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**Modification of the myometrial relaxing effect of  
nifedipine *in vitro* and *in vivo***

Ph.D. Thesis

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## List of abbreviations

**PTB:** preterm birth

**$\beta_2$ -AR:**  $\beta_2$ -adrenergic receptor

**Ca<sup>2+</sup>:** calcium ion

**PGs:** prostaglandins

**PTL:** preterm labour

**VGCCs:** Voltage-gated calcium channels

**MLCK:** myosin light chain kinase

**DHP:** dihydropyridine

**BK<sub>Ca</sub> channels:** Large-conductance calcium-activated potassium channels

**TEA:** tetraethyl ammonium

**cAMP:** cyclic adenosine monophosphate

**PK:** protein kinase

**P4:** progesterone

**E2:** oestrogen

**KCl:** potassium chloride

## 1. Introduction

### 1.1. Epidemiology and consequences of preterm birth

Preterm birth (PTB), defined as childbirth between 20 and 37 weeks of gestation by WHO, is a major determinant of neonatal mortality and morbidity and has long term adverse consequences for health (Beck *et al.*, 2010). In the USA, the preterm delivery rate is 12-13%, in Europe and other developed country, reported rates are generally 5-9% (Goldenberg *et al.*, 2008). According to the College of Hungarian Obstetricians and Gynaecologists (Egészségügyi Közlöny, 2008) the incidence of preterm labour is still around 8% in Hungary. The most common reason of a pregnant woman's hospitalization before delivery is threatening PTB.

The exact causes and aetiologies of PTB are not known. Several risk factors for PTB have been established: previous low birth weight or preterm delivery, repeated second trimester abortion, uterine and cervical anomalies, *in vitro* fertilization, multiple pregnancy, maternal medical complications, gestational bleeding, abnormal placentation, urogenital infection, low socio-economic status, smoking, and low body mass index (BMI) before conception (Moutquin, 2003; Pennel *et al.*, 2007; Haas, 2006). Bacterial vaginosis is also a risk factor and associated with a higher incidence of PTB of infants with low birth weight (Hillier *et al.*, 1995). Other factors such as maternal age, parity, infertility, drug abuse, heredity, sexual activities, physical workload, psychosocial stress, inadequate prenatal care, and maternal weight gain are still under evaluation (Berkowitz *et al.*, 1998).

The incidence of PTB has not decreased over the years despite major improvements in medical, especially perinatal care facilities and extensive medical research. Children who are born prematurely have higher rates of cerebral palsy, sensory deficits, learning disabilities and respiratory illnesses compared with children born at term. The morbidity associated with PTB often extends to later life, resulting in enormous physical, psychological and economic costs (Papatsonis, 2005; Petrou, 2005; Petrou *et al.*, 2003).

In view of the enormity of the social, economic and emotional costs of premature birth, there has been an intensive effort to understand its pathology and to develop treatment strategies.

## 1.2. Tocolytic therapy

There are no clear first line tocolytic drugs to manage PTB. Despite research directed to the development of drugs to inhibit myometrial contractions, there has been no reduction in the incidence of PTB for more than 30 years. Present therapies cannot prevent preterm delivery, but at best provide sufficient delay in order to attempt treatments that ameliorate the consequences of prematurity (Mohan and Bennett, 2006). A number of agents are used clinically as tocolytics, including magnesium sulphate, indometacin,  $\beta_2$ -adrenergic receptor ( $\beta_2$ -AR) agonists, atosiban, progesterone (P4), prostaglandin (PG) synthesis inhibitors, nitric oxide donors and calcium ( $\text{Ca}^{2+}$ ) channel blockers, but the efficacy of the current modes of pharmacological treatment has been questioned (Kim *et al.*, 2006). The main rationale for use of these drugs is to delay delivery for at least 48 hours in order to allow time for the treatment effect of corticosteroids, or transfer of the pregnant mother to a specialized high-risk obstetrical unit (Husslein *et al.*, 2003).

Intravenous magnesium sulphate has been used in the United States as treatment for eclamptic convulsions, and for seizure prophylaxis in the setting of suspected preeclampsia, since 1906 (Chesley, 1978). It has been widely used as a tocolytic in the USA and for more than 30 years was often the first line tocolytic, but it has been rarely used in Europe for this purpose. Various mechanisms of action have been proposed, such as magnesium competes with  $\text{Ca}^{2+}$  and thereby affects multiple intracellular pathways, but the exact role remains controversial (Mohan and Bennett, 2006). According to a 2009 Cochrane review magnesium sulphate is not effective at delaying birth or preventing PTB, because there was not enough evidence to show any difference between magnesium maintenance therapy and either placebo or no treatment (Arrowsmith *et al.*, 2010).

PGs play an important role in the onset and maintenance of labour. The use of indometacin for tocolysis was first reported in 1974 (Zukerman *et al.*, 1974), but despite the favourable results, most studies have limited the duration of indometacin use because of the development of oligohydramnios, constriction of the ductus arteriosus and an increased risk of necrotizing enterocolitis (Giles and Bisits, 2007; Mohan and Bennett, 2006).

Nitric oxide donors have been employed for cervical ripening, labour induction and tocolysis. NO increases levels of cyclic guanosine monophosphate and protein kinase (PK) G and can thereby affect several pathways associated with relaxation. According to a 2002

intervention review nitric oxide donors did not delay labour or improve neonatal outcome compared to placebo or alternative tocolytic (Arrowsmith *et al.*, 2010)

Atosiban – a competitive antagonist of oxytocin – has been shown to completely inhibit the uterotonic action of oxytocin in a competitive and dose-dependent manner and to downregulate oxytocin receptors and to inhibit oxytocin-mediated PG release. Although atosiban has been extensively studied in randomized, controlled trials, there is still controversy about its effectiveness and long-term safety (Kim *et al.*, 2006; Kinsler *et al.*, 1996).

$\beta_2$ -AR agonists were considered the drugs of choice to treat threatened PTL based on randomised controlled trials and several subsequent meta-analyses, which showed  $\beta$ -agonists to delay PTL for at least the required 48 hours. Although there are convincing data indicating effective prolongation of pregnancy,  $\beta$ -mimetics have the most undesirable side effect profile of all currently employed tocolytics (Oei, 2006; Pryde *et al.*, 2001).

Supplemental treatment with P4 has been studied to prevent preterm labour (PTL) and birth (Meis *et al.*, 2003; da Fonseca *et al.*, 2003) and as an adjunct to treat acute PTL (Noblot *et al.*, 1991). It has been shown to reduce the risk of recurrent PTB when used prophylactically but has not been thoroughly investigated as an adjunct to tocolytic drugs on human myometrium. Gálik *et al.* (2008) investigated the combination of P4 and  $\beta_2$ -AR agonists on rat myometrium. They showed that gestagens can enhance the effect of  $\beta_2$ -AR agonists.

In recent years,  $\text{Ca}^{2+}$  channel antagonists have been used increasingly as tocolytic agents. These agents act to inhibit  $\text{Ca}^{2+}$  influx across the cell membrane, thereby decreasing the tone in the smooth muscle vasculature (Tan *et al.*, 2006). These agents were originally introduced to treat hypertension. Comparative trials with  $\beta$ -agonists have shown more favourable neonatal outcomes and better prolongation of gestation (Koks *et al.*, 1998; Papatsonis *et al.*, 1997), although no placebo-control trials have addressed the acute management of PTB, and there is uncertainty about the optimal form, dose and route of administration for Ca channel blockers (Arrowsmith *et al.*, 2010).

With a view to decreasing the potentially maternal and foetal adverse events and improving the perinatal outcome, there is growing interest in experimental studies of the possible use of different tocolytic combinations.

### 1.3. The role of L-type $\text{Ca}^{2+}$ channels in uterine contraction

Voltage-gated calcium channels (VGCCs) play a major role both in the normal functioning and also in various pathological processes that occur in neuronal, neurosecretory and muscle cells.

VGCCs mediate  $\text{Ca}^{2+}$  influx in response to membrane depolarization and regulate intracellular process such as contraction, secretion, neurotransmission, and gene expression in many different cell types. Their activity is essential to couple electrical signals in the cell surface to physiological events in cells (Catterall *et al.*, 2005).

Uterine contractility is generated by contractions of the myometrial smooth muscle cells that comprise most of the myometrial layer of the uterine wall (Bursztyń *et al.*, 2007). Depolarization of the cell membrane initiates  $\text{Ca}^{2+}$  entry into the cells.  $\text{Ca}^{2+}$  binding to calmodulin activate the myosin light chain kinase (MLCK) and therefore initiate the phosphorylation and subsequent cross-bridge cycling. There are two sources for the increase in activator  $\text{Ca}^{2+}$ : entry across the surface membrane through VG L-type  $\text{Ca}^{2+}$  channels and/or release from the sarcoplasmic reticulum. In those phasic smooth muscles, such as uterus, where action potential occurs, the resulting depolarisation and consequent opening of L-type  $\text{Ca}^{2+}$  channels make this the major source of  $\text{Ca}^{2+}$  for contraction. Each phasic contraction is accompanied by a  $\text{Ca}^{2+}$  transient in the uterus, and both the transients and contractions are abolished if L-type channels are blocked (Noble *et al.*, 2009; Wray, 2007; Dolphin, 2006).

The  $\text{Ca}^{2+}$  channels are complex proteins composed of five distinct subunits ( $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ ,  $\delta$  and  $\gamma$ ) encoded by multiple genes (Catterall *et al.*, 2005). The  $\alpha_1$  protein was identified as the component that bound 1,4-dihydropyridines (DHPs). Four of the 10 VGCC  $\alpha_1$ -subunits characterized constitute the family of L-type  $\text{Ca}^{2+}$  channels, namely  $\text{Ca}_v1.1$ ,  $\text{Ca}_v1.2$ ,  $\text{Ca}_v1.3$  and  $\text{Ca}_v1.4$  (Liao *et al.*, 2004). The  $\text{Ca}_v1.2$  L-type  $\text{Ca}^{2+}$  channels are localized mainly in the brain, cardiac and smooth muscles and a large number of alternative splicing sites of the  $\alpha_1$ -subunit have been reported in various tissues (Liao *et al.*, 2009).

DHP compounds, such as nifedipine, bind to the inside of the VG L-type channels, inhibiting the action potential and the contractility. The DHPs are the most potent inhibitors of uterine tension development among the  $\text{Ca}^{2+}$  entry blockers and are therefore of considerable interest for both therapeutic and experimental purposes (Garfield, 1990). Nifedipine and its analogs have recently been considered as tools for tocolytic therapy

(Moynihan *et al.*, 2008; Oei, 2006). To date, the changes in myometrial contractility to nifedipine during pregnancy have not been investigated in experimental studies.

