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Title Page

Effects of different antibiotics on the uterine contraction and the expression of aquaporin 5 in term pregnant rat

Short title: Effects of antibiotics on AQP5 in uterus

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Highlights

- The pre-treatment with amoxicillin or fosfomycin decreased the AQP5 protein level and enhanced the oxytocin-induced contractions in the last day of pregnancy in rat.
- Amoxicillin and fosfomycin may sensitize the uterus to oxytocin via the reduction of AQP5 expression. This synergetic effect must be considered in pharmacotherapy during pregnancy.

Abstract

Aquaporin (AQP) water channels are small hydrophobic integral membrane proteins. AQP5 expression, which is regulated by oxytocin, showed a dramatic down-regulation at the term and preterm uterus. Since antibiotics are among the drugs to treat intrauterine infections, our aim was to study the effects of antibiotics on AQP5 and uterine contractility on 22-day pregnant rats.

The change in uterine AQP5 expression was investigated by PCR and Western blot techniques. Uterine contractility was tested in an organ bath system.

7 days of pre-treatment with amoxicillin or single dose of fosfomycin decreased the AQP5 protein level, while 7 days of treatment with doxycycline had no effect. Fosfomycin or amoxicillin pre-treatments enhanced, while doxycycline pre-treatment did not alter the oxytocin-induced contractions.

Amoxicillin and fosfomycin may sensitize the uterus to oxytocin via the reduction of AQP5 expression. This synergism might have importance during the pharmacotherapy of infection-related preterm birth.

Keywords: amoxicillin; aquaporin 5; doxycycline; fosfomycin, pregnancy

1 Introduction

Water molecules are essential to the functioning of most known life-forms and aquaporins (AQPs) are integral membrane proteins that form channels to facilitate the rapid movement of water and a few small molecules across cell membranes. So far 13 mammalian AQPs have been identified and all but one are expressed in mammalian reproductive systems (1). These small, hydrophobic integral membrane proteins are divided into three subtypes. Classical AQPs, including AQP0, 1, 2, 4, 5, 6 and 8 are selectively permeable to water (2). Aquaglyceroporins are AQP3, 7, 9 and 10; these are non-selective water channels, permeable not only to water but also to glycerol, urea and other small solutes (3,4). Unorthodox aquaporins include AQP11 and 12; their functions are not clearly identified (5,6).

AQPs have an important role during pregnancy. The reduction in the amount of luminal fluid at the time of implantation is essential for successful implantation (7). In humans, AQP2 and 3 were found in epithelial cells with an increased expression during the mid-secretory phase, which corresponds with the time of implantation (8, 9). Sun XL et al. proved that female Aqp4$^{-/-}$ mice had subfertility with defective folliculogenesis (10). The presence of AQP5 in the mouse and rat uterus was observed in the luminal epithelial cells at the time of implantation (11, 12). *Aqp1, 3* and *8* genes were found in the second half of pregnancy in murine, ovine and human placenta (13). Escobar et al. found high mRNA expression for *Aqp1, 3, 9* and *11*, low for *Aqp4, 5* and 8, while *Aqp2, 6* and *7* were undetectable in chorionic villi between the 10th and 14th weeks of human gestation (14). At the end of pregnancy, during the process of cervical ripening, the cervical water content increases. The presence of *Aqp3, 4, 5* and *8* was determined in mice pregnant cervix, which influences this ripening process (15).

Our earlier study demonstrated the presence of AQP1, 2, 3, 5, 8, and 9 in the late-pregnant rat uterus with the predominance of AQP5 before parturition. AQP5 expression was dramatically reduced on the last day of pregnancy and was down-regulated by oxytocin, suggesting its

participation in the initiation of delivery (16). It was confirmed by microarray analysis, in which the significant down-regulation of *Aqp5* mRNA was observed during delivery, underscoring their potential role in parturition in the rat uterus (17). We also proved that AQP5 expression was significantly decreased by hormonally-induced preterm delivery in pregnant rat uterus similarly to the last day of pregnancy (18). These findings suggest that the reduced AQP5 expression in the uterus may be linked to the onset of preterm labour.

Preterm delivery increases prenatal morbidity and mortality, which often leads to long-term neurological impairments and respiratory and gastrointestinal complications in children (19). There are multiple mechanisms in the background of preterm labour, such as infection, ischemia, uterine over-distension, cervical disease, abnormal allograft reaction, allergic phenomena or endocrine disorder (20). It is well-known that genital tract infection is one of the factors that increases the incidence of preterm birth (21). A possible mechanism for the link between infection and preterm delivery is the bacterial stimulation of the biosynthesis of prostaglandins, either directly via phospholipase A2 and C (22), or indirectly via substances such as interleukin-1, tumor necrosis factor and platelet activating factor, all of which may be found in infected amniotic fluid (23). Antibiotics are often used to reduce the risk of infection-related preterm birth, especially in case of the premature rupture of the membranes (PROM) (24). A meta-analysis demonstrated that prophylactic antibiotics, e.g. amoxicillin after PROM can decrease the incidence of sepsis and intraventricular haemorrhage among newborns (25). Despite the lack of evidence of longer-term benefit in childhood, the advantages of short-term morbidities are such that we recommend antibiotics should be routinely prescribed. The antibiotic of choice is not clear but co-amoxiclav should be avoided in women due to the increased risk of neonatal necrotizing enterocolitis (24). Amoxicillin and fosfomycin are also widely used antibiotics against bacteriuria and bacterial vaginosis during gestation (26,27,28,29). The usage of these antibiotics is allowed during pregnancy but limited information is available about their effect on AQP5 expression and thereby on uterine contraction. AQP5 expression was increased 7 days after treatment with doxycycline in cuboidal and squamous epithelial cells in the lung periphery as well as in bronchiolar epithelial cells (30), but there is no scientific data about its uterine action (or that of other antibiotics).

Therefore our aim was to investigate the effect of these antibiotics on the expression of AQP5 and uterine contraction on the day of birth in rats.

2 Methods

2.1 Animals

The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) 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 number: IV/198/2013).

Mature female (180-200 g) and male (240-260 g) Sprague-Dawley rats (INNOVO Ltd., Gödöllő, Hungary) were mated in a special mating cage. It had a time-controlled movable metal door separating male and female animals. This door was opened before dawn, because rats are active at night. Within 4-5 hours after the potential copulation, vaginal smears were taken and were examined by a microscope at a magnification of 1200 x. Female rats were separated and regarded as being on the first-day of gestation in the presence of copulation plug or the presence of sperms in the native vaginal smear.

The animals were kept at a controlled temperature of 20-23°C, in relative humidity of 40%-60% and under a 12 h light/dark cycle. The rats were fed a standard rodent pellet diet (INNOVO Ltd., Isaszeg, Hungary), with tap water available *ad libitum*.

2.2 Treatments of the Rats

The first group of pregnant rats was treated with amoxicillin from gestational days 16 to 22. The animals were treated orally by oral gavage with Ospamox granulates for suspension (Sandoz Ltd. Kundl, Austria) in a dose of 40 mg/kg of body weight once a day. (31,32). The suspension was prepared with purified water. On the last day of gestation (day 22) uterine samples were collected and molecular studies were carried out.

The second group of pregnant rats was treated with fosfomycin on day 21 of gestation. On gestational day 22 uterine samples were collected and molecular studies were carried out. 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 (33,34). The solution was prepared with purified water.

The third group of pregnant rats was treated with doxycycline from gestational days 16 to 22. The animals were treated orally by oral gavage with Doxycyclin AL (Aliud Pharma Ltd. Laichingen, Germany) (100 mg hard capsule) in a dose of 30 mg/kg of body weight once a day (35,36). The suspension was prepared by opening the capsules and grinding the powder with methyl cellulose. On the last day of gestation (day 22), uterine samples were collected and molecular studies were carried out. The durations of the therapies were determined based

on human therapeutic protocols (37,38,39). The control groups (one group as control for amoxicillin and doxycycline groups with 7 days of treatment and another group for fosfomycin groups with a single treatment) were treated orally by oral gavage with tap water.

RT-PCR Studies

2.2.1 Tissue Isolation

The pregnant rats were terminated by CO2 inhalation, while newborn pups were sacrificed by cervical dislocation (decapitation) immediately after caesarean section. The uterine tissues from pregnant rats (n=6) (tissue between two implantation sites) 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.

2.2.2 Total RNA Preparation

Total cellular RNA was isolated by extraction with guanidinium thiocyanate-acid-phenol-chloroform according to the procedure of Chomczynski and Sacchi (40). After precipitation with isopropanol, the RNA was washed with 75% ethanol and then re-suspended 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.

2.2.3 Real-Time Reverse-Transcription PCR

Reverse transcription and amplification of the PCR products were performed by using the TaqMan RNA-to-C_T-Step One Kit (Life Technologies, Budapest, Hungary) and an ABI StepOne Real-Time cycler. Reverse-transcriptase PCR amplifications were performed as follows: at 48°C for 15 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and at 60°C for 1 min. The generation of specific PCR products was confirmed by melting curve analysis. The samples of PCR experiments contained “no-template” control and “absolute” control or RNA samples from non-treated and treated uteri. The following primers were used: assay ID Rn00562837_m1 for *Aqp5* water channel and Rn00667869_m1 for *β-actin* as endogenous 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).

2.3 Western Blot Analysis

The uterine tissues from pregnant animals (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 the supernatant were determined by a 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 XCellSureLock Mini-Cell Units (Life Technologies). Proteins were transferred from gels to nitrocellulose membranes using the iBlot Gel Transfer System (Life Technologies). Ponceau S (Sigma-Aldrich) was used to check the standard running and transfer conditions. After the blocking, the membrane has been cut and the antibodies were used on the two part of membrane in the same time and conditions. The blots were incubated overnight on a shaker with AQP5 (24 kDa) and β-actin (43 kDa) polyclonal antibodies (ThermoFisher Scientific, 1ug/ml, host: rabbit, specificity: mouse, rat and human) in blocking buffer. Antibody binding was detected with the WesternBreeze® Chromogenic immunodetection kit (ThermoFisher Scientific), which contains alkaline phosphatase secondary antibody solution. 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. β-actin was used for protein normalization for this semi-quantitative method. Optical densities were calculated as arbitrary units after local area background subtraction.

2.4 In Vitro Contractility Studies

Uteri were removed from the 22-day-pregnant rats. 5-mm-long muscle rings were sliced from both horns of the uterus and mounted vertically in an organ bath containing 10 ml of de Jongh solution (composition: 137 mMNaCl, 3 mMKCl, 1 mM CaCl2, 1 mM MgCl2, 12 mM NaHCO3, 4 mM NaH2PO4, 6 mM glucose, pH = 7.4). The temperature of the organ bath was maintained at 37 °C, and carbogen (95% O2 + 5% CO2) was perfused 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. Results were analysed statistically with the Prism 4.0 (Graphpad Software Inc. San Diego, CA, USA) computer program.

2.5 Statistical Analysis

All experiments were carried out on six animals and 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 multiple comparison test (for contractility studies) or two-tailed unpaired *t* test (for RT-PCR and Western-blot studies) were used. $P < 0.05$ was considered as a level of significance.

3 Results

3.1 RT-PCR and Western Blot Studies

The *Aqp5* mRNA (Fig. 1A) and protein (Fig. 1B) expressions significantly decreased ($p < 0.05$) on the last day of gestation as a result of 7 days of amoxicillin pre-treatment. A single dose of fosfomycin on gestational day 21 significantly increased the *Aqp5* mRNA levels on day 22 of gestation ($p < 0.001$), compared to the non-treated animals (Fig. 2A). Contrary to the mRNA level, the protein level of AQP5 decreased on day 22 of gestation ($p < 0.01$) (Fig. 2B).

7 days of doxycycline pre-treatment did not cause significant changes either in the *Aqp5* mRNA or the protein levels on the last day of gestation ($p > 0.05$), compared to the non-treated uterus (Fig. 3).

3.2 Isolated Organ Studies

Oxytocin (10^{-12}–10^{-8} M) induced rhythmic contractions in the 22-day pregnant rat uterine rings. In low doses (10^{-12}-10^{-10} M) the oxytocin effect was moderated, while from concentration of 10^{-9} M a relatively steep elevation was found in the uterine contraction on the concentration-response curve reaching an approximately 3-fold increase in contraction as compared with spontaneous activity. Fosfomycin or amoxicillin pre-treatments significantly enhanced the uterine contracting effects of oxytocin, but only at the highest concentration (10^{-}

^{8}M). However, doxycycline pre-treatment did not alter the oxytocin-induced contractions (Fig. 4).

4 Discussion

Preterm delivery has several risk factors, such as intrauterine inflammation or infection (41,42). The use of antibiotics in pregnancy with PROM could increase the period between the rupture of membranes and the onset of labour (44). We presume that the reduction of the oxytocin-regulated AQP5 protein may lead to the initiation of uterine contractions (16).

In our study we have investigated the uterine actions of three different antibiotics on AQP5 expression and contractions. Amoxicillin and fosfomycin are widely used in the therapy for pregnant women, while doxycycline is contraindicated (46). However, it was proven that doxycycline induces AQP5 expression in the lungs of transgenic mice (40), therefore it is the only one known AQP5-influencing antibiotic. Based on our earlier studies, AQP5 expression is inversely proportional to uterine contractility (16,18). Amoxicillin treatment caused significant decreases in the AQP5 level in the pregnant uterus on the day of labour. In the *in vitro* isolated organ experiment, the uterine tissues showed enhanced contractility responses to oxytocin as a result of amoxicillin pre-treatment. Three randomized controlled studies have focused on penicillins and PTB. No significant benefit was associated with the use of penicillins. There was no difference in the mean gestational age at delivery, and other perinatal outcomes were also similar (43-45). Our results might explain the ineffectiveness of amoxicillin and other beta-lactams in PTB (47), or even warn of the potential risk of enhancement in myometrial contractility.

In the case of fosfomycin, there were significant increases in the *Aqp5* mRNA expression but the protein level was decreased. This difference in the mRNA and protein level is not rare in molecular biology studies. Several biological factors were identified to influence the translation of information from RNA to protein, so a strong correlation between mRNA and protein expression does not always exist. With regard to the pharmacological effect, protein expression is perceived as a determining factor for ligand action. Several studies proved that the correlation between the mRNA and protein expression is frequently quite weak. One of the putative reasons for this discordance can be the complicated and varied post-translational processes that have not been defined yet (48,49). Fosfomycin pre-treatment reduced the AQP5 protein expression and increased the sensitivity of uteri to oxytocin stimulation in the

10^{-8}M concentration. Doxycycline had an effect neither on the AQP5 expression nor on the uterine contracting effects of oxytocin.

Several studies have reported that antibiotic therapy is able to directly modulate the contractility of smooth muscles, too (50,51,52). Piccino et al. also investigated the modulator effect of three antibiotics (amoxicillin, enrofloxacin, and rifaximin) on the contractility of the non-pregnant bovine uterine tissue. Amoxicillin induced a relaxing concentration-dependent effect on basal contractility in the lutheal and follicular phases of the cycle (53).

Based on these results, we have found further evidence for the cross-talk between AQP5 and oxytocin in the regulation of uterine contraction. Although the exact 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. The positive correlation between AQP5 and intracellular calcium concentration was proved in kidney cell lines (54,55). The calcium-dependent activation of calmodulin and myosin light chain kinase is considered the canonical pathway of uterine stimulation by oxytocin. It is well-known that the oxytocin receptor density increases at the onset of labour, which is assumed to mediate an increase in the sensitivity of the myometrium to oxytocin at term.

Antibiotics are used not only in PROM but also in pregnancy-associated urinary tract infections (PAUTI). Untreated PAUTIs can have serious consequences, including pyelonephritis, preterm labor, low birth weight, and sepsis (56). Based on our finding, the therapy with amoxicillin and fosfomycin requires increased attention, especially if there are other factors which enhance the potential for preterm birth.

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.

Conflict of Interest Statement:

The authors declare that there are no conflicts of interest.

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Figure legends

Figure 1. Changes in mRNA (**A**) and protein (**B**) expression of AQP5 after 7 days of amoxicillin (A) pre-treatment in rat uterus on the last day of pregnancy. *$p < 0.05$ as compared with the non-treated uterus; Ax: amoxicillin. Each bar denotes the mean ± S.D. $n = 6$.

Figure 2. Changes in mRNA (**A**) and protein (**B**) expression of AQP5 after fosfomycin (F) pre-treatment in pregnant rat uterus on gestation day 22. **$p < 0.01$, ***$p < 0.001$ as compared with non-treated uterus. F: fosfomycin. Each bar denotes the mean ± S.D. $n = 6$.

Figure 3. Changes in mRNA (**A**) and protein (**B**) expression of AQP5 after doxycycline (D) pre-treatment in pregnant rat uterus on gestation day 22. ns $p > 0.05$ as compared with non-treated uterus. ns: non-significant, D: doxycycline. Each bar denotes the mean ± S.D. $n = 6$.

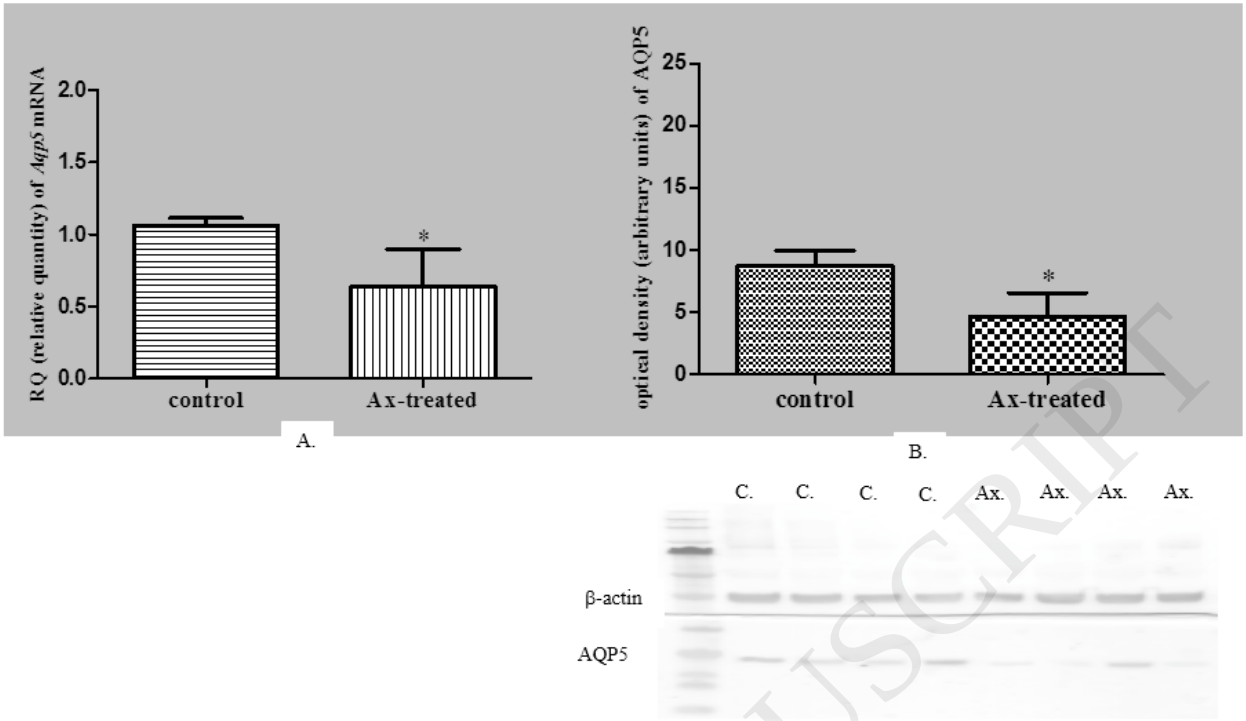
Figure 4. Representative traces of oxytocin-induced *in vitro* contractions in non-treated (**A**) and fosfomycin- (**B**), amoxicillin- (**C**), or doxycycline-treated (**D**) pregnant uteri.

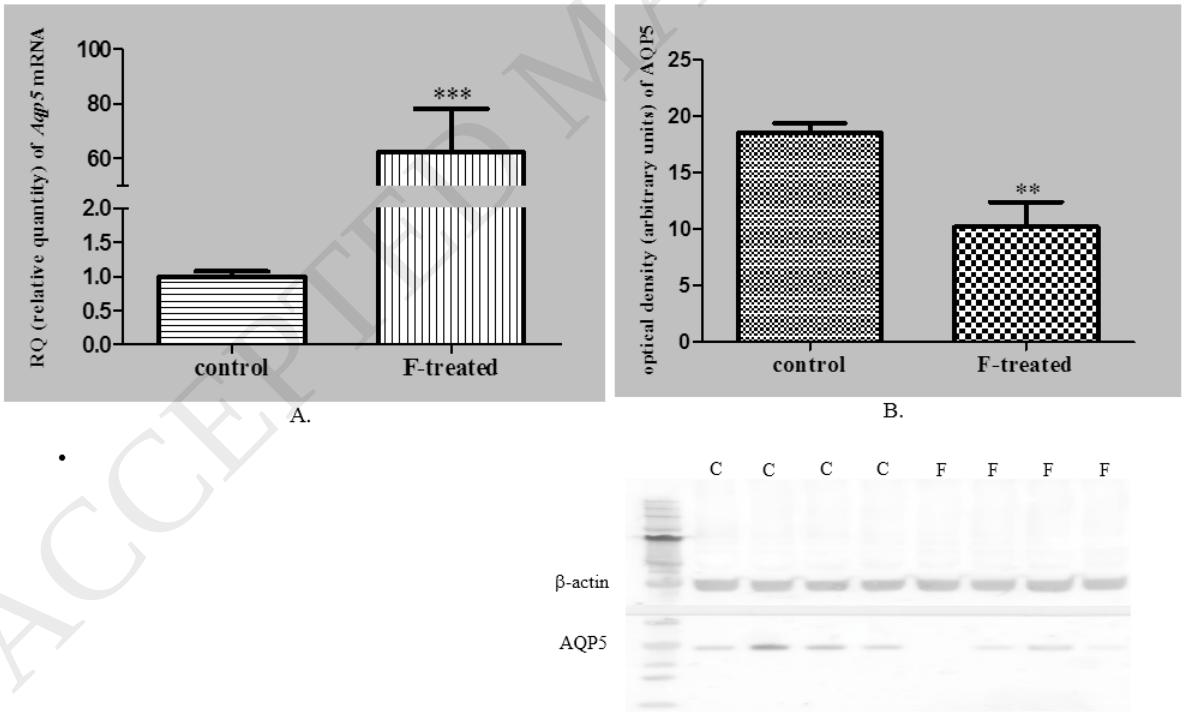
The change in contraction was calculated by the area under the curve (AUC) alteration and expressed in % ± SD as compared with AUC of control contractions (**E**).

"F" diagram shows the changes of the AUC of spontaneous uterus contractions after the pretreatment with antibiotics compared to the non-treated uterus.

Each value denotes the mean ± S.E.M, n = 6. *ns*: non-significant; ns $p > 0.05$; *$p < 0.05$; **$p < 0.01$. b.c.: basal contraction, OT: oxytocin.

Figure 1.





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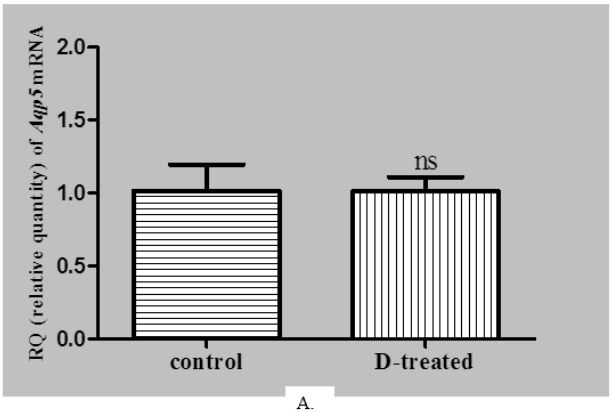

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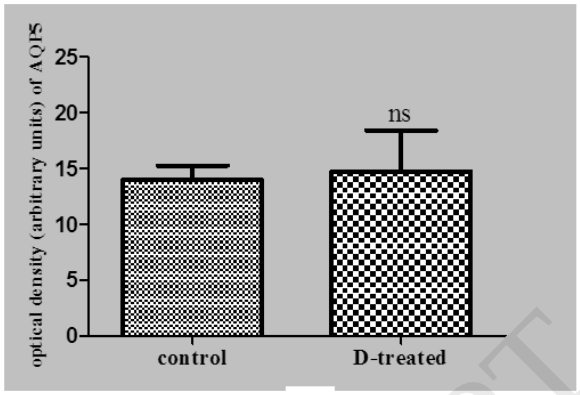

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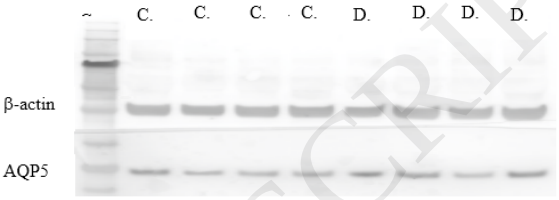

Figure 3.

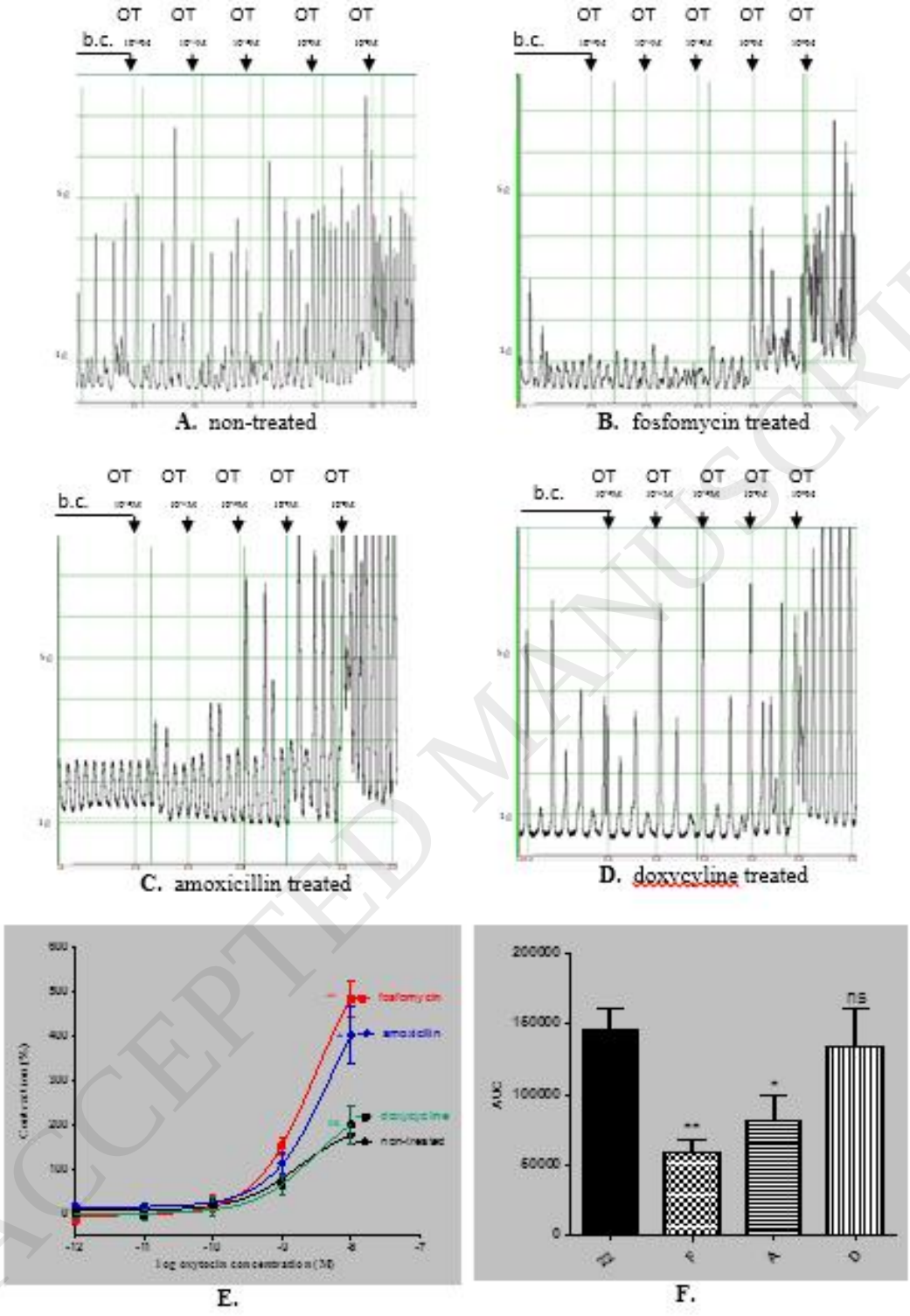

Figure 4.