Investigation of potential tocolytic combinations of terbutaline with magnesium sulfate and sildenafil in pregnant rat model

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List of publications

Full papers related to the Thesis

I. Tamara Barna, Kalman F. Szucs, Annamaria Schaffer, Mohsen Mirdamadi, Judit Hajagos-Toth, Robert Gaspar (2023). Combined uterorelaxant effect of magnesium sulfate and terbutaline: Studies on late pregnant rat uteri in vitro and in vivo.
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Life. 12 (6) Paper 794. 17 p.
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III. Mohsen Mirdamadi, Annamária Schaffer, Tamara Barna, Reza Samavati, Kálmán F. Szűcs, Edina Szűcs, Sándor Benyhe, Mihály Szécsi, Róbert Gáspár (2022). Non-genomic uterorelaxant actions of corticosteroid hormones in rats: An in vitro and in vivo study.
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List of abbreviations

MgSO4	magnesium-sulfate
FDA	Food and Drug Administration
cAMP	cyclic adenosine monophosphate
PDE5-Is	phosphodiesterase type 5 inhibitors
cGMP	cyclic guanosine monophosphate
NO	nitric oxide
KCl	potassium chloride
SMEMG	smooth muscle electromyography
AUC	area under the curve
E_{max}	maximal inhibitory effect
EC_{50}	half maximum effective concentration
FFT	fast Fourier transformation
PsD_{max}	power spectrum density maximum
cpm	cycles per minute

1. Introduction

1.1 Epidemiology and consequences of preterm birth

Preterm birth prevention and treatment have always been a difficult challenge in obstetrical practice. Despite the percentage of premature birth decreasing over the last decade in the U.S., its global incidence is over 15 million each year, and the rates of neonatal morbidity remain high, which also means an economic burden for the families due to medical costs and events that arise later [1][2]. Our current knowledge of the mechanisms underlying preterm labor is still restricted, despite significant advancements in our understanding of myometrial physiology. Contributing factors include infections, stress, uteroplacental thrombosis and intrauterine vascular lesions associated with fetal stress or decidual hemorrhage, uterine overdistension, and cervical insufficiency [3][4]. Previous research has shown that several environmental risk factors can also contribute to preterm birth, such as older and younger maternal age, a short inter-pregnancy interval, primiparity and grand multiparity, foreign origin, a low educational level and other measures of social disadvantage [5][6].

1.2 Tocolytic therapy

A wide range of drugs (tocolytics) are applied to inhibit myometrial contractions, including β-adrenergic receptor agonists (betamimetics), calcium channel blockers, prostaglandin inhibitors, oxytocin receptor antagonists, nitric oxide donors and magnesium sulfate ($MgSO_4$) (Fig. 1.)[7]. The main goal of using these tocolytic drugs is to delay delivery long enough to allow for the administration of prophylactic corticosteroids to decrease the severity of lung disease of prematurity, prolong gestation to achieve fetal maturation and allow time to transfer the mother to a high-level health care facility [8][9]. Medical therapies for acute tocolysis (maximum 48 hours) appear to be beneficial. However, American College of Obstetricians and Gynecologists (ACOG) has not recommended medical therapy for maintenance tocolysis (treatment beyond 48 hours) and the U.S. Food and Drug Administration (FDA) has not approved any medication for this indication [10]. Despite the lack of sanctioning, treatments are often employed on an individual basis in clinical practice [11].

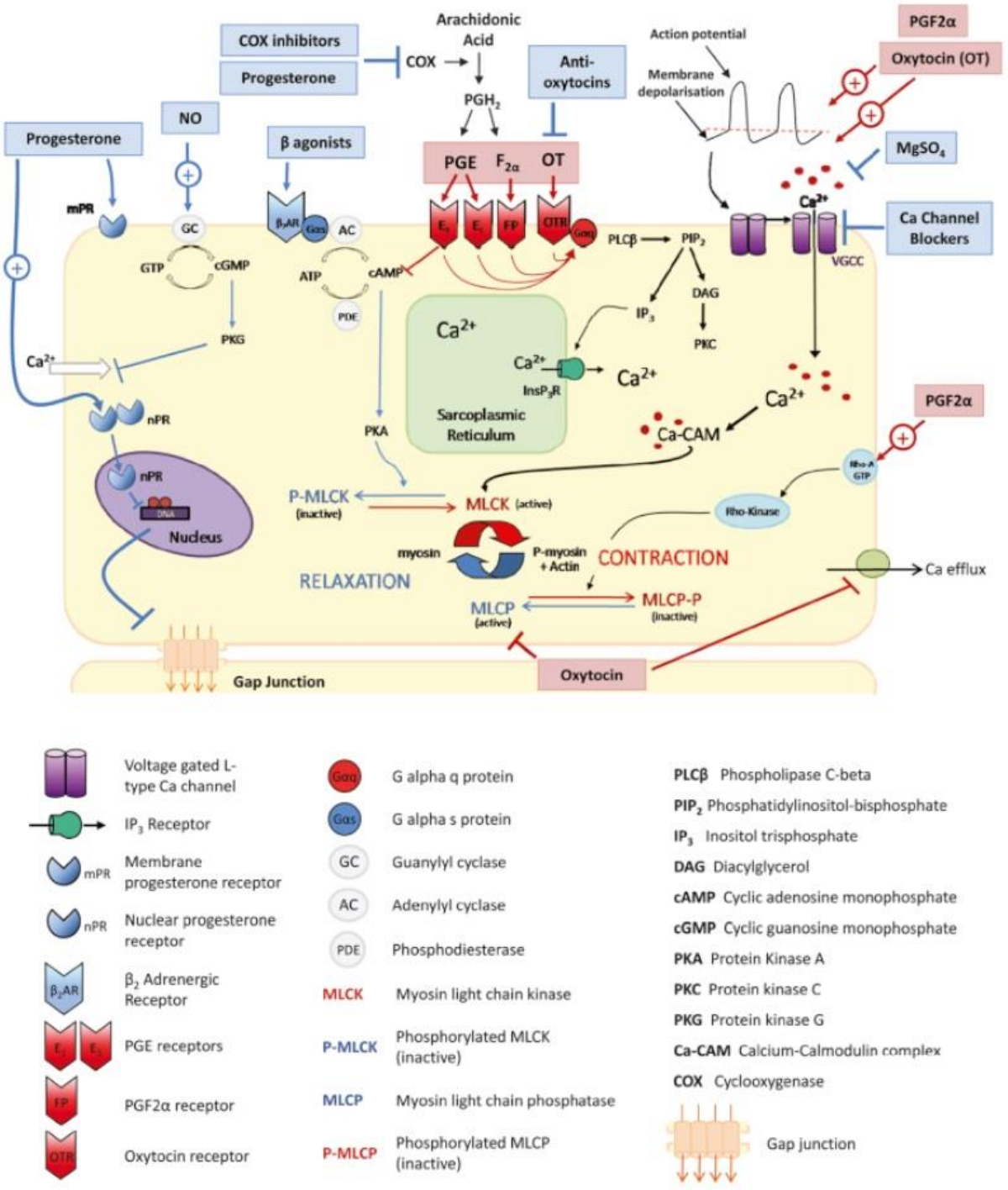


Figure 1.: Mechanism of action of the most common tocolytic agents [12]

Unfortunately, despite the well-known smooth muscle relaxant effects of many different classes of drugs, no new medications have achieved widespread clinical use for the treatment preterm labor in recent decades [13]. This treatment gap is likely related to dose restrictions that are necessary because of unacceptable effects of smooth muscle relaxants on vascular tone or that directly affect the heart (Fig. 2.)[14][15][16][17]l. One possible approach to maximizing tocolytic effects while minimizing cardiovascular effects would be to identify combinations of

drugs that function synergistically in the uterus. Therefore, it is necessary to either conduct research on new active compounds or investigate existing drugs as potential tocolytic agents. As noted above, combination therapy may offer benefit, although a wide range of possible cardiovascular side effects require scrutiny. For example, combining Ca-channel blockers and β-adrenergic receptor agonists are associated with pulmonary hypertension [18]. On the other hand, there are several evidence in the literature regarding the synergistic effect of tocolytic drugs, for example with sildenafil and nifedipine [19][20] or terbutaline and nifedipine [21]. Based on these, the importance of combination therapy is confirmed.

Drug class	Individual drugs in the class commonly used or studied	Major side effects	Comments
Betamimetics[18]	Ritodrine, terbutaline, hexoprenaline, salbutamol	Cardiac arrhythmias (tachycardia), hypotension, hyperglycemia, pulmonary edema	Long-term use recently given an FDA "black box" warning
Calcium channel blockers[19]	Nifedipine, nicardipine	Maternal hypotension, dizziness	Initial loading dose common
Magnesium sulfate[25]	Magnesium sulfate	Flushing, respiratory suppression, cardiac arrest	Currently utilized for neuroprotection protocols
Oxytocin receptor blockers[28]	Atosiban, barusiban	Gastrointestinal upset	Not utilized in the USA
Prostaglandin inhibitors[29]	Indomethacin, sulindac, celecoxib	Maternal gastrointestinal disturbance, oligohydramnios, premature constriction of the ductus	Concern about ductal constriction limits use generally to <32 weeks' gestation
Nitrates and others[30]	Nitroglycerin, nitric oxide	Headache, flushing, maternal hypotension, tachycardia	Currently limited to research trials

Figure 2.: Major side effects of the most commonly used tocolytic agents [22].