#### **1.4. The role of BK<sub>Ca</sub> channels, terbutaline and P4 in the regulation of the L-type Ca<sup>2+</sup> channels**

The activity of L-type Ca<sup>2+</sup> channels is regulated by several factors (Kobayashi, 2007).

Relaxation of the myometrium follows a reversal of the Ca<sup>2+</sup>-calmodulin - MLCK pathway. The uterus contains Ca<sup>2+</sup>-activated potassium channels (BK<sub>Ca</sub>), and their expression and distribution have been shown to be gestationally regulated (Khan *et al.*, 2001). BK<sub>Ca</sub> channels are a diverse group of K<sup>+</sup> channels participating in the repolarization and hyperpolarization of action potentials. The increased activity of BK<sub>Ca</sub> channels serves as a negative feedback mechanism to limit the Ca<sup>2+</sup> influx in excitable cells (Wu *et al.*, 2006). Thus, if Ca<sup>2+</sup> sparks or BK channels were to be inhibited, and then Ca<sup>2+</sup> transients and force would be predicted to increase. The BK<sub>Ca</sub> channel antagonists iberiotoxin and tetraethyl ammonium (TEA) have already been used in combination with nifedipine to investigate the role of this channel in the effects of nifedipine in the human uterus. It emerged that the BK<sub>Ca</sub> channel blockers significantly antagonized the relaxant effect of nifedipine (Moynihan *et al.*, 2008).

The adrenergic system plays an important role in the control of uterine contractility. Currently, β<sub>2</sub>-AR agonists are still one of the most frequently used tocolytics, although their therapeutic significance in PTL is constantly questioned.

β-adrenergic stimulants are known to produce smooth muscle cell relaxation by raising the intracellular level of cyclic adenosine monophosphate (cAMP), which activates protein kinase A. This activated form leads to phosphorylation of the Ca<sup>2+</sup> channels. This mechanism in the heart muscle (Kamp and Hell, 2000) might be similar to that in the pregnant myometrium. Investigation of the effects of combinations of β<sub>2</sub>-agonists and Ca<sup>2+</sup> channel blockers have in the isolated trachea demonstrated that both isradipine and nifedipine potentiated the relaxant action of terbutaline and salmeterol, respectively (Thirstrup *et al.*, 1997; Lever *et al.*, 1984). The efficacy of a β<sub>2</sub>-agonist and a Ca<sup>2+</sup> channel blocker has not been investigated *in vitro* nor tested *in vivo*.

Another factor which regulates the L-type Ca<sup>2+</sup> channel is the P4/oestrogen (E2) ratio (Helguera *et al.*, 2002). P4 is a key component in the complex regulation of normal

female reproductive function. It plays a central role in the maintenance of pregnancy and the initiation of parturition by modulating myometrial contractility and excitability. P4 supports pregnancy and prevents parturition by promoting myometrial quiescence (Graham *et al.*, 1997; Thijssen, 2005).

P4 normally declines at term prior to the development of labour. It was shown, that if P4 levels are maintained by injections of the hormone, animals (rats and rabbits) do not go into labour (Garfield, 1990). Mackenzie *et al.* (2006) has found in clinical trials that progestational agents, initiated in the second trimester of pregnancy, reduce the risk of delivery less than 37 weeks' gestation for women at increased risk of spontaneous PTB.

It was shown that mRNA expression of the pore-forming  $\alpha_{1C}$  subunit of the L-type channel is regulated by glucocorticoid hormones but tissue-specific changes may occur (Takimoto *et al.*, 1997). Biochemical experiments have detected the presence of two forms of the L-type  $\text{Ca}^{2+}$  channel in native tissues: a short form ( $\alpha_{1C\text{-short}}$ ) and a long ( $\alpha_{1C\text{-long}}$ ) form. Helguera *et al.* (2002) established that a P4-mediated mechanism favours the expression of the long form, in the presence of which the channel has lower activity.

## 2. Aims

The main focus of our study was to enhance the tocolytic effect of nifedipine. Since no extensive experiments have been carried out to investigate the *in vivo* and *in vitro* uterus-relaxing effect of a combination of nifedipine and  $\beta_2$ -mimetics or P4 in the rat and also on human myometrium, the following aims were set:

1. As the changes in myometrial contractility during pregnancy to nifedipine have not been investigated, the first aim of the study was to investigate the effects of nifedipine on the potassium chloride (KCl)-evoked rat uterine contractions on different days (15, 18, 20, and 22) of pregnancy *in vitro*.
2. It is known, that the activity of the  $Ca_v1.2$  channel is regulated by several factors. Our further aim was to alter the effect of nifedipine on rat myometrium by applying a combination with terbutaline, P4 or  $BK_{Ca}$ -channel inhibitors, which can have effects on the  $Ca_v1.2$  channel.
3. The efficacy of the  $Ca_v1.2$  channel blocker –  $\beta_2$ -agonist combination has not been investigated on isolated human myometrium. In our study we investigate how terbutaline and nifedipine modify the contractions of the isolated human myometrium.
4. And finally, our last aim was to investigate the effects of nifedipine – salmeterol and nifedipine – P4 combination in hormone-induced PTB model *in vivo*, whether these drug interactions apply under *in vivo* circumstances.

### **3. Materials and methods**

#### **3.1. Animal studies**

##### ***Housing and mating of the animals***

The animals were treated according to the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv.32.§). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: IV/1758-2/2008). Sprague-Dawley rats (Charles River Laboratories, Hungary) were kept at  $22 \pm 3^\circ\text{C}$ ; the relative humidity was 30-70% and the light/dark cycle was 12 hours/12 hours. They were maintained on a standard rodent pellet diet (Charles River Laboratories), with tap water available *ad libitum*.

Mature female (180-200 g) and male (240-260 g) Sprague-Dawley rats were mated in a special mating cage. Vaginal smears were taken from the female rats and a sperm search was performed under a microscope at a magnification of 1200 x. When the smear provided positive, the female rats were separated as first-day pregnant animals.

##### **3.1.1. *In vitro* studies**

###### ***Uterus preparation***

Uteri were removed from rats (250-350 g) on day 15, 18, 20 or 22 of pregnancy. Muscle rings 5 mm long were sliced from the uterine horns and mounted vertically in an organ bath containing 10 ml de Jongh solution (composition: 137 mM NaCl, 3 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 12 mM NaHCO<sub>3</sub>, 4 mM NaH<sub>2</sub>PO<sub>4</sub>, 6 mM glucose, pH=7.4). The organ bath was maintained at 37 °C and carbogen (95% O<sub>2</sub> + 5% CO<sub>2</sub>) was bubbled through it. After mounting, the rings were equilibrated for about 1 h before experiments were undertaken, with a solution change every 15 min. The initial tension of the preparation was set to about 1.25 g, which was relaxed to about 0.5 g at the end of equilibration. 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).

### ***Nifedipine studies***

Contractions were elicited with 25 mM or 100 mM KCl, and noncumulative dose–response curves were constructed in each experiment in the presence of nifedipine ( $10^{-11}$ – $10^{-6}$  M) (Sigma-Aldrich, Budapest, Hungary). Following the addition of each concentration of nifedipine, recording was performed for 300 s. Concentration–response curves were fitted, and areas under curves (AUCs) were evaluated and analyzed statistically with the Prism 4.0 (Graphpad Software Inc. San Diego, CA, USA) computer program. From the AUC values, the maximal inhibitory effect of nifedipine on a given day of pregnancy ( $E_{\max}$ ) and the concentration of nifedipine eliciting 50% of the maximal inhibition of uterine contraction ( $EC_{50}$ ) were calculated. For statistical evaluations, data were analyzed by the ANOVA Neuman-Keuls test.

### ***Investigation the effect of $BK_{Ca}$ -channel blockers on nifedipine action***

Uteri were removed from rats (250-350g) on day 22 of pregnancy and mounted vertically in the organ bath as described above. Contractions were elicited with  $10^{-6}$  M oxytocin and cumulative nifedipine ( $10^{-11}$  –  $10^{-6}$  M) dose-response curves were constructed in each experiment in the presence of paxilline (5  $\mu$ M) (Sigma-Aldrich, Budapest, Hungary) or TEA ( $10^{-3}$  M) (Sigma-Aldrich, Budapest, Hungary). For statistical evaluations, data were analyzed by the ANOVA Neuman-Keuls test.

### ***Nifedipine combination with terbutaline***

Uteri were removed from rats (250-350 g) on day 22 of pregnancy and mounted vertically in the organ bath as described above. Contractions were elicited with 25 mM KCl, and non-cumulative dose–response curves were constructed in each experiment in the presence of nifedipine ( $10^{-11}$ – $10^{-6}$  M) and terbutaline (Sigma-Aldrich, Budapest, Hungary) ( $10^{-7}$  M) or terbutaline ( $10^{-10}$ – $10^{-4}$  M) and nifedipine ( $10^{-7}$  M). The effects of the nifedipine – terbutaline combination were also investigated in the absence of  $Ca^{2+}$  ion *in vitro*. De Jongh solution containing 0.5 mM  $Ca^{2+}$  ion was used to induce a low  $Ca^{2+}$  environment. After the equilibration period, the normal De Jongh solution was changed to the low  $Ca^{2+}$ -containing solution. The  $E_{\max}$  and  $EC_{50}$  values of the curves obtained with the

combinations were calculated. For statistical evaluations, data were analyzed through the unpaired t test.

#### ***P4 treatment***

The P4 treatment of the pregnant animals was started on day 15 of pregnancy. P4 (Sigma Aldrich, Budapest, Hungary) was dissolved in corn oil and injected subcutaneously every day up to day 21 in a dose of 0.5 mg/0.1 ml. On day 22, the uterine samples were collected and the contractility studies (25 mM KCl) were carried out with nifedipine as described above.

#### **3.1.2. *In vivo* studies**

##### ***Induction of PTL***

PTL was induced according to Rechberger *et al* (1996). Briefly, the animals were treated with mifepristone (3 mg per 0.1 ml) (donated by Richter Gedeon NyRt, Budapest, Hungary) and PGE<sub>2</sub> (0.5 mg/animal) (Sigma Aldrich, Budapest, Hungary) on day 19 of pregnancy. Mifepristone was suspended in olive oil and given as a subcutaneous injection at 9:00 <sub>A.M.</sub>. At 4:00 <sub>P.M.</sub>, PGE<sub>2</sub> was applied intravaginally. The delivery time of the first foetus was noted as the duration in hours from the time of mifepristone administration.

