapy during pregnancy.

(**4**) Co-expression was demonstrated between the AQP5 and TRPV4 channels in the late pregnant rat uterus. The uterus relaxant effect of TRPV4 antagonist was proved (**5**) on the day of birth as a new target for the tocolysis. Based on our findings we presume the decreased AQP5 expression triggers an osmotic stress, which activates TRPV4 and increases uterus contraction on the day of labor.

With these studies, we hope to give basic knowledge about the role of AQP5 in delivery and to obtain new information about the mechanism of preterm birth. Although the results stem from rats, the data may provide a basis for further studies in humans.

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