1.3 Magnesium sulfate

$MgSO_4$ is commonly used in obstetric practise. It was first described as a tocolytic agent, but nowadays it is mostly used in the treatment of pre-eclampsia, eclampsia and for fetal neuroprotection [23][24][25]. However, the exact mechanism of action of $MgSO_4$ in preterm birth and eclampsia is not completely defined. It is suggested that magnesium acts both

intracellularly and extracellularly. It is able to inhibit the transport of Ca^{2+} through the voltage-gated calcium channels and to decrease Ca^{2+} release from the sarcoplasmic reticulum. Competing with intracellular calcium, it can also inhibit myosin light-chain kinase activity [26], resulting in decreased contraction and arterial relaxation. This effect may lower peripheral and cerebral vascular resistance, relieve vasospasm, and drop arterial blood pressure which contribute to the prevention of eclamptic seizures [27]. The neuroprotective effect of $MgSO_4$ also has several potential mechanisms, such as non-competitive NMDA receptor antagonist effect, excitotoxicty reducing effect and anti-inflammatory properties [28][29] [30][31].

The therapeutic use of $MgSO_4$ is controversial regarding the benefits and maternal and fetal adverse effects. Common maternal side effects such as bradycardia, flushing, nausea, headache, and hypothermia, as well as fetal side effects like lethargy, hypotonia, hypocalcemia, and respiratory depression limit the applicability of $MgSO_4$ [32][33]. The FDA Drug Safety Communication from 2013 states that the prolonged use of $MgSO_4$ is not recommended for preterm birth as the decreased calcium level causes potential bone changes in the foetus and mother [34]. However, it remains popular in case of acute tocolytic therapy. In spite of these unfavorable side effects, magnesium can be used successfully in cardiac arrhythmias due to its important role in cardiac metabolism and its electrophysiological properties [35].

1.4 Terbutaline

β-adrenergic receptor agonists (β-mimetics), such as terbutaline, were among the most potent and widely used inhibitors of uterine contractility. It is known that terbutaline had been considered for first-line clinical use, especially in resource-poor countries. However, the applied high doses and the presence of β-adrenergic receptors in the cardiovascular system caused a number of severe maternal side effects, such as reflex tachycardia, which compensate for the hypotension caused by vasodilation. Moreover, other adverse effects like headache, tremor, dyspnoea or pulmonary oedema are responsible for its limited use in maintenance tocolysis, which is also recommended by FDA [36][37]. Its smooth muscle relaxant effect is linked to the increased intracellular level of cyclic adenosine monophosphate (cAMP) as myosin light-chain kinase activity is inhibited [32]. It is also known about β_2-mimetics, that β_2-adrenergic receptor desensitization could worsen the efficacy during tocolytic therapy due to the overstimulation of β_2-receptor agonists [38][39]. However, the use of terbutaline in acute tocolytic therapy and in combination with other agents remains popular.

1.5 Sildenafil citrate

Sildenafil citrate is a selective inhibitor of phosphodiesterase type 5 (PDE5-Is) and is known for being efficacious as a treatment for erectile dysfunction. On the other hand, there is limited clinical knowledge on the effect of sildenafil in pregnancy [40]. One study suggests that sildenafil-nifedipine combination may be beneficial in preterm birth [41]. PDE-5 is the main enzyme responsible for the degradation of cyclic guanosine monophosphate (cGMP) following the release of nitric oxide (NO) from nitrergic nerves or vascular endothelial cells, leading to smooth muscle relaxation [42]. Although the presence of PDE5 has been proven in the uterus, only few studies have been carried out to investigate the effect of PDE-Is on uterine contractility [43]. It was found that sildenafil dose-dependently reduces the contractions of isolated pregnant human myometrium, which action is associated with the putative role of BKCa (large-conductance calcium-activated potassium channels) channels [44]. Moreover, the relaxant effect of sildenafil also appears to be related to the H_2S pathway in mouse uteri [41]. These findings imply that the potential tocolytic effect of sildenafil and other PDE5-Is may be mediated through various pathways.

2. Aims

Although numerous well-known tocolytic drugs are used in the clinical therapy, there is limited literature of the potentiating effect of different agents. The main focus of our study was to investigate the effect of different tocolytic drugs and their combinations in the late pregnant rat uterus. The following aims were set:

- The first aim of the study was to investigate the effects of terbutaline and $MgSO_4$ on the potassium chloride (KCl)-evoked rat uterine contractions on the last day (22^{nd}) of pregnancy in vitro. Since there is limited knowledge of sildenafil citrate in tocolytic therapy, the same contractility studies were carried out on different days (5, 15, 18, 20, and 22) of pregnancy on non-pregnant uterine tissue in vitro too. The combination of terbutaline and $MgSO_4$ and terbutaline and sildenafil citrate were also investigated.

- Our further aim was to detect the changes in levels of second messengers (cAMP, cGMP) during the sildenafil citrate in vitro studies on days 20 and 22.

- In our study we also performed in vivo smooth muscle electromyographic (SMEMG) studies with terbutaline, $MgSO_4$ and sildenafil citrate alone and their combinations (terbutaline – $MgSO_4$; terbutaline – sildenafil citrate).

- Finally, we also investigated whether the tachycardia-inducing effect of terbutaline can be reduced by the presence of $MgSO_4$, due to the opposite heart rate modifying effects of the two agents.

3. Materials and methods

3.1 Housing and handling of the animals

The animals were housed in rooms with controlled temperature (22 ± 3 °C), humidity (30%–70%), and light (12 h light/dark cycle), with tap water and standard rodent pellet (Animalab Hungary Ltd, Vác, Hungary) available *ad libitum*.

The animals were treated in accordance with the European Communities Council Directive (2010/63/EU) and the Hungarian Act for the Protection of Animals in Research (Article 32 of Act XXVIII). All experiments involving animal subjects were carried out with the approval of the National Scientific Ethical Committee on Animal Experimentation (registration number: XIII./72/2020.; XIII./735/2023.).

3.2 Mating of the animals

Male (240–260 g) and female (180–200 g in the estrus phase) Sprague-Dawley rats were mated in a special mating cage in the early morning hours. The estrus cycle was measured by an Estrus Cycle Monitor (IM-01, MSB-MET Ltd., Balatonfüred, Hungary). Rats with vaginal impedance values between 5.0-8.0 kΩ are in the proestrus phase and were chosen for the mating process. Intercourse was confirmed by the presence of a copulation plug or sperm in the vaginal smears. Positive cases were separated and were regarded as first-day pregnant animals.

3.3 Isolated organ bath studies

3.3.1 Uterus preparation

On days 5, 15, 18, 20 and 22 of pregnancy or in the estrus phase, the animals were terminated by inhalation of carbon dioxide. Uterine horns were removed from the rats, 5-mm-long muscle rings were sliced and cleaned of connective tissue and fat, then immediately placed in an organ bath filled with 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). Temperature of the organ bath was maintained at 37 °C and carbogen (95% O_2+ 5% CO_2) was bubbled through it. After the tissue rings were mounted vertically, 1-hour equilibrium incubation period started with a solution change every 15 min. The initial tension of the uterus samples was set to about 1.5 g, which was measured with a gauge transducer (SG-02; MDE GmbH., Walldorf, Germany) and recorded and analysed with a SPEL Advanced ISOSYS Data Acquisition System (MDE GmbH, Walldorf, Germany).

3.3.2 Magnesium sulfate studies

The uteri were placed in the organ bath as described above. Equilibrated contractions were elicited with 25 mM KCl (7–10 min), and cumulative concentration–response curves were constructed in each experiment in the presence of $MgSO_4$ (10^{-8} – 10^{-2} M) (Molar Chemicals Kft.). Recording was performed for 5 min after each concentration of $MgSO_4$. Concentration–response curves were fitted, and the areas under the curves (AUCs) were evaluated and analyzed statistically. From the AUC values, the maximal inhibitory effect (E_{max}) of $MgSO_4$ and the concentration of $MgSO_4$ eliciting 50% of the maximal inhibition of uterine contraction (EC_{50}) were calculated.

The drug combination studies were carried out by using the method described above, and the contractions were induced by KCl. The cumulative-concentration response curves were elicited in the presence of $MgSO_4$ in combination with terbutaline (Sigma-Aldrich, Budapest, Hungary). After one dose of $MgSO_4$ (10^{-7} M), terbutaline (10^{-9}–10^{-5} M) was administered every 5 min. The uterus-relaxing effects of terbutaline were also investigated alone.

The uterus relaxant effects of the combination of $MgSO_4$ and terbutaline were also investigated in Ca^{2+}-reduced buffer in vitro. As opposed to the normal 1 mM Ca^{2+}-containing buffer, a solution containing 0.1 mM Ca^{2+} was used to induce a low Ca^{2+} environment. During the incubation time, normal De Jongh solution was used for 1 h, and after this equilibration period, the buffer was changed to the low Ca^{2+}-containing buffer.

3.3.3 Sildenafil citrate studies

During the sildenafil citrate studies, the same methodology was used as in the previous experiments. Rhythmic contractions were elicited with 25 mM KCl (7-10 min), and concentration–response curves were constructed in each experiment in the presence of sildenafil-citrate (10^{-10} - 10^{-4} M) (Sigma-Aldrich, Budapest, Hungary) in a cumulative manner for 5 minutes after each concentration. In case of the terbutaline – sildenafil combination studies, after one dose of terbutaline (10^{-8} M), sildenafil was administered every 5 minutes.

3.4 Measurement of uterine cAMP and cGMP accumulation

As the part of the sildenafil citrate studies, uterine tissue samples from 20- and 22-day pregnant rats were incubated in de Jongh solution, under the same conditions as detailed above. The tissues were incubated for 5 min in KCl (25 mM), then isobutylmethylxanthine (10−4 M) and different concentrations of sildenafil (from 10−10 until 10-4 M) without or with terbutaline (10-

8 M) were added for a further 20 min. At the end of the incubation period, forskolin (10−5 M) was added for 10 min. The samples were immediately frozen in liquid nitrogen after stimulation and stored at -70 °C until cAMP and cGMP extraction. Frozen tissue samples were then ground, weighed, homogenized in 10 volumes of ice-cold 5% trichloroacetic acid and centrifuged at 600 g for 10 min. The supernatants were extracted with 3 volumes of water-saturated diethyl ether, repeated 3 times, while the ether supernatants were removed. After drying, the extracts were stored at – 70 °C until the cAMP/cGMP assays. The accumulation of uterine second messengers was measured with commercial cAMP Enzyme Immunoassay Kit (Enzo Life Sciences, USA) and cGMP Enzyme Immunoassay Kit (DRG Instruments GmbH, Germany). The levels of cAMP and cGMP were expressed in pmol/mg and pmol/g tissue.