##### ***Treatments of the animals***

Nifedipine was dissolved in a 6:6:4 polyethylene glycol: ethanol: physiologic saline mixture. Salmeterol xinafoate (Sigma Aldrich, Budapest, Hungary) was dissolved in a 1:1 methanol-water mixture. Alzet osmotic pumps (model 2ml1; DURECT Corp, Cupertino, CA) were loaded with nifedipine, salmeterol xinofoate solution or vehicle. In combination studies two different osmotic pumps were inserted subcutaneously into the back skin of rats on days 16 or 18 of pregnancy (which may correlate to gestation weeks 30-35 in humans) under isoflurane anesthesia (Burton's narcotic apparatus). The dose of nifedipine was 3.89 mg/day per animal and the dose of salmeterol xinofoate was 0.13 mg/day per animal.

The P4 treatment of the pregnant animals was started on day 15 of pregnancy. P4 was dissolved in corn oil and injected subcutaneously every day up to delivery (days 19 or 20) in a dose of 0.5 mg/0.1 ml.

Group A was the control group, while group B was treated with vehicle, group C with nifedipine, group D with the nifedipine–salmeterol combination and group E with the nifedipine–P4 combination. There were 8 rats in each group. Statistical analyses were carried out with the analysis of Dunnett's Multiple Comparison Test.

### **3.2. Human myometrial studies**

#### ***Tissue collection***

Biopsy specimens of human myometrial tissue were obtained at caesarean section in the third trimester of pregnancy. Uterine smooth muscle tissue samples were collected at 37 to 40 weeks of gestation from 13 women who were undergoing caesarean delivery because of foetal distress, growth restriction, a previous caesarean delivery, breech presentation, or suspected cephalopelvic disproportion. None of the women was treated with any tocolytic agent. The parity of the women varied from 0 to 3, and their mean age was 29.8 years, range 26-37 years. In all cases, the operation was performed under spinal anaesthesia. The Ethical Committee of Albert Szent-Györgyi Clinical Center approved the clinical protocol for the use of human tissue from fully informed and consenting women (registration number: 114/2009). Each tissue sample (10x10x20 mm) was obtained from the upper edge of a lower-segment transverse incision, after delivery of the child, but before oxytocin was given to the mother. Tissues were stored in Krebs-Henseleit solution at 4 °C, and were used within 12 hours of collection.

#### ***Isolated organ studies***

Longitudinal myometrial strips (measuring approximately 3x5x10 mm) were mounted vertically in an organ bath containing 10 mL Krebs-Henseleit (composition: 118 mM sodium chloride, 5 mM potassium chloride, 2 mM calcium chloride, 0.5 mM magnesium sulphate, 1 mM potassium sulphate, 25 mM sodium bicarbonate, 10 mM glucose; pH 7.4) solution. The organ bath was maintained at 37 °C and carbogen (95% O<sub>2</sub> + 5% CO<sub>2</sub>) was bubbled through it. After mounting, the rings were equilibrated for ~ 2 hours before

experiments were undertaken, with a solution change every 15 min. The initial tension of the preparation was set to  $\sim 3.00$  g, which was relaxed to  $\sim 1.5$  g at the end of equilibration. The tension of the myometrial rings was measured with an isometric force transducer (SG-02; Experimetria Ltd., Budapest, Hungary) and recorded with a SPEL Advanced ISOSYS Data Acquisition System (Experimetria Ltd., Budapest, Hungary).

After eliciting contractions with  $10^{-6}$  M oxytocin, noncumulative dose–response curves were constructed in each experiment with nifedipine ( $10^{-11}$ – $10^{-5}$  M) and terbutaline ( $10^{-7}$  M) or terbutaline ( $10^{-11}$ – $10^{-5}$  M) and nifedipine ( $10^{-7}$  M). Drugs were used from stock solutions and stored at  $-20$  °C. Concentrations–response curves were fitted, and areas under curves (AUCs) were evaluated and analyzed statistically with the Prism 4.0 (Graphpad Software Inc. San Diego, CA, USA) computer program. The maximal inhibitory effect ( $E_{\max}$ ) and  $EC_{50}$  values in the curves obtained with the combinations were calculated. For statistical evaluations, data were analyzed by use of the unpaired t test.

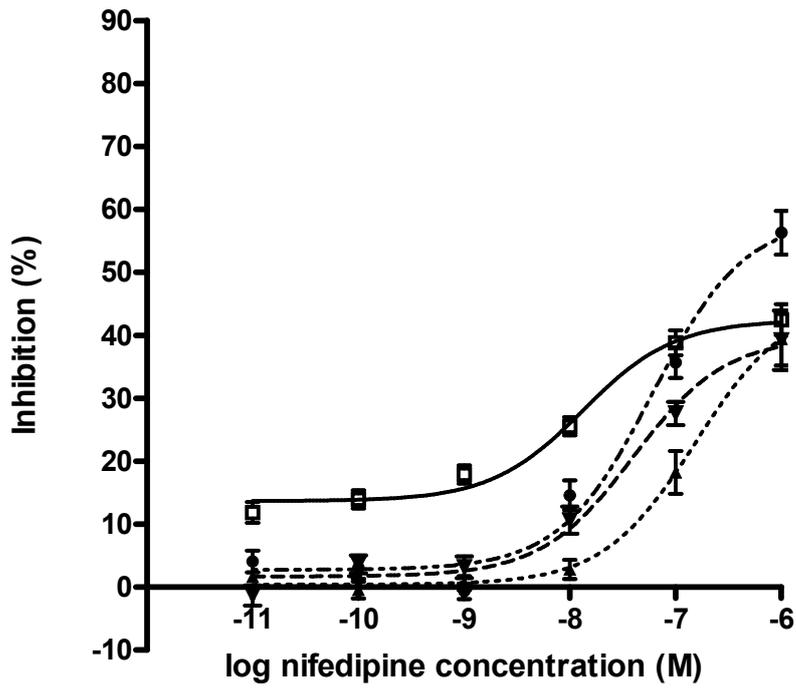
## 4. Results

### 4.1. Organ bath studies

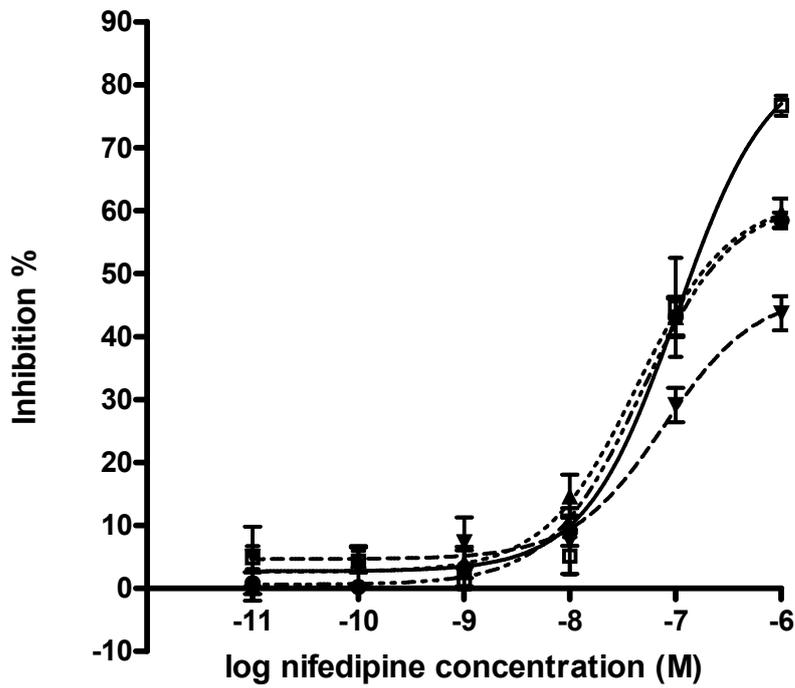
#### 4.1.1. The effect of nifedipine on isolated pregnant rat uteri on different days (15, 18, 20 and 22) of pregnancy

The 25 mM and 100 mM KCl-stimulated uterine contractions were inhibited concentration-dependently by nifedipine in the range of  $10^{-11}$ – $10^{-6}$  M (Fig. 1a, b). As concerns the contractions induced by 100 mM KCl, the calculated  $EC_{50}$  was lower on day 18 than on day 15 (Table 1), but there were no changes on the other days. There were significant changes in  $E_{max}$  on days 18, 20 and 22 as compared that on day 15. In the presence of 25 mM KCl, the maximal relaxing effect of nifedipine was significantly greater on days 20 and 22 than on day 15. There were no significant changes in  $EC_{50}$  (Table 2).

a



b



**Fig. 1.**

Contraction inhibiting effect of nifedipine on isolated pregnant rat uteri evoked by 100 mM (a) and 25 mM (b) potassium chloride *in vitro* on different days of pregnancy.

(● day 15, ▲ day 18, ▼ day 20, □ day 22).

**Table 1.**

The EC<sub>50</sub> and E<sub>max</sub> values of uterine relaxation curves induced by nifedipine (10<sup>-11</sup>-10<sup>-6</sup> M) in the presence of 100 mM KCl on different days (15, 18, 20 and 22) of pregnancy.

On each day, the level of significance relates to the comparison with the values on day 15.

S.E.M.: standard error mean; ns: not significant, \*p<0.05, \*\*\*p<0.001

Day of pregnancy	EC <sub>50</sub> (M ± S.E.M.)	E <sub>max</sub> (% ± S.E.M.)
15	6.8 X 10 <sup>-8</sup> ± 1.1 X 10 <sup>-8</sup>	62.7 ± 2.7
18	1.5 X 10 <sup>-7</sup> ± 4.7 X 10 <sup>-8</sup> ***	46.9 ± 3.6 *
20	3.9 X 10 <sup>-8</sup> ± 1.1 X 10 <sup>-8</sup> ns	39.6 ± 4.4 *
22	1.2 X 10 <sup>-8</sup> ± 1.1 X 10 <sup>-8</sup> ns	41.7 ± 4.3 *

**Table 2.**

The EC<sub>50</sub> and E<sub>max</sub> values of uterine relaxation curves induced by nifedipine (10<sup>-11</sup>-10<sup>-6</sup> M) in the presence of 25 mM KCl on different days (15, 18, 20 and 22) of pregnancy.

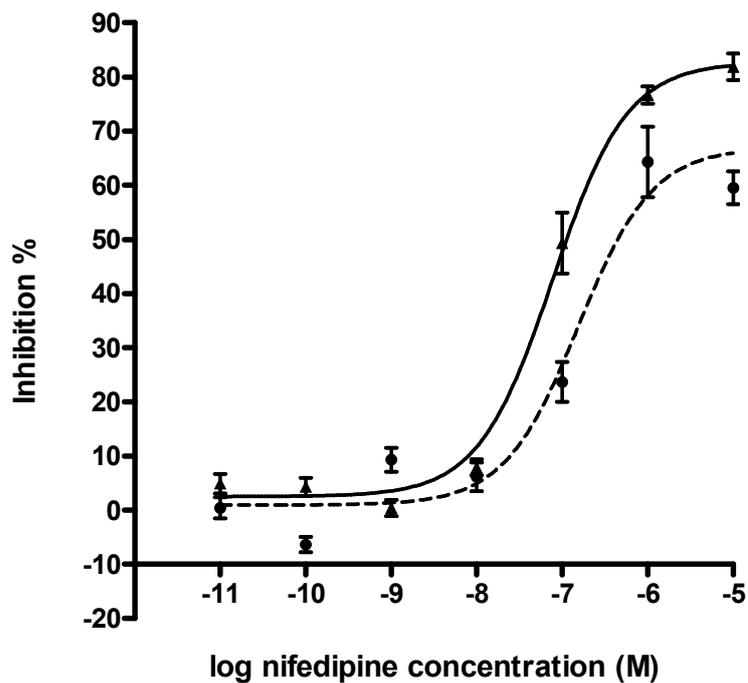
On each day, the level of significance relates to the comparison with the values on day 15.

S.E.M.: standard error mean; ns: not significant, \*\*p<0.01, \*\*\*p<0.001

Day of pregnancy	EC <sub>50</sub> (M ± S.E.M.)	E <sub>max</sub> (% ± S.E.M.)
15	4.8 X 10 <sup>-8</sup> ± 1.4 X 10 <sup>-7</sup>	61.7 ± 1.9
18	4.7 X 10 <sup>-8</sup> ± 1.1 X 10 <sup>-8</sup> ns	61.8 ± 2.9 ns
20	7.4 X 10 <sup>-8</sup> ± 2.4 X 10 <sup>-8</sup> ns	47.0 ± 3.1 **
22	1.03 X 10 <sup>-7</sup> ± 3.6 X 10 <sup>-8</sup> ns	84.6 ± 2.6 ***

#### 4.1.2. The effect of nifedipine after P4 pre-treatment on isolated pregnant rat uteri on pregnancy day 22

The P4 pre-treatment, was started on day 15 of pregnancy and injected subcutaneously every day up to day 21, decreased the maximal inhibitory effect of nifedipine on day 22 and more than doubled its  $EC_{50}$  (Fig. 2, Table 3).



**Fig. 2.**

Contraction inhibiting effect of nifedipine on isolated pregnant rat uteri evoked by 25 mM KCl on day 22 after the 7-day-long P4 pre-treatment (from day 15 to day 21).

▲nifedipine, ●after P4 pre-treatment.

**Table 3.**

The EC<sub>50</sub> and E<sub>max</sub> values of uterine relaxation curves induced by nifedipine (10<sup>-11</sup>–10<sup>-5</sup> M) in the presence of 25 mM KCl on day 22 after P4 pre-treatment (started on day 15 of pregnancy and injected subcutaneously every day up to day 21).

S.E.M.: standard error mean; ns: not significant, \*\*p<0.01

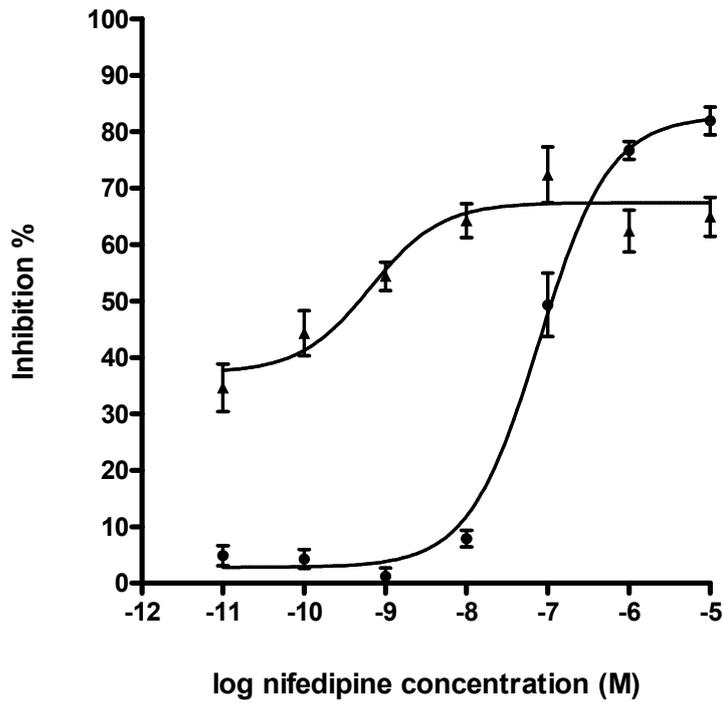
	EC <sub>50</sub> (M ± S.E.M.)	E <sub>max</sub> (% ± S.E.M.)
nifedipine	9.7 X 10 <sup>-8</sup> ± 2.8 X 10 <sup>-8</sup>	87.3 ± 2.2
nifedipine + P4	2.8 X 10 <sup>-7</sup> ± 4.9 X 10 <sup>-8</sup> **	81.9 ± 8.7 <sup>ns</sup>

#### **4.1.3. The effect of nifedipine – terbutaline combination on isolated pregnant rat uteri on pregnancy day 22**

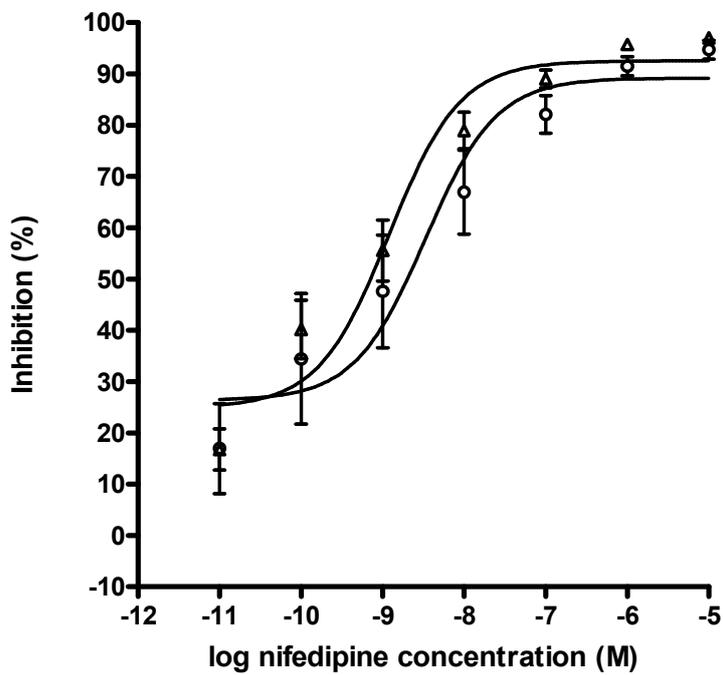
The concentration–response curves for nifedipine in the presence of  $10^{-7}$  M terbutaline were shifted to the left and a decrease in the maximal inhibitory effect was observed (Fig. 3a, Table 4a). In the presence of 0.5 mM  $\text{Ca}^{2+}$  ( $\text{Ca}^{2+}$ -poor buffer), terbutaline did not alter the effect of nifedipine (Fig. 3b, Table 4b).

The concentration–response curves for terbutaline in the presence of  $10^{-7}$  M nifedipine were also shifted to the left, but this shift was greater than that of the nifedipine curve by terbutaline. Nifedipine also significantly increased the  $E_{\text{max}}$  of terbutaline (Fig. 4a, Table 5a). In the  $\text{Ca}^{2+}$ -poor buffer, the presence of nifedipine increased the  $\text{EC}_{50}$  of terbutaline, but did not alter its  $E_{\text{max}}$  (Fig. 4b, Table 5b).

a



b



**Fig. 3**

Effect of nifedipine-terbutaline combination on the 25 mM KCl evoked contraction *in vitro* on day 22.

(a) ● nifedipine; ▲ nifedipine and terbutaline (10<sup>-7</sup> M) in 1 mM Ca<sup>2+</sup> containing buffer

(b) ○ nifedipine; Δ nifedipine and terbutaline (10<sup>-7</sup> M) in 0.5 mM Ca<sup>2+</sup> containing buffer.

**Table 4**

The EC<sub>50</sub> and E<sub>max</sub> values of uterine relaxation curves induced by nifedipine – terbutaline combination in the presence of 25 mM KCl on day 22 *in vitro* in case of 1mM (a), 0.5mM (b) Ca<sup>2+</sup> - concentration.

The level of significance relates to the comparison with the values of nifedipine and the values of terbutaline. S.E.M.: standard error mean; ns: not significant, \*p<0.05, \*\*p<0.01

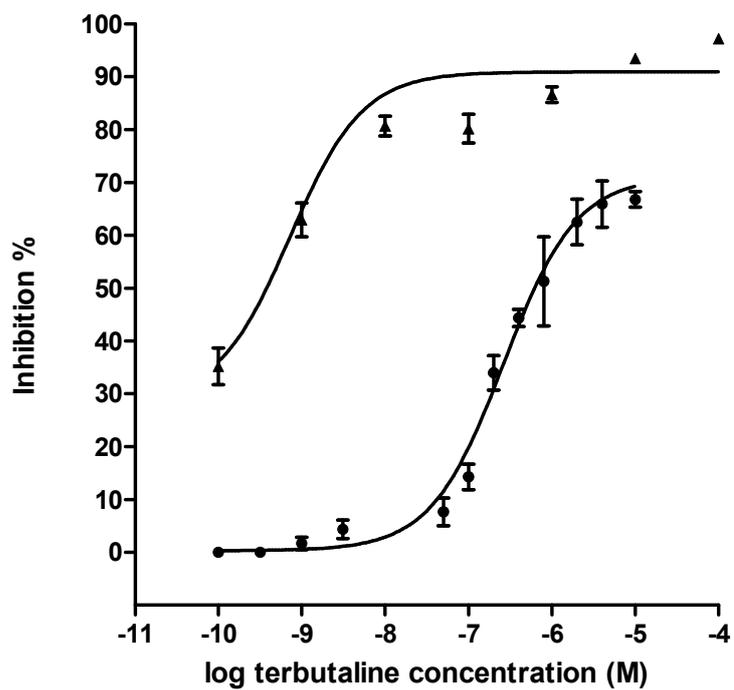
**a**

	EC <sub>50</sub> (M ± S.E.M.)	E <sub>max</sub> (% ± S.E.M.)
nifedipine	7.9 X 10 <sup>-8</sup> ± 1.3 X 10 <sup>-8</sup>	82.9 ± 1.4
nif + terb (10 <sup>-7</sup> M)	7.0 X 10 <sup>-10</sup> ± 1.2 X 10 <sup>-10</sup> *	68.0 ± 4.5**

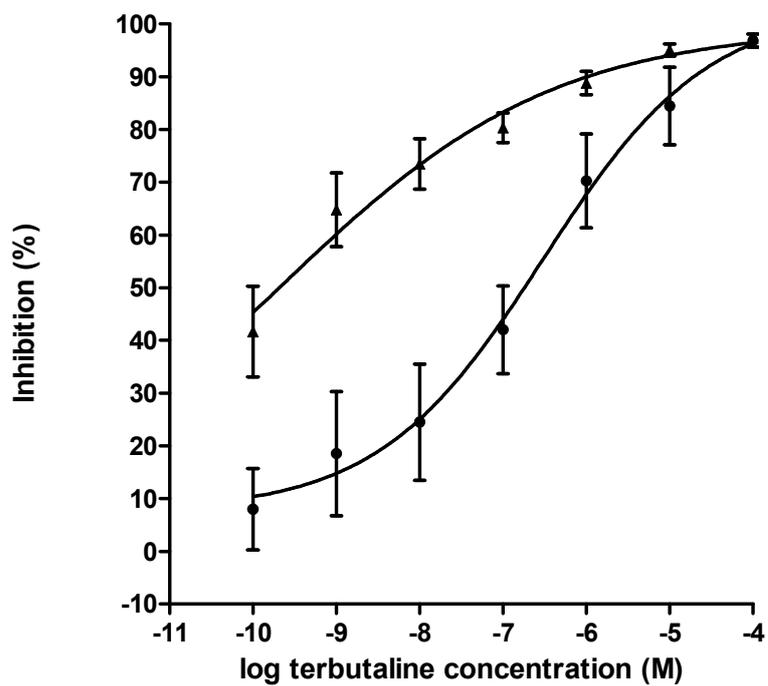
**b**

	EC <sub>50</sub> (M ± S.E.M.)	E <sub>max</sub> (% ± S.E.M.)
nifedipine	4.0 X 10 <sup>-9</sup> ± 2.1 X 10 <sup>-9</sup>	89.2 ± 2.1
nif + terb (10 <sup>-7</sup> M)	1.6 X 10 <sup>-9</sup> ± 7.3 X 10 <sup>-10</sup> ns	92.6 ± 0.4 <sup>ns</sup>

a



b



**Fig. 4**

Effect of nifedipine – terbutaline combination on the 25 mM KCl evoked contraction *in vitro* on day 22.

(a) ● terbutaline; ▲ terbutaline and nifedipine 10<sup>-7</sup>M

(b) in 0.5 mM Ca<sup>2+</sup> containing buffer (● terbutaline; ▲ terbutaline and nifedipine 10<sup>-7</sup>M).

**Table 5**

The EC<sub>50</sub> and E<sub>max</sub> values of uterine relaxation curves induced by nifedipine – terbutaline combination in the presence of 25 mM KCl on day 22 *in vitro* in case of 1 mM (a), 0.5 mM (b) Ca<sup>2+</sup> - concentration.

The level of significance relates to the comparison with the values of nifedipine and the values of terbutaline. S.E.M.: standard error mean; ns: not significant, \*p<0.05, \*\*p<0.01

\*\*\*p<0.001

**a**

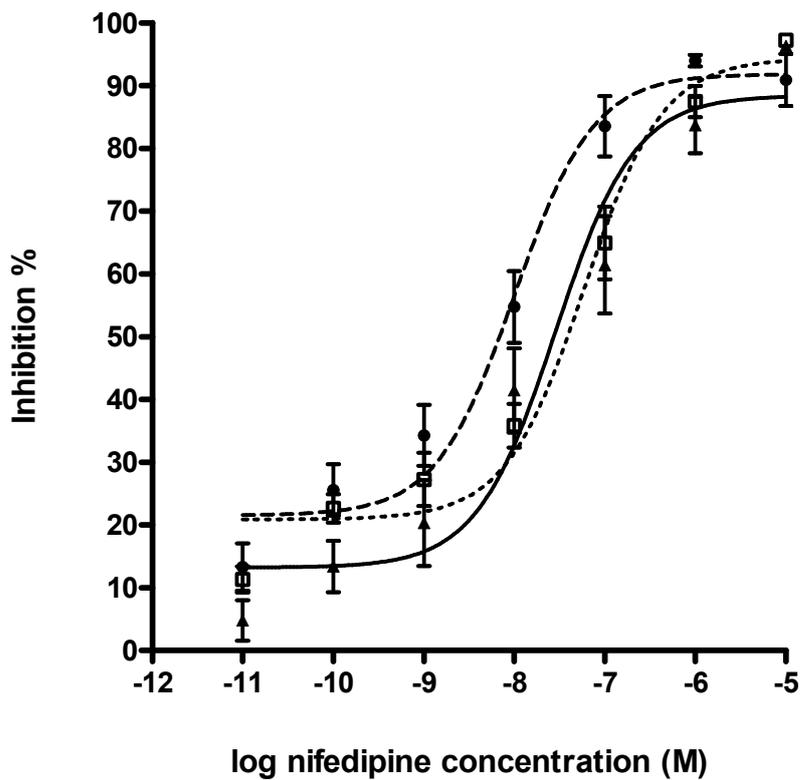
	EC <sub>50</sub> (M ± S.E.M.)	E <sub>max</sub> (% ± S.E.M.)
terbutaline	6.9 X 10 <sup>-7</sup> ± 1.7 X 10 <sup>-7</sup>	74.6 ± 2.8
terb + nif (10 <sup>-7</sup> M)	8.3 X 10 <sup>-10</sup> ± 1.6 X 10 <sup>-10</sup> **	90.8 ± 0.8 ***

**b**

	EC <sub>50</sub> (M ± S.E.M.)	E <sub>max</sub> (% ± S.E.M.)
terbutaline	1.5 X 10 <sup>-7</sup> ± 1.6 X 10 <sup>-10</sup>	97.6 ± 1.1
terb + nif (10 <sup>-7</sup> M)	5.3 X 10 <sup>-9</sup> ± 2.1 X 10 <sup>-9</sup> **	95.4 ± 1.1 ns

#### 4.1.4. The effect of nifedipine – BK<sub>Ca</sub>-channel blocker combination on the oxytocin-evoked pregnant rat myometrium on pregnancy day 22 *in vitro*

The presence of selective or non-selective BK<sub>Ca</sub>-channel inhibitors (paxilline or TEA) had no effect on the maximal relaxing effect of nifedipine and did not modify the EC<sub>50</sub> values of nifedipine. Although, paxilline shifted the nifedipine curve to the left, but it was not significant (Fig. 5).



**Fig. 5.**

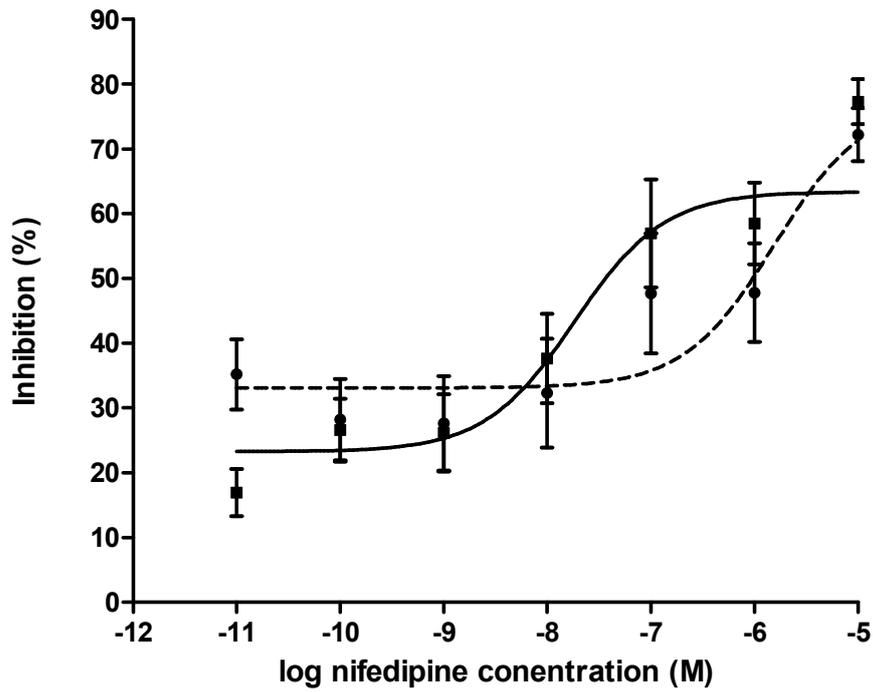
The effects of treatment with the nifedipine – TEA and nifedipine – paxilline combination on oxytocin-evoked myometrial contractions *in vitro* on pregnancy day 22

□: TEA (10<sup>-3</sup> M); ●: paxilline (5μM); ▲: nifedipine (10<sup>-11</sup> – 10<sup>-5</sup> M)

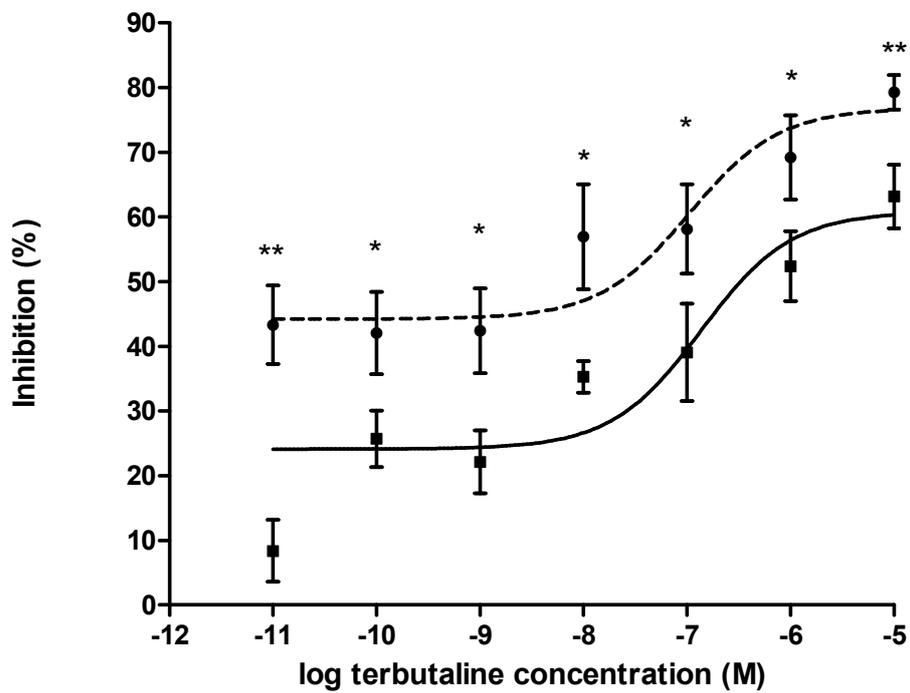
#### **4.1.5. The effect of nifedipine – terbutaline combination on the oxytocin-evoked human myometrial contractions *in vitro***

The  $10^{-6}$  M oxytocin-stimulated human uterine contractions were inhibited concentration-dependently by nifedipine and terbutaline in the range  $10^{-5}$ – $10^{-11}$  M (Fig. 6. a, b). The addition of  $10^{-7}$  M terbutaline to nifedipine did not alter the  $E_{\max}$  of nifedipine, but decreased  $EC_{50}$  from  $1.8 \times 10^{-8}$  M to  $1.5 \times 10^{-6}$  M ( $p < 0.05$ ) (Fig. 6a). The addition of  $10^{-7}$  M nifedipine to terbutaline increased the  $E_{\max}$  of terbutaline (Fig. 6b), from 60.8% to 76.8% ( $p < 0.01$ ) but  $EC_{50}$  was not changed.

a



b



**Fig. 5.**

The effects of treatment with the nifedipine – terbutaline combination on oxytocin-evoked human myometrial contractions *in vitro*. **a:** ■ nifedipine ( $10^{-11}$ - $10^{-5}$  M); ● terbutaline ( $10^{-7}$  M) + nifedipine ( $10^{-11}$ - $10^{-5}$  M) **b:** ■ terbutaline ( $10^{-11}$ - $10^{-5}$  M); ● nifedipine ( $10^{-7}$  M) + terbutaline ( $10^{-11}$ - $10^{-5}$  M)

## 4.2. *In vivo* studies

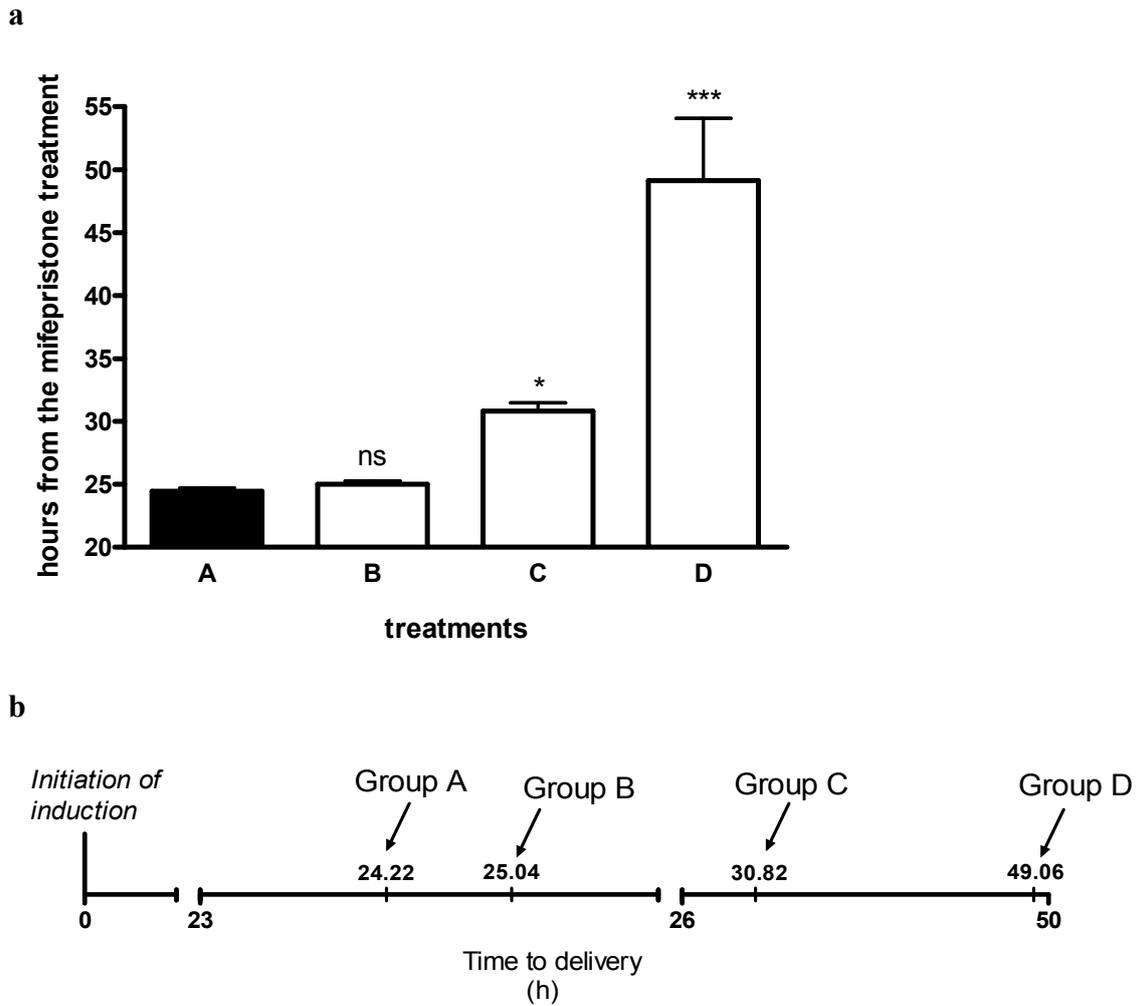
### 4.2.1. The effect of nifedipine, salmeterol and P4 in the hormone-induced PTL model

In group A (control), preterm labour occurred within 24 hours after mifepristone treatment, at about 9:00 a.m. on pregnancy day 20. The vehicle (group B) did not alter the time of delivery relative to that in group A.

Nifedipine (group C) treatment started on pregnancy day 16 was effective in delaying the hormone-induced preterm delivery by 6.6 hours. In group D (nifedipine–salmeterol combination), the treatment was extremely effective; preterm birth was delayed by ~ 24 hours as compared with group A (Fig. 6a, b).

When started on day 18 of pregnancy (Fig. 7a, b), nifedipine treatment (group C) was not effective. In contrast in group D (combination therapy) the treatment was effective; labour was delayed by ~ 25 hours. The difference in efficacy between groups C and D was most expressed for the treatment started on day 18.

With the nifedipine – P4 combination (Fig. 8a, b), the P4 pre-treatment (group E) abolished the effect of nifedipine (group C).



**Fig. 6.**

The effects of treatment with nifedipine (3.89 mg/day) or the nifedipine – salmeterol combination on hormone-induced preterm delivery in the rat (n = 8 for each group). Treatment was started on gestation day 16.

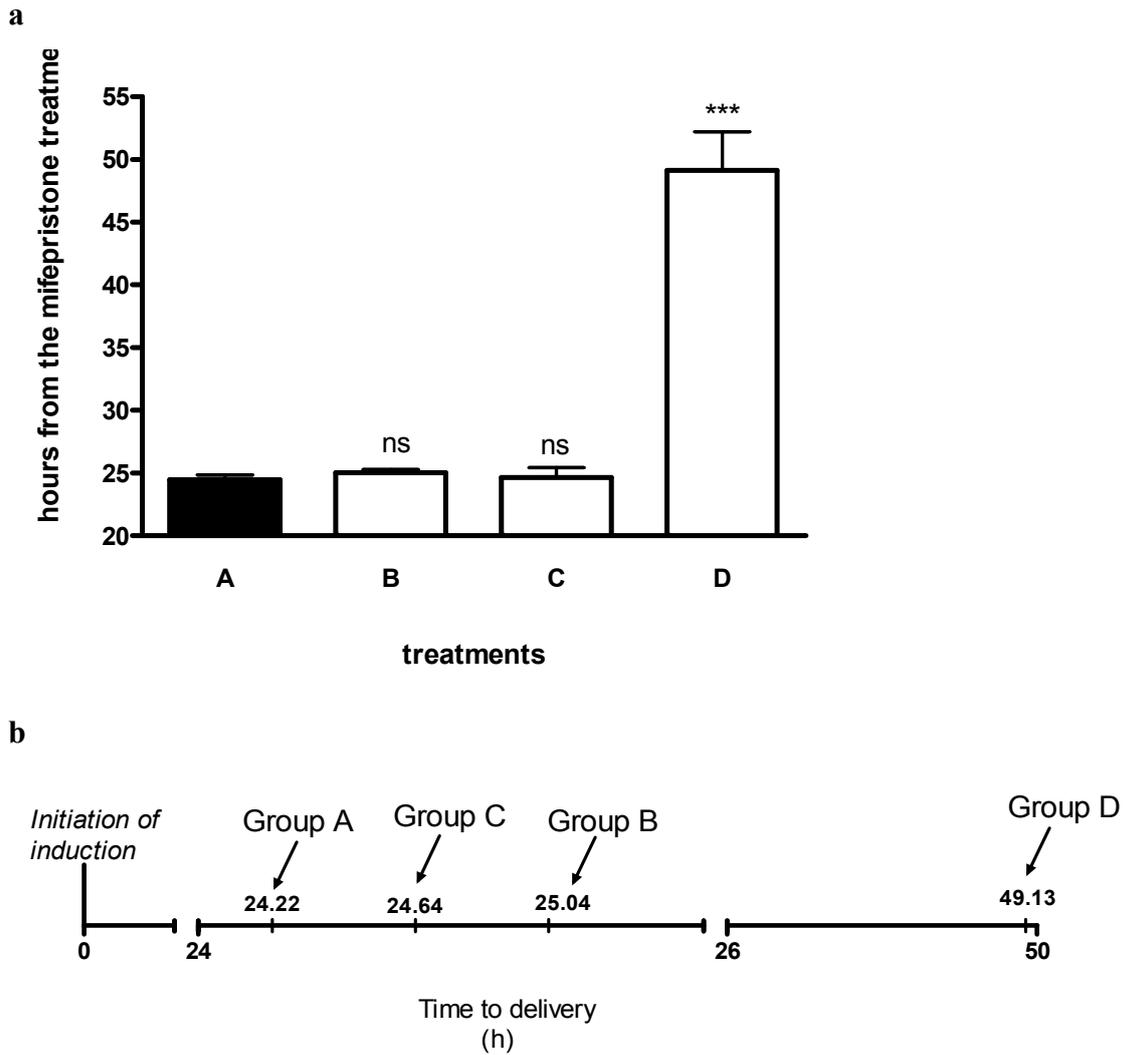
- a.** A: control group; B: vehicle-treated (PEG400 : ethanol : physiological salt solution = 6:6:4 + methanol : water =1:1 in osmotic minipump); C: nifedipine-treated; D: nifedipine – salmeterol combination-treated animals.

The *bar graphs* show means ± SD. The effects were compared with the results on group A.

ns: not significant; \*: p<0.05; \*\*\*: p<0.001.

The difference in efficacy between the treatments in groups C and D was significant (p<0.001).

- b.** The time to delivery from the initiation of PTL (mifepristone treatment). The longest delay was caused by the combination of nifedipine and salmeterol (49.06 h).



**Fig. 7.**

The effects of treatment with nifedipine (3.89 mg/day) or the nifedipine – salmeterol combination on hormone-induced preterm delivery in the rat (n = 8 for each group). Treatment was started on gestation day 18.

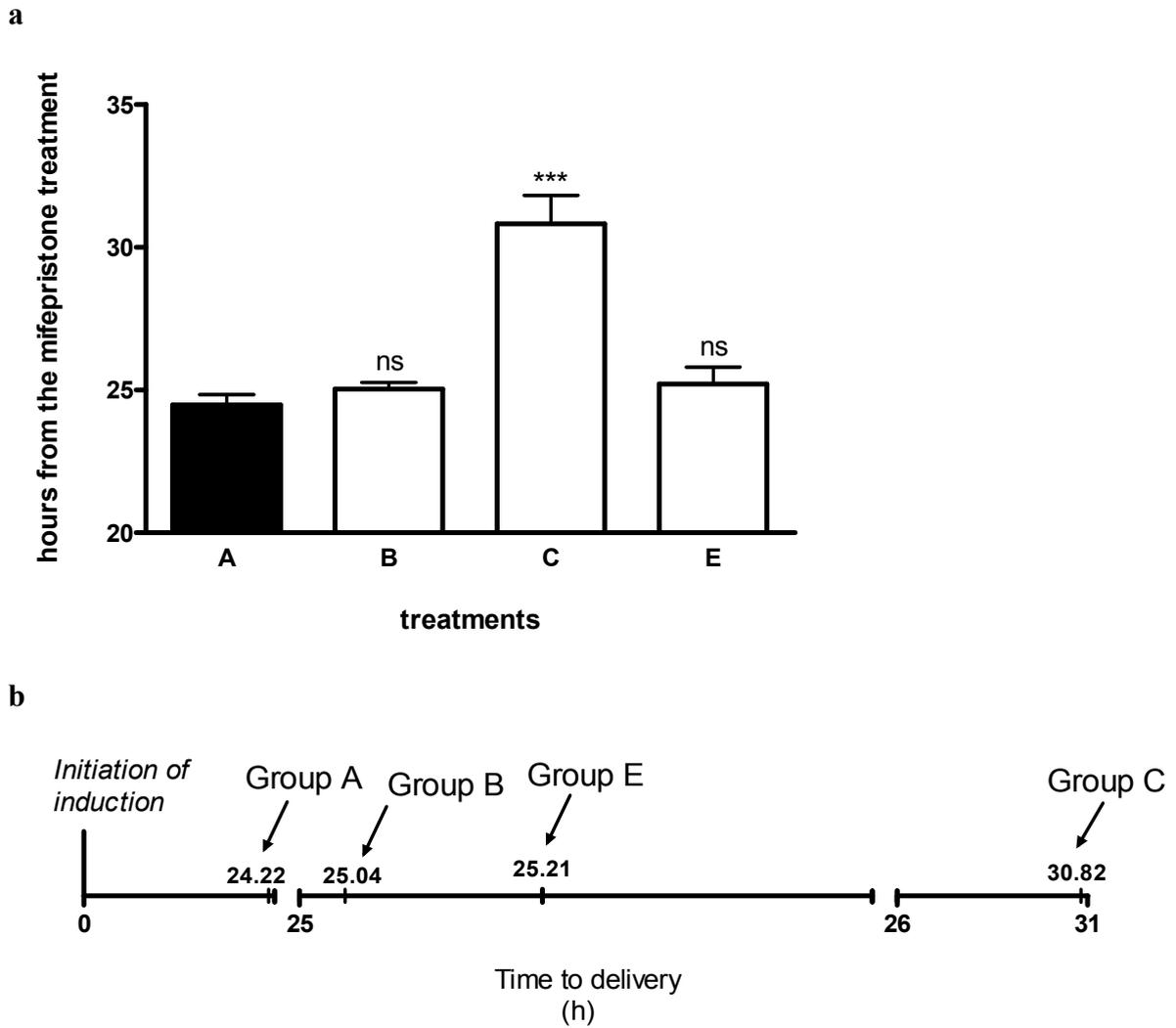
- a.** A: control group; B: vehicle-treated (PEG400 : ethanol : physiological salt solution = 6:6:4 + methanol : water =1:1 in osmotic minipump); C: nifedipine-treated; D: nifedipine – salmeterol combination-treated animals.

The *bar graphs* show means ± SD. The effects were compared with the results on group A.

\*\*\*: p<0.001.

The difference in efficacy between the treatments in group C and D was significant (p<0.001).

- b.** The time to delivery from the initiation of PTL (mifepristone treatment). The longest delay was caused by combination of nifedipine and salmeterol (49.13 h)



**Fig. 8.**

The effects of treatment with nifedipine (3.89 mg/day) or the nifedipine – P4 combination on hormone-induced preterm delivery in the rat (n = 8 for each group). Treatments were started on gestation day 16.

**a.** A: control group; B: vehicle-treated (PEG400 : ethanol : physiological salt solution = 6:6:4 in osmotic minipump + corn oil s.c.); C: nifedipine treated; E: nifedipine – P4 combination-treated animals.

The *bar graphs* show means ± SD. The effects were compared with the results on group A. *ns*: not significant; \*\*\*:  $p < 0.001$ . The difference in efficacy between the treatments in group C and E was significant ( $p < 0.001$ ).

**b.** The time to delivery from the initiation of PTL (mifepristone treatment). The longest delay was caused by nifedipine-treatment alone.

## 5. Discussion

PTB is the one of the greatest challenges in obstetrical practice. The currently used medications are not able to stop or sufficiently delay the process of PTB, therefore there is growing interest in experimental studies of the possible use of different tocolytic combinations to decrease the potentially maternal and foetal adverse events and improving the perinatal outcome.

The most important factor controlling force in the myometrium is the concentration of intracellular  $\text{Ca}^{2+}$ , and membrane potential is the major factor governing  $\text{Ca}^{2+}$  entry into the cell (Wray *et al.*, 2003). High  $\text{K}^+$  stimulation, which provokes membrane depolarization and uterine contractions, is the most common method for the introduction of  $\text{Ca}^{2+}$  into cells without receptor stimulation. There are a number of data relating to the use of different concentrations of KCl (from low to high  $\text{K}^+$ ) to evoke contraction in vitro by opening VG  $\text{Ca}^{2+}$  channels, though it is not clear which of these concentrations causes rhythmic contractions of the uterus providing an appropriate model for investigation of the pregnant uterus-relaxing effects.

We found that in the presence of 25 mM KCl the uterine contractions were rhythmic and the relaxing effect of nifedipine was the highest on the last day of pregnancy. With 100 mM KCl, however, the contractions became spastic and the inhibitory action of nifedipine was highest on day 15, but was later quite weak. These results led us to conclude that stimulation with 25 mM KCl is much more appropriate for investigations of the action of the  $\text{Ca}^{2+}$  channel blocker nifedipine. As the relaxing effect of nifedipine was highest on the last day of pregnancy, further experiments were carried out on day 22.

It was earlier demonstrated that the myometrial DHP binding is increased more than 3-fold at the end of pregnancy in the rat (Mershon *et al.*, 1994), and this result was supported by our findings. However, others found that the protein expressions and mRNA levels of the pore-forming  $\alpha_1$ -subunits of the  $\text{Ca}^{2+}$  channels were not altered by pregnancy either in rats or in humans (Helguera *et al.*, 2002; Batra and Popper, 1989). It is known that the  $\text{Ca}_v1.2$  channels possess splice variants in the cardiac muscle, which have different pharmacological and electrophysiological properties (Liao *et al.*, 2005). So far, such variants have not been discovered in the myometrium, but the apparent contradictions observed between the above-mentioned papers and our result might be explained by the existence of putative channel splice variants in the pregnant uterus.

The activity of the Ca<sub>v</sub>1.2 channel is regulated by several factors (Kobayashi *et al.*, 2007). In our study, three of them (BK<sub>Ca</sub> channels, adrenergic system and P4/E2 ratio) were investigated with regard to how to influence the uterus-relaxing effect of nifedipine *in vitro*.

The studies with the selective BK<sub>Ca</sub> channel blocker paxilline and non-selective blocker TEA were carried out by oxytocin-induced contractions, because in these cases the stimulation with KCl was not useful. The results revealed that BK<sub>Ca</sub> channels and any other K<sup>+</sup> channels, contrary to human myometrium (Moynihan *et al.*, 2008), are not involved in the relaxing effect of nifedipine in pregnant rat myometrium.

P4 is regarded as a preventive drug against PTB, especially in late-preterm birth (Borna and Sahabi, 2008). Unfortunately, the *in vivo* P4 pre-treatment decreased the maximal inhibitory effect of nifedipine and increased its EC<sub>50</sub>. The uterine smooth muscle possesses  $\alpha_{1C-long}$  and  $\alpha_{1C-short}$  isoforms of the L-type Ca<sup>2+</sup> channel. In the presence of the long isoform, the channel has lower activity than with the short isoform. In the pregnant rat uterus, P4 and E2 enhance the expressions of the  $\alpha_{1C-long}$  and  $\alpha_{1C-short}$  isoforms, respectively, of the L-type Ca<sup>2+</sup> channels (Helguera *et al.*, 2002). These facts explain why P4 pre-treatment worsened the relaxing effect of nifedipine.

The other investigated factor which regulates the Ca<sub>v</sub>1.2 channel was the  $\beta_2$ -adrenergic system. We found synergism in the uterus-relaxing effect of nifedipine and the  $\beta_2$ -AR agonist terbutaline, although the extent of potentiation depended on the sequence of administration of the two compounds. When terbutaline was added first in a single dose, synergism was found in EC<sub>50</sub> (the nifedipine curve was shifted to the left), but the maximal inhibitory effect of nifedipine was lower. When nifedipine was administered first, the relaxing effect of terbutaline was obviously stronger.

It is known that stimulation of  $\beta_2$ -ARs activates G-proteins and increases the intracellular cAMP level. cAMP activates PK A the activated form of which phosphorylates the Ca<sub>v</sub>1.2 channels. This mechanism is well known in the heart muscle (Kamp and Hell, 2000) and it is very probably similar in the pregnant myometrium. The entry of the Ca<sup>2+</sup> into the cells through the VG Ca<sup>2+</sup> channel is one of the crucial factors in the generation of smooth muscle contraction. Terbutaline possibly activates the Ca<sub>v</sub>1.2 channels and decreases the maximal relaxing effect of nifedipine. The resultant effect of the increase of cAMP level and activation of Ca<sub>v</sub>1.2 channels causes a weaker smooth muscle relaxation. In the

opposite case, when nifedipine is administered first, the Ca<sub>v</sub>1.2 channels are blocked; hence, there is only a low possibility that terbutaline can activate them.

To check on the above-mentioned hypothesis, the synergism between the two compounds was investigated in Ca<sup>2+</sup>-poor buffer. A Ca<sup>2+</sup>-poor environment theoretically decreases the terbutaline-induced Ca<sup>2+</sup> influx and may alter the extent of the synergism. The Ca<sup>2+</sup>-poor environment shifted the nifedipine dose-response curve to the left, and the maximal inhibitory effect of nifedipine was so high that its effect could not be enhanced by terbutaline. In contrast, the Ca<sup>2+</sup>-poor environment shifted the terbutaline dose-response curve to the left, but nifedipine was able to enhance the shift. However, it could not increase the maximal uterus-relaxing effect of terbutaline, possibly because of the very strong blocking effect of the β-mimetic. These results indicate that, in a Ca<sup>2+</sup>-poor environment, terbutaline is not able to worsen the maximal effect of nifedipine, which suggests the role of the Ca<sup>2+</sup> inflow in the weakening effect of terbutaline.

Synergism between nifedipine and the β<sub>2</sub>-agonist terbutaline was also investigated on human myometrium tissue, which revealed that both nifedipine and terbutaline inhibit the oxytocin-induced myometrial contractions dose-dependently. When terbutaline was added first, it decreased the EC<sub>50</sub> of nifedipine (the nifedipine curve was shifted to the right), though it did not alter the E<sub>max</sub> of nifedipine. In the opposite case, nifedipine administered first increased the relaxing effect of terbutaline, though there was no difference in EC<sub>50</sub>. The results suggest a parallelism between the human situation and that in the rat myometrium.

In view of these results, the effects of nifedipine – salmeterol and nifedipine – P4 combination were investigated also in hormone-induced PTB model *in vivo*, whether these drug interactions apply under *in vivo* circumstances.

The effect of combined salmeterol – gestagen treatment in hormone-induced preterm delivery in rats *in vivo* was investigated earlier (Gálik *et al.*, 2008): salmeterol treatment started on pregnancy day 16 delayed preterm birth by 2.8 hours and the salmeterol – P4 combination caused a 4.5-hour delay.

Preterm delivery was induced by the deprivation of P4 using P4 antagonist combined with PGE<sub>2</sub> to accelerate cervical ripening on pregnancy day 19. At this stage of pregnancy the function of corpus luteum declines which is mainly attributed to the luteolytic effect of PGF<sub>2</sub>α (Hernandez *et al.*, 2009). It is also known that P4 induces the metabolism of PGs, thus substitution of P4 is anticipated to improve relaxation response in late-pregnant rat uterus (Farina *et al.*, 2004).

We used the same *in vivo* salmeterol dose as was effective in delaying PTB, and the P4 dose that had increased the effect of salmeterol. The *in vivo* dose for the tocolytic effect was calculated by using the pharmacokinetic parameters reported for nifedipine in pregnant rats by Downing *et al.* (Downing and Hollingsworth, 1998). We planned administration of a dose regimen via osmotic pumps which provided a plasma nifedipine concentration of  $\sim 1.5 \mu\text{g/ml}$  (estimated). Downing and Hollingsworth (1998) concluded that this plasma level of the drug did not cause significant changes in heart rate or blood pressure, but elicited a well-defined uterus-relaxing effect. We found that treatment with nifedipine alone started on pregnancy day 16 was more effective in delaying delivery than salmeterol treatment alone (in the earlier study) (Gálik *et al.*, 2008). Similarly, the nifedipine – salmeterol combination also had a greater effect than the salmeterol – gestagen combination (Gálik *et al.*, 2008).

The short-term effects of nifedipine and its combination with salmeterol were also tested in our experiments. Nifedipine treatment started on day 18 did not delay labour, but the nifedipine – salmeterol combination was as effective as the combination started on the pregnancy day 16. The ability of nifedipine to delay labour was tripled by its combination with the  $\beta_2$ -agonist. In our *in vivo* study two osmotic pumps were implanted subcutaneously in the rat and the administration of the two agents was simultaneous and continuous. The results suggested that the parallel administration of the two compounds may lead to a similar benefit as that of nifedipine-potentiated terbutaline treatment.

P4 pre-treatment also abolished the ability of nifedipine to delay labour in hormone-induced preterm delivery in rats *in vivo*. These results correlate with the hypothesis that progesterone decreases the activity of the L-type  $\text{Ca}^{2+}$  channels (Helguera *et al.*, 2002). Accordingly we presumed that a  $\text{Ca}^{2+}$  channel blocker + P4 combination might not have any benefit in clinical practice.

A weakness of this study is that the experiments do not provide data relating to the prompt effect of the drugs in the onset of hormone-induced preterm birth, but the investigation of this effect is almost impossible in rats. The first visual sign of the onset of labour is vaginal bleeding. From this time on, at most only 10-15 min is available until the delivery of the first foetus. This short period is not sufficient for the absorption of drugs administered. On the other hand, intravenous drug administration to delivering rats would be very difficult and might well cause severe stress for the animal, altering the delivery process. Despite this weakness, our study reports the first attempt to delay antigestagen – PG-induced PTB *in vivo* with a  $\text{Ca}^{2+}$  antagonist or a  $\text{Ca}^{2+}$  antagonist – gestagen combination. Additionally,

the effects of Ca<sup>2+</sup> antagonist –  $\beta_2$ -agonist combinations were on the pregnant human myometrium proved.

## 6. Conclusion

In the light of our results, we can conclude that P4 pre-treatment abolished the ability of nifedipine to delay labour in hormone-induced preterm delivery in rat. These results correlate with the hypothesis that P4 decreases the activity of the L-type Ca<sup>2+</sup> channels.

There is growing interest in nifedipine as a potentially effective and well-tolerated form of tocolysis. Nifedipine has been demonstrated (Tsatsaris *et al.*, 2002) to have fewer side-effects and leads to a better neonatal outcome than  $\beta_2$ -mimetics. We presume that the effect of nifedipine in tocolytic therapy might be intensified through combination with  $\beta_2$ -adrenergic agonists and enhanced by low concentrations of  $\beta_2$ -mimetics. However, our results indicate that the administration of  $\beta_2$ -adrenergic agonists can not precede that of nifedipine. The significance of these experimental findings remains to be validated in clinical trials, including human side effects.

## 7. References

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## 8. Appendix

### 8.1. List of publication

#### 8.1.1. Publications related to the Ph.D. thesis

**Hajagos-Tóth J**, Falkay G, Gáspár R. Modification of the effect of nifedipine in the pregnant rat myometrium: The influence of progesterone and terbutaline. *Life Sci* 2009; 85:568–572. **IF: 2.56**

**Hajagos-Tóth J**, Kormányos Zs, Falkay G, Pál A, Gáspár R. Potentiation of the uterus-relaxing effects of beta-adrenergic agonists with nifedipine: studies on rats and the human myometrium. *Acta Obstetricia et Gynecologica* 2010; 89:1284-1289 **IF: 1.618**

**Hajagos-Tóth J**, Kormányos Zs, Falkay G, Pál A, Gáspár R. Nifedipin myometrium relaxáló hatásának vizsgálata terbutaline és  $K^+$ -csatorna gátlók jelenlétében *in vitro*. *Acta Pharm Hung* 2010;3: 109-114

#### 8.1.2. Abstracts

**Hajagos-Tóth J**, Gáspár R, Falkay G. Investigation of uterine-relaxing effect of nifedipine on late-pregnant rat myometrium *in vitro*. The First Conference of PhD Students in Medicine and Pharmacy, July 9-11, 2008, Targu Mures, Romania (presentation)

**Hajagos-Tóth J**, Falkay G, Gáspár R. Nifedipin uterusz kontrakciót gátló hatásának vizsgálata *in vitro*. A Magyar Tudomány Ünnepe; Szeged, 2008. November 28. (presentation)

**Hajagos-Tóth J**, Falkay G, Gáspár R. Investigation of uterus-relaxing effect of nifedipine on late pregnant myometrium *in vitro*. 2nd Meeting of the Egon & Ann *Diczfalusy* Foundation; Szeged, 2008.09.30-10.01. (poster)

**Hajagos-Tóth J**, Falkay Gy, Gáspár R. Nifedipin uterusz kontrakciót gátló hatásának módosítása ptogeszteronnal és terbutalinnal *in vitro*. XIV. Congressus Pharmaceuticus Hungaricus; Budapest, 2009. november 13-15. (poster)

**Hajagos-Tóth J**, Kormányos Zs, Falkay Gy, Gáspár R. A nifedipin tocolyticus hatásának módosítása béta-mimetikumokkal. A Magyar Élettani Társaság (MÉT) LXXIV. Vándorgyűlése és a Magyar Kísérletes és Klinikai Farmakológiai Társaság (MFT) II. Közös tudományos konferenciája; Szeged, 2010. június 16-18. (presentation)

**Hajagos-Tóth J**, Kormányos Zs, Falkay G, Gáspár R. Potentiation of uterus-relaxing effects of nifedipine with  $\beta$ -adrenergic agonists: Studies on rats and human myometrium. 16<sup>th</sup> World Congress on Basic and Clinical Pharmacology , 17-23 July, 2010, Copenhagen, Denmark (poster)

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