3.5 Smooth muscle electromyographic studies

22-day-pregant rats were used for the SMEMG measurements. Anaesthesia was induced by the intraperitoneal (i.p.) injection of ketamine (36 mg/kg)-xylazine (4 mg/kg) and maintained by the inhalation of isoflurane (0.5-1%) (R550 Multi-output Animal Anesthesia Machine, Animalab Hungary Ltd, Vác, Hungary). The jugular vein was cannulated for intravenous (i.v.) drug administration, and a bipolar disk electrode (SEN-15-2; MSB-Met Ltd., Balatonfüred, Hungary) was inserted subcutaneously above the uterus to detect the myoelectric signals of contractions in the pregnant uterus (the distance between the two electrodes was 20 mm). Control myoelectric signals were registered for 45 minutes, and a 1: 100 mixture of heparin-Na and physiological saline was injected into the jugular vein every 15 minutes to prevent coagulation.

The animals were treated with $MgSO_4$ (0.1 - 30 mg/kg) or terbutaline (0.05 – 50 μg/kg) alone in a cumulative way every 15 minutes. The effect of the co-administration of $MgSO_4$-terbutaline was also registered. In each dose, the animals were treated with constant $MgSO_4$ i.v. (0.3 mg/kg) while increasing doses of terbutaline (from 0.05 to 50 μg/kg every 15 min) were added in a cumulative manner.

The experiment described above was also repeated in the presence of sildenafil alone (0.42 μg/kg – 4.2 mg/kg) and in combination with terbutaline. The effect of co-administered sildenafil and terbutaline was monitored as the animals were treated with constant terbutaline i.v. (0.15 μg/kg) along with increasing doses of sildenafil (from 0.42 μg/kg to 4.2 mg/kg) every 15 min in a cumulative manner.

The myoelectric signals of the pregnant uterus, representing the strength of contractions, were detected by an online computer system (SPEL Advanced ISOSYS Data Acquisition System). Uterine contractility was evaluated by fast Fourier transformation (FFT), then the power spectrum density maximum (PsD_{max}, the highest peak of PsD) of the SMEMG activity was determined by characterizing the spectrum of the activity in the frequency range of 1-3 cycles per minute (cpm). The uterine relaxant effect of the drugs was expressed as a percentage compared to the control PsD_{max} value.

3.6 Heart rate measurements

As the part of the $MgSO_4$ studies, a bipolar disk electrode (SEN-15-2; MSB-Met Ltd., Balatonfüred, Hungary) was placed subcutaneously on the surface of the abdominal wall to detect the heart rate signals of rats on day 22 of pregnancy under anaesthesia as described above. After control signals were measured (15 min), terbutaline (50 μg/kg or 500 μg/kg) was injected into the cannulated jugular vein to detect the changes in frequency for 15 minutes. The combination of terbutaline (50 μg/kg or 500 μg/kg) with $MgSO_4$ (2.1 mg/kg, which is equivalent to 7 doses of $MgSO_4$ used during the SMEMG experiments) was also registered. Heart rate signals were detected with an SMEMG/HR/BT Holter system (MSB-MET Ltd., Balatonfüred, Hungary).

3.7 Statistical analysis

All data were analysed using Prism version 5.01 (GraphPad Software, San Diego, CA) computer program. The values of the isolated organ bath combination studies and the in vivo uterine studies were statistically evaluated with unpaired *t*-test (two-tailed) and the isolated organ bath studies on different days of pregnancy and the values of the cAMP/cGMP studies were evaluated with one-way ANOVA-test (Dunett's post hoc test). Shapiro-Wilk test was used to assess normality of distribution.

4. Results

4.1 *In vitro* contractility studies

4.1.1 Magnesium sulfate and terbutaline

Myometrial activity was determined by the AUC of the concentration-response curves of 22-day-pregnant Sprague-Dawley rat uterine strips. (Fig. 3.) Both $MgSO_4$ and terbutaline inhibited the KCl-evoked (25mM) contractions in a concentration-dependent manner. The maximal inhibitory effect (E_{max}) of $MgSO_4$ reached almost 100% in the range of 10^{-8} – 10^{-1} M (Fig. 4. A) (Table 1.). The E_{max} of terbutaline alone was also over 90% in the range of 10^{-10} – 10^{-4} M. Pre-treatment with $MgSO_4$ (10^{-7} M) significantly enhanced the relaxant effect of terbutaline, especially in the lower range, however, it could not improve the E_{max} of terbutaline. (Fig. 4.B) The maximal uterus relaxant effect (E_{max}) and the EC_{50} of terbutaline compared with the combination ($MgSO_4$ pre-treatment (10^{-7} M) + terbutaline) were not significant in reduced Ca^{2+} buffer (0.1 mM Ca^{2+}). (Fig. 4.C) (Table 1.)

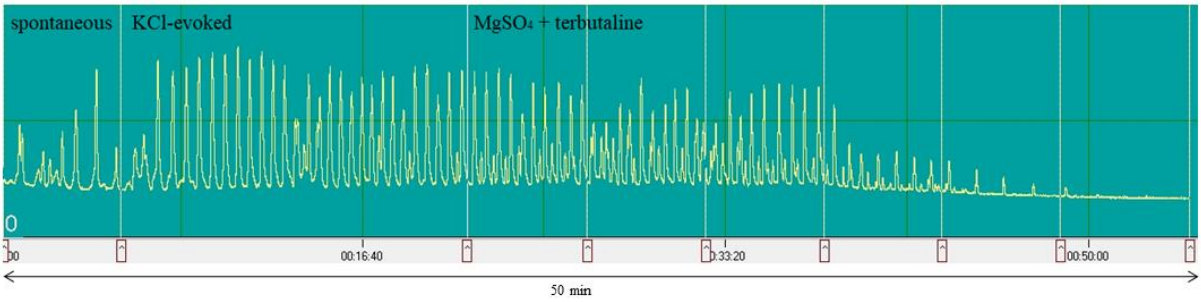


Figure 3.: Recorded signals of the 22-day-pregnant uterus in vitro: spontaneous contractions, KCl-evoked contractions and contractions in the presence of $MgSO_4$ and terbutaline.

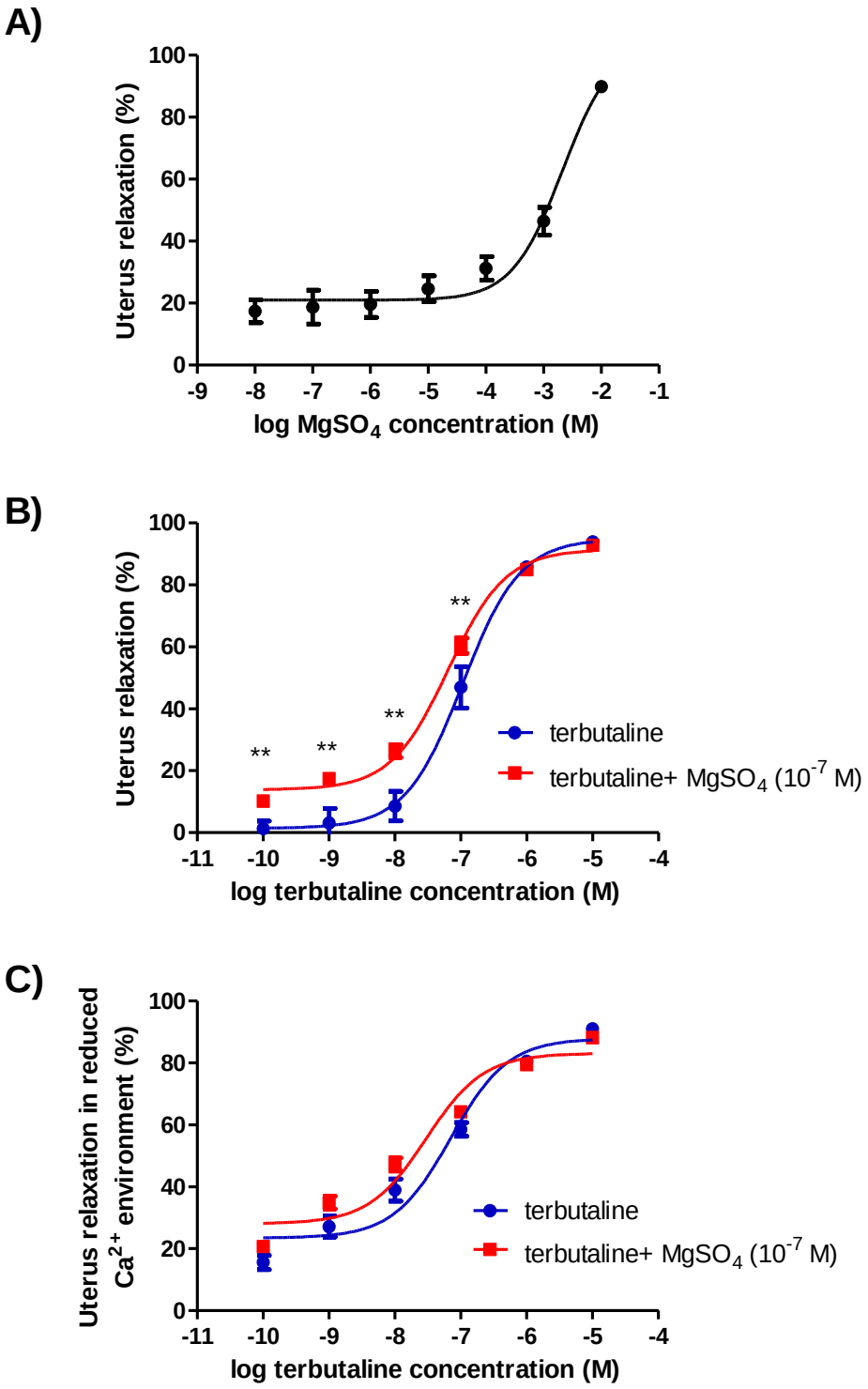


Figure 4.: Inhibitory effect of $MgSO_4$ alone (A), terbutaline alone (B) and in combination with $MgSO_4$ (10^{-7} M) (B) in normal Ca^{2+}-containing buffer. Also, inhibitory effect of terbutaline alone (C) and in combination with $MgSO_4$ (C) in reduced Ca^{2+} environment on contractions evoked by 25 mM KCl on pregnancy day 22. ***: $p < 0.01$ as compared with the values of terbutaline*

	EC_{50} (M± S.E.M.)	E_{max} (%± S.E.M.)
A) $MgSO_4$	$1.6\pm 0.7x10^{-3}$	89.8 ± 1.1
B) terbutaline terbutaline + $MgSO_4$	$1.3\pm 0.6x10^{-7}$ $7.2\pm 3.1x10^{-8}$ *	95.9 ± 2.3 92.0 ± 4.9
C) terbutaline terbutaline + $MgSO_4$	$7.4\pm 5.4x10^{-8}$ $3.6 \pm 2.7x10^{-8}$ *	87.5 ± 4.6 83.2 ±3.3

Table 1. EC_{50} and E_{max} values of curves of uterine relaxation induced by $MgSO_4$ (A), terbutaline (10^{-10}–10^{-5} M) alone or in the presence of $MgSO_4$ (10^{-7} M) at 1 mM (B) or 0.1 mM (C) Ca^{2+} level-containing buffer. *: $p < 0.05$ *as compared with the values of terbutaline S.E.M.: standard error of the mean.*

4.1.2 Sildenafil citrate and terbutaline

Contractions of uterine strips were determined based on the AUC values of the raw traces (Fig. 5.). The uterine relaxant effect of the drugs was expressed as a percentage compared to the KCl-evoked equilibrium activity. Sildenafil inhibited the KCl-evoked contractions in a concentration-dependent manner on each investigated gestational day and on non-pregnant uterine strips (Fig. 6.). There were significant differences in the lower concentration range (10^{-9}-10^{-7} M) on day 20 as compared with non-pregnant uteri. The maximal relaxing effect (E_{max}) of sildenafil was greater at the beginning of pregnancy and on non-pregnant uteri than on gestational days 18, 20 and 22 (Table 2.).

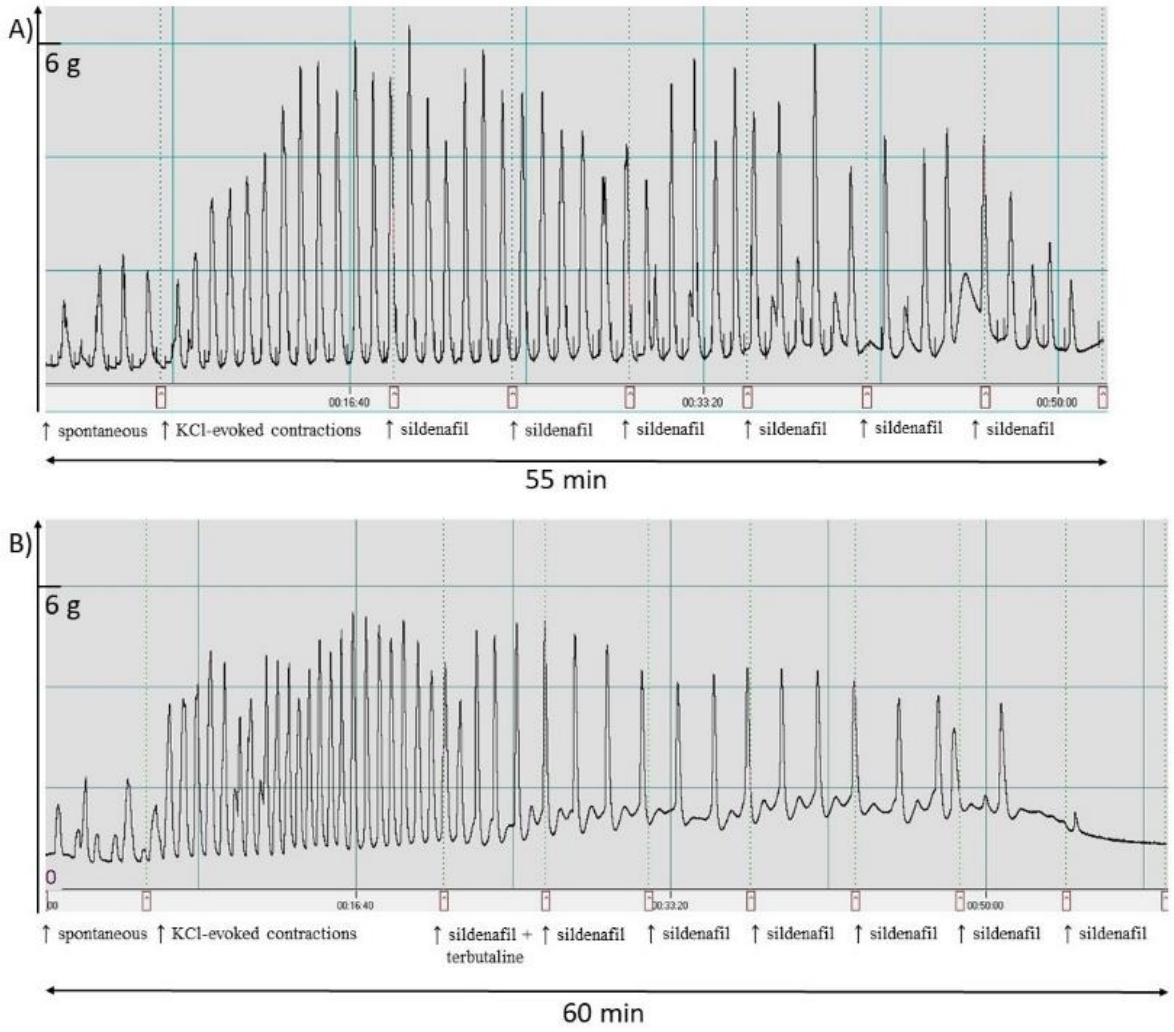


Figure 5.: Representative raw traces of the 22-day pregnant uterine contractions in vitro: spontaneous contractions, KCl-evoked contractions and contractions in the presence of sildenafil alone (A) or in combination with terbutaline (B).

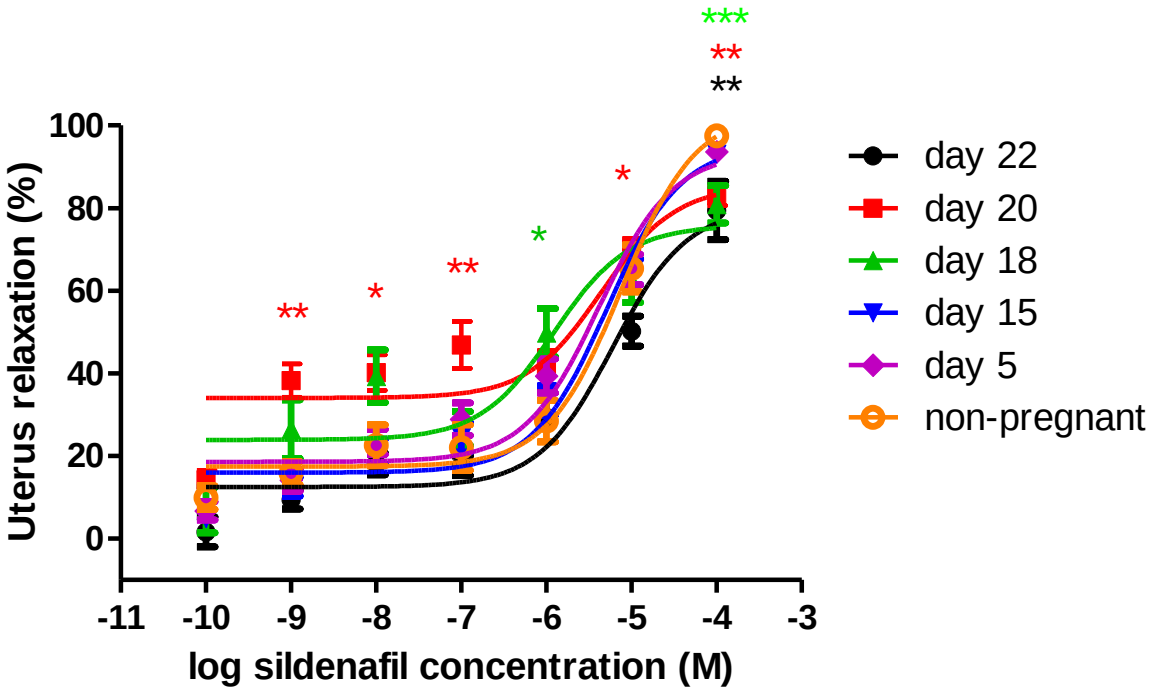


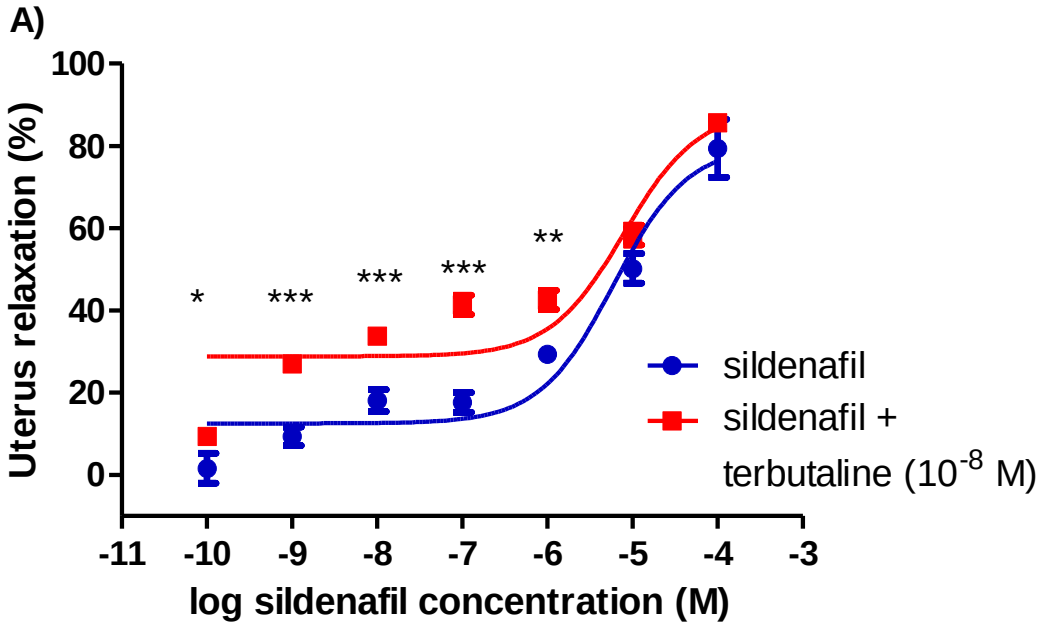
Figure 6.: Inhibitory effect of sildenafil (10^{-10} – 10^{-4} M) on pregnant (days 22/20/18/15/5) and non-pregnant uterine contractions stimulated with KCl (25 mM) (n=8/day). **: p < 0.05; **: p < 0.01; ***: p < 0.001 as compared with the values of non-pregnant uteri.*

	EC_{50} (M± S.E.M.)	**E_{max} (%± S.E.M.)**
non-pregnant	$7.6 \pm 3.9 \times 10^{-6}$	98.9 ± 0.9
day 5	$4.2 \pm 2.5 \times 10^{-6}$	93.5 ± 3.7 ^{ns}
day 15	$5.2 \pm 1.9 \times 10^{-6}$	95.3 ± 4.3 ^{ns}
day 18	$1.2 \pm 3.4 \times 10^{-6}$	75.8 ± 11.2 ***
day 20	$4.5 \pm 1.8 \times 10^{-6}$	85.4 ± 8.9 **
day 22	$4.1 \pm 4.3 \times 10^{-6}$	82.3 ± 16.7 **

Table 2.: EC_{50} and E_{max} values of curves of uterine relaxation induced by sildenafil on days 5/15/18/20/22 or on non-pregnant animals. *ns: non-significant **: p < 0.01; ***: p < 0.001 as compared with the values of non-pregnant uteri.*

As sildenafil, terbutaline also caused dose-dependent myometrial relaxation (Fig. 7. A; B) in 22-day pregnant rats. The maximal inhibitory effect (E_{max}) of terbutalin alone was over 90% at

10^{-5} M. The presence of terbutaline (10^{-8} M) significantly enhanced the cumulative relaxant effect of sildenafil, especially in the lower concentration range, however, it could not improve E_{max} (Table 3.). In the opposite case, sildenafil treatment followed by increasing doses of terbutaline, the presence of sildenafil (10^{-6} M) did not affect the cumulative uterine relaxing effect of terbutaline (Fig. 7. B). Since this combination had no benefit in the potentiating effect, no further in vivo testing was done with this kind of arrangement.



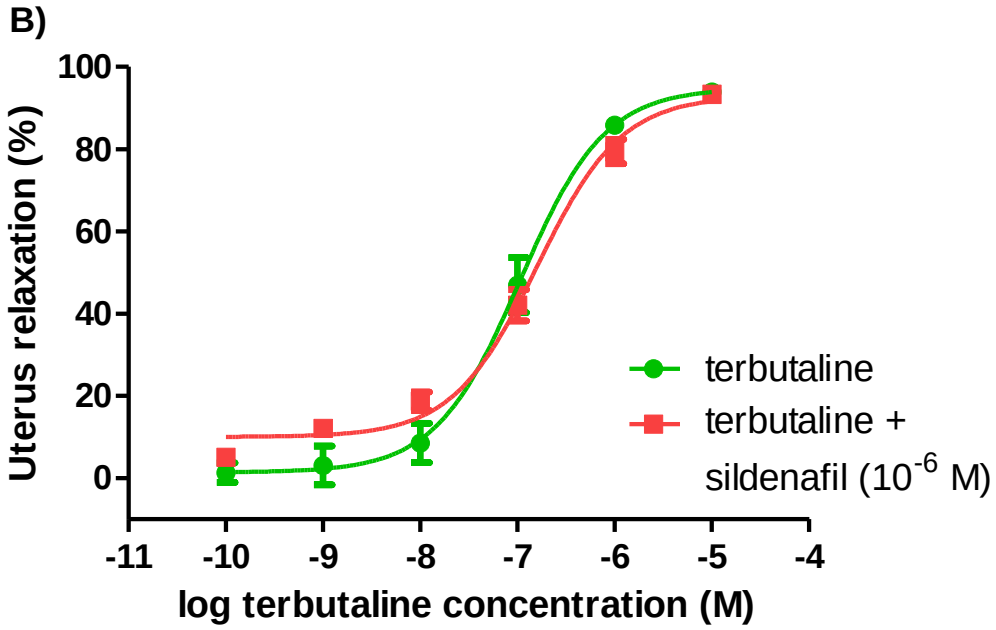


Figure 7.: A): Inhibitory effect of sildenafil (10^{-10} – 10^{-4} M) alone or in combination with terbutaline (10^{-8} M) on 22-day pregnant uterine contractions stimulated with KCl (25 mM). **: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ as compared with the values of sildenafil alone.*

B): Inhibitory effect of terbutaline (10^{-10} – 10^{-5} M) alone or in combination with sildenafil (10^{-6} M) on 22-day pregnant uterine contractions stimulated with KCl (25 mM).

	EC_{50} (M± S.E.M.)	E_{max} (%± S.E.M.)
sildenafil	$4.1 \pm 4.3 \times 10^{-6}$	82.3 ± 16.7
sildenafil + terbutaline (10^{-8} M)	$8.8 \pm 6.3 \times 10^{-6}$ ns	86.5 ± 10.3 ns
terbutaline	$1.3 \pm 0.6 \times 10^{-7}$	95.9 ± 2.3
terbutaline + sildenafil (10^{-6} M)	$2.3 \pm 2.0 \times 10^{-7}$ ns	94.5 ± 4.2 ns

Table 3.: EC_{50} and E_{max} values of curves of uterine relaxation induced by sildenafil alone and in combination with terbutaline, terbutaline alone and in combination with sildenafil on gestational day 22 (n=8/group*). ns: non-significant as compared with sildenafil or terbutaline alone.*

4.2 The effects of sildenafil and terbutaline on uterine cAMP and cGMP levels

The cGMP and cAMP levels of 20-day pregnant uterine tissue samples were measured in the presence of 10^{-10} – 10^{-4} M sildenafil (Fig. 8.). A concentration-dependent increase in cGMP levels was induced by sildenafil, however, it could not affect the levels of cAMP substantially. The levels of the second messengers were also measured on day 22 in the presence of 10^{-10} – 10^{-4} M sildenafil alone or in combination with 10^{-8} M terbutaline (Fig. 9.). Sildenafil also increased the cGMP levels in a concentration-dependent manner similarly to day 20. However, the effect of the lowest concentration of sildenafil on raising cGMP on day 20 is equivalent to the effect of 10^{-6} M sildenafil on day 22, which means that on day 20, compared to day 22, sildenafil has a greater ability to enhance the level of cGMP. Nevertheless, terbutaline significantly enhanced the levels of cGMP in the case of lower concentrations (10^{-10} – 10^{-6} M) of sildenafil, but it was not able to modify the levels of cAMP.

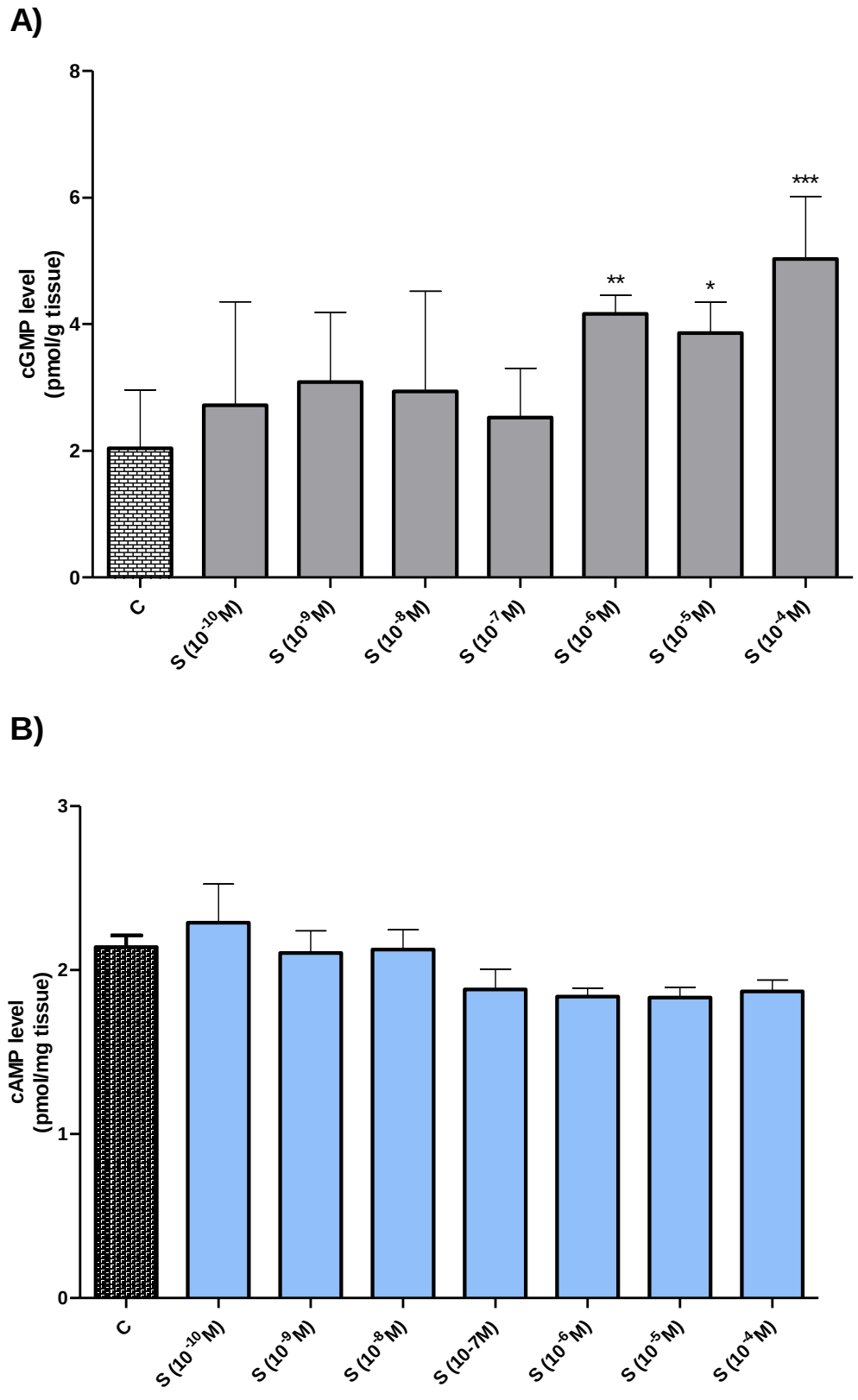


Figure 8.: The levels of cGMP (A) and cAMP (B) in the presence of different concentrations of sildenafil (S) on day 20 (n=10). **: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$* as compared with the level of non-treated control uterus (C).

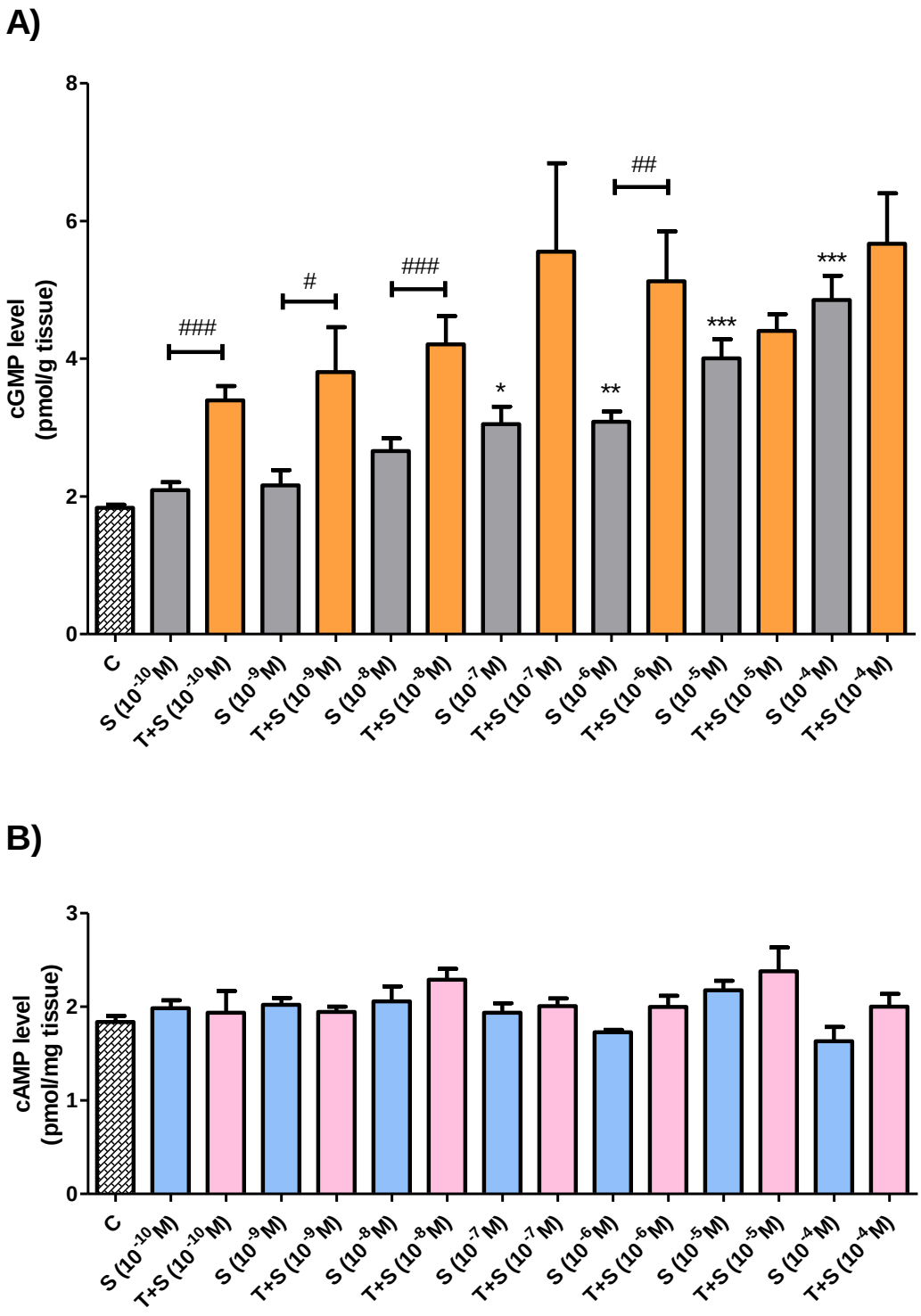


Figure 9.: The levels of cGMP (A) and cAMP (B) in the presence of different concentrations of sildenafil alone (S) or in combination with terbutaline (T+S) on day 22 (n=10). **: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$* as compared with the level of non-treated control uterus (C), #: *$p < 0.05$; ##: $p < 0.01$; ###: $p < 0.001$* as compared with the levels of sildenafil alone.

4.3 *In vivo* contractility studies

4.3.1 Magnesium sulfate and terbutaline

The myoelectric signals of the pregnant uterus, representing the strength of contractions, were detected and evaluated (Fig. 10.). Both $MgSO_4$ and terbutaline caused dose-dependent myometrial relaxation on 22-day-pregnant rats *in vivo*. The E_{max} of $MgSO_4$ was about 70% relaxation in the range of 0.1 - 30 mg/kg (Fig. 11. A), while the relaxing E_{max} of terbutaline (94.3 ± 1.8 %) was similar to the *in vitro* results in the range of 0.05 – 50 μg/kg. The maximal inhibition of the co-administration was not statistically different from the administration of terbutaline alone, however, the curve was shifted to the left. (Fig. 11. B) (Table 4.)

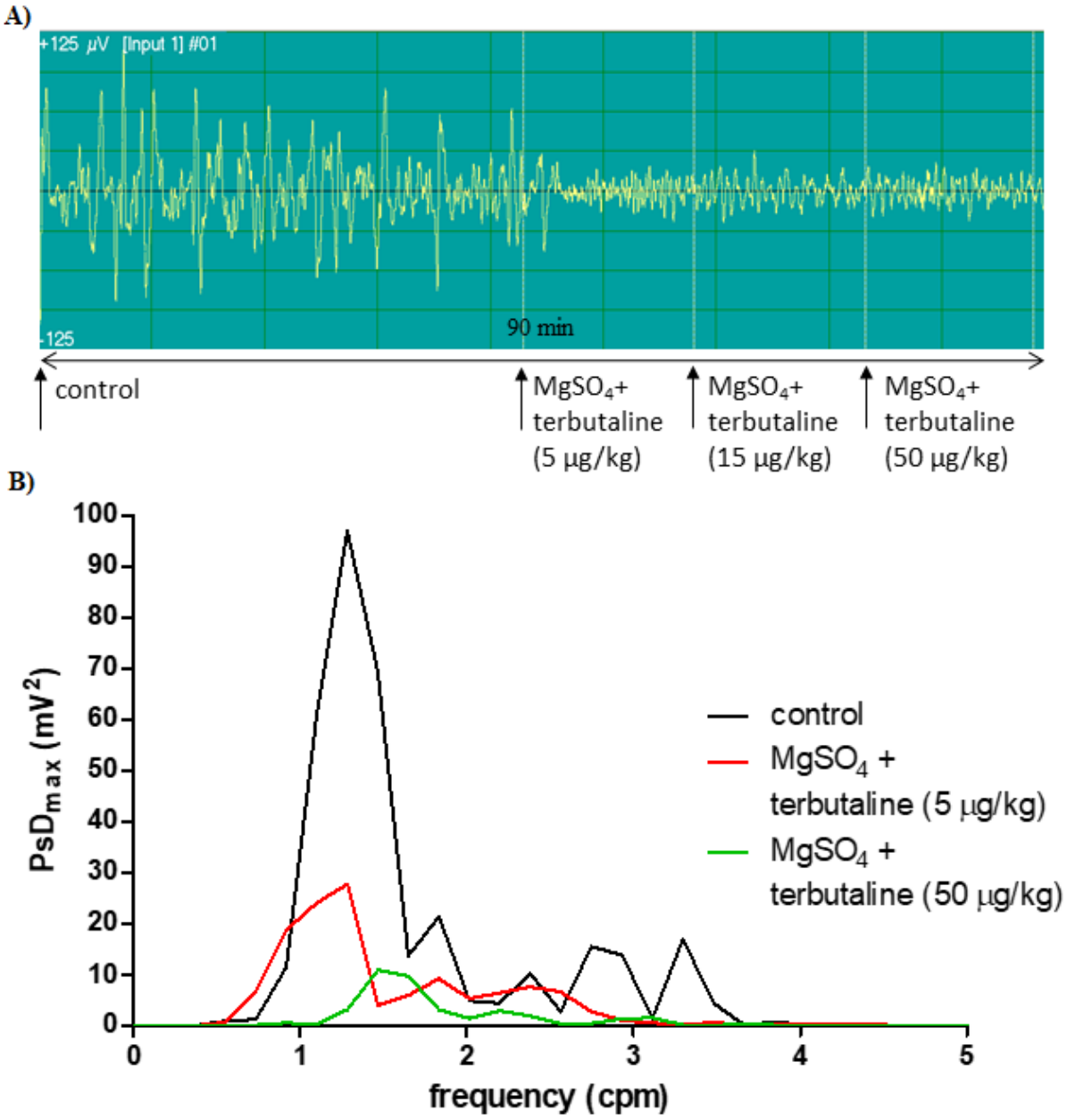


Figure 10.: Myoelectric signals of the pregnant uterus of rat detected with a bipolar disk electrode under anaesthesia. A) Raw trace of control contractions and contractions in the presence of the co-administration of $MgSO_4$ and terbutaline every 15 minutes. B) FFT analysis of myoelectric signals. The spectrum of the SMEMG activity was characterized in the frequency range of 1-3 cpm. The highest PsD_{max} value in the spectrum correlates with the contractility of the tissues. PsD_{max}: Power spectrum density maximum.

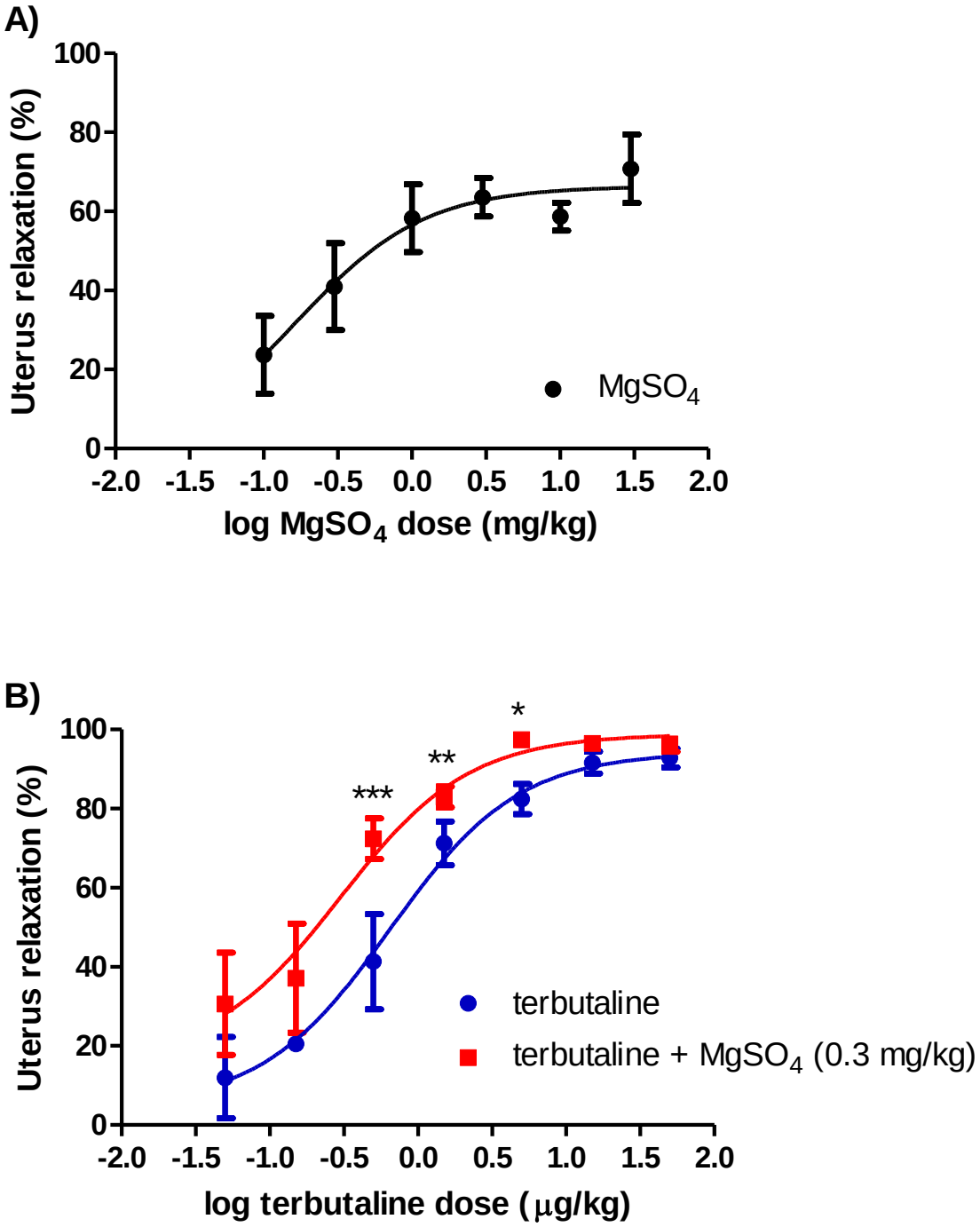


Figure 11.: Inhibitory effect of $MgSO_4$ alone (A), terbutaline alone (B) and in combination with $MgSO_4$ (0.3 mg/kg) (B) on pregnancy day 22 *in vivo*. *:P<0.05; **: P< 0.01, ***:P<0.001

	ED_{50} (± S.E.M.)	**P-value**	**E_{max} (%± S.E.M.)**	**P-value**
A) $MgSO_4$	0.20 ± 0.08 mg/kg	-	69.9 ± 12.2	-
B) terbutaline	0.65 ± 0.19 μg/kg		94.3 ± 1.8	
terbutaline + $MgSO_4$	0.30 ± 0.10* μg/kg	0.036	98.7 ± 2.2	0.106

Table 4. ED_{50} and E_{max} values of curves of uterine relaxation induced by $MgSO_4$, terbutaline (10^{-10}–10^{-5} M) alone or in the presence of $MgSO_4$ (0.3 mg/kg) in vivo. The level of significance is related to the comparison with the values for terbutaline. S.E.M.: standard error of the mean.

Both the lower dose (50 μg/kg) and the higher dose (500 μg/kg) of terbutaline alone increased heart rate, especially at the beginning of the experiment. In the case of the lower dose of terbutaline (Fig. 12. A), the changes in cardiac frequency were below 10% in each minute, however, in the presence of $MgSO_4$ (2.1 mg/kg), the heart rate-increasing effect of terbutaline was significantly decreased between the 1st and 5th minutes (**:P<0.05, **: P< 0.01*). Similarly to the lower dose, the higher dose of terbutaline also increased the heart rate (Fig. 12. B). From the 2nd minute, the changes in cardiac frequency were over 10%. In combination with $MgSO_4$, the heart rate was significantly reduced (**:P<0.05, **: P< 0.01*), especially from the 3rd minute. In both cases, there were no changes in cardiac frequency after the 5th minute.

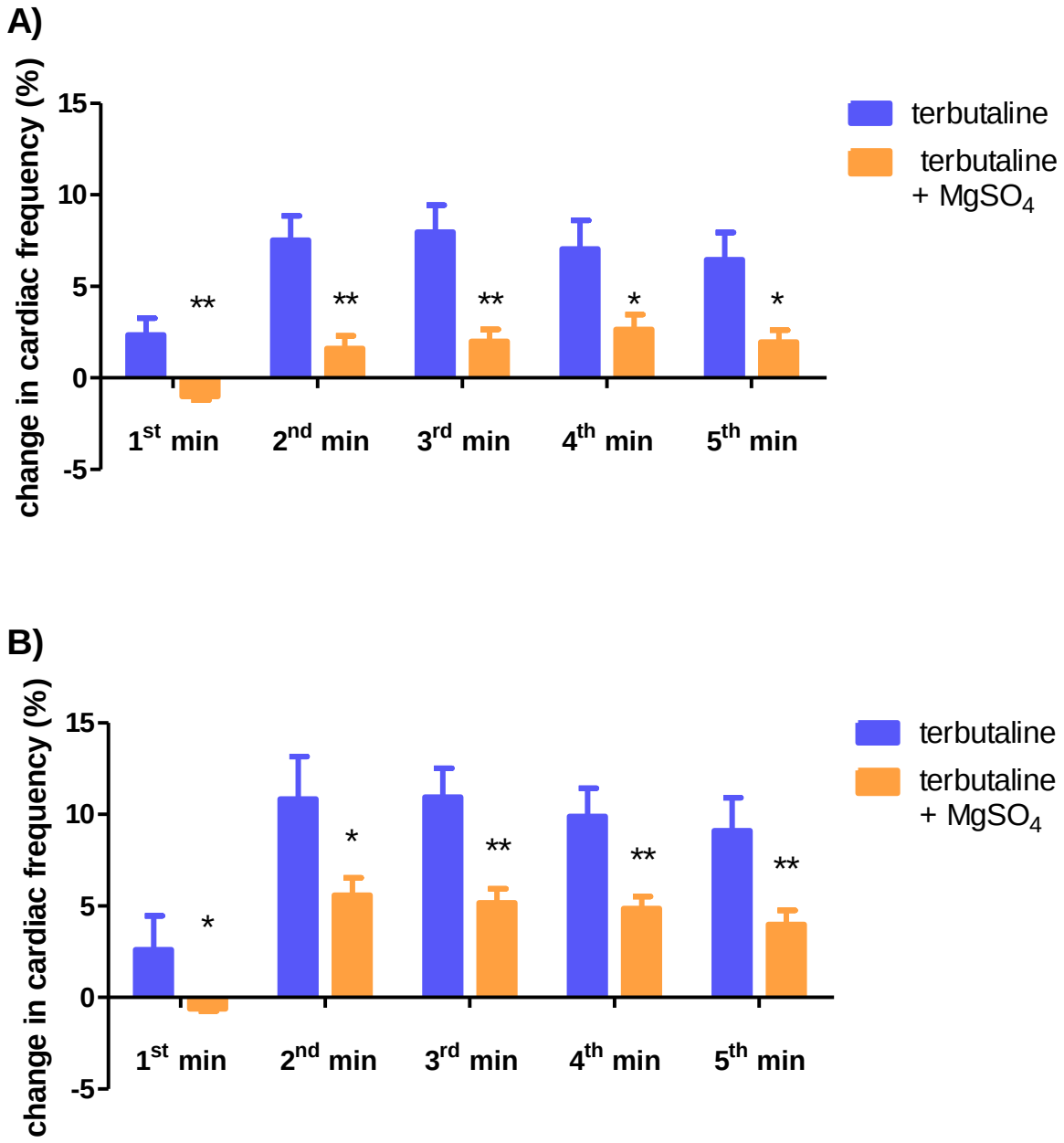


Figure 12.: Changes in cardiac frequency during treatment with 50 µg/kg (A) or 500 µg/kg (B) terbutaline alone or in combination with $MgSO_4$ on pregnancy day 22. **:P<0.05*.

4.3.2 Sildenafil citrate and terbutaline

Similarly, to the organ bath contractility studies, uterus contractions were inhibited by both compounds in a dose-dependent manner in 22-day pregnant rats (Fig. 13.). The maximal relaxing effects (E_{max}) of terbutaline and sildenafil were around 95% in the range of 0.05 – 50 μg/kg, and around 80% in the range of 0.42μg/kg – 4.2 mg/kg, respectively (Fig.13.; Table 5.). The action of sildenafil was potentiated by terbutaline significantly at lower doses, however, the maximal inhibitions achieved with terbutaline + sildenafil were not statistically different, similarly to in vitro results.

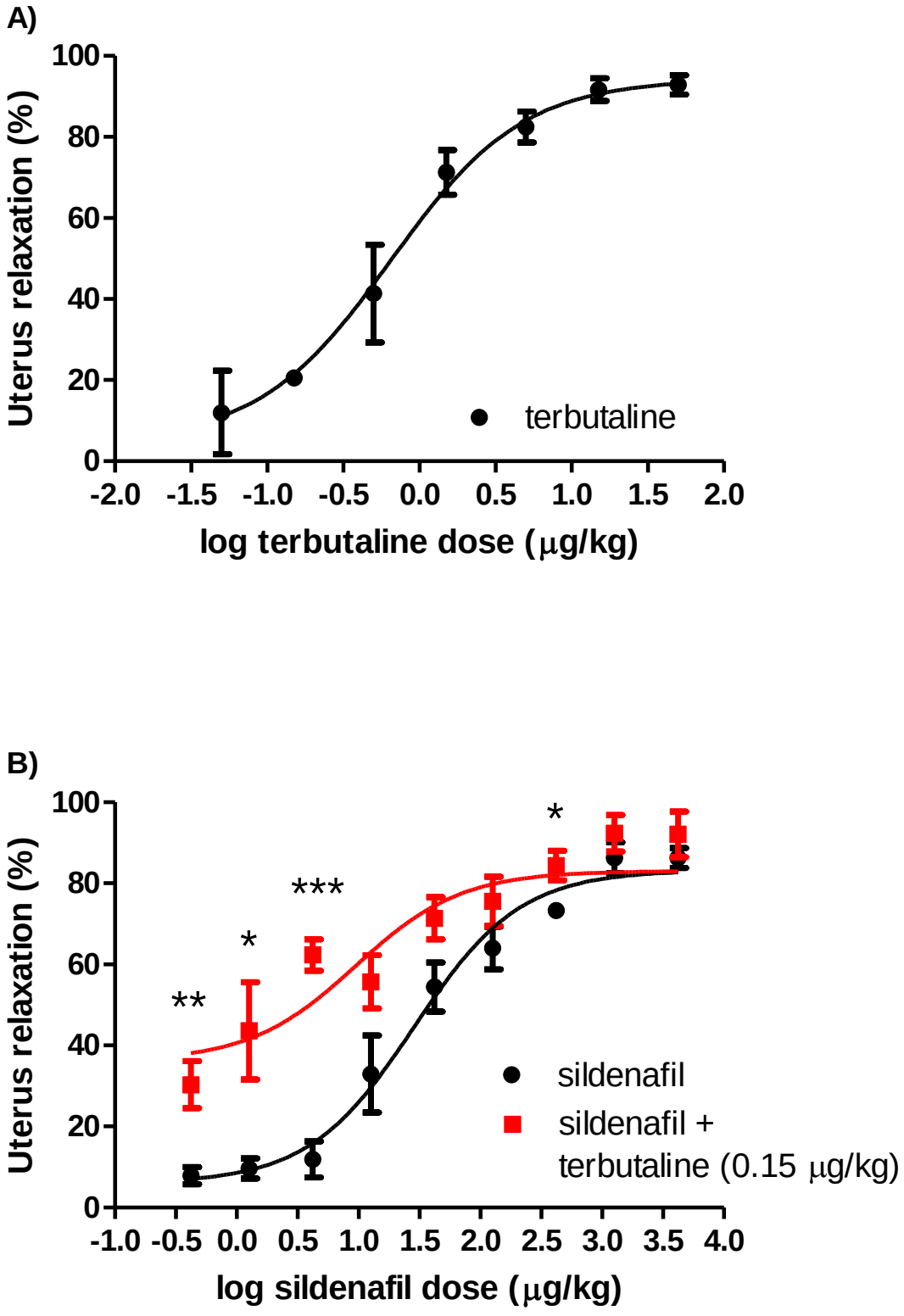


Figure 13.: Inhibitory effect of terbutaline (0.05-50 μg/kg) alone (A), sildenafil (0.42 μg/kg – 4.2 mg/kg) alone (B) or in combination with terbutaline (0.15 μg/kg) on 22-day pregnant uterine contractions in vivo. **: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$* as compared with sildenafil values.

	ED_{50} (± S.E.M.)	**E_{max} (%± S.E.M.)**
terbutaline	0.65 ± 0.19 µg/kg	94.3 ± 1.8
sildenafil	28.7 ± 2.11 µg/kg	83.2 ± 2.3
sildenafil + terbutaline (0.15 µg/kg)	9.5 ± 1.3 µg/kg **	83.6 ± 1.7 ^{ns}

Table 5.: ED_{50} and E_{max} values of curves of uterine relaxation induced by terbutaline, sildenafil alone or in the presence of terbutaline (0.15 µg/kg) in vivo (n=8 rats/group). *ns: non-significant, **: $p < 0.01$* as compared with sildenafil values.

5. Discussion

The purpose of the current study was to investigate the effect of different tocolytic drugs and their combinations in the late pregnant rat uterus. In view of the efficiency of the currently used tocolytic therapy, combinations of different smooth muscle relaxant agents should be considered as an option to decrease adverse maternal and foetal events and enhance efficacy. Previous research has already proven the importance of certain combinations, like progestogen treatment was found to enhance the tocolytic effect of salmeterol in hormone-induced preterm labour in rats in vivo [45], and the addition of terbutaline to nifedipin also increased the uterorelaxant effect of nifedipin on pregnant rat myometrium in vitro and in vivo [21]. Since terbutaline and $MgSO_4$ are widely used tocolytic agents, even though their efficacy and side effect profiles are not completely satisfying, one of our main goals was to investigate the uterus relaxant effect of the combination of these drugs. Furthermore, due to its smooth muscle relaxant effect, selective inhibitor of phosphodiesterase type 5 sildenafil citrate might be considered as a new, tocolytic agent. In the present study, in addition to the uterus relaxant effect of sildenafil, we also investigated the efficacy of its combination with terbutaline. As these two agents act through different pathways in uterine relaxation, we hypothesized that they would demonstrate a synergistic tocolytic effect on the uterus. Furthermore, the synergistic effect may be unique to the uterus, which would then allow reduced dosing of either or both drugs and provide a mechanism for reducing maternal and fetal side effects.

In isolated organ bath studies we investigated uterine ring contractions that involved both longitudinal and transverse muscle layers responses. In the first series of our experiment, we found synergism in the uterorelaxant effect of terbutaline and $MgSO_4$, although $MgSO_4$ was not able to enhance the maximal inhibitory effect of terbutaline. However, we observed a significant potentiating effect at lower concentrations, which may have importance in the reduction of adverse effects. We also proved that Ca^{2+} plays a significant role in this process. It is known that the entry of Ca^{2+} into the cells through the voltage-dependent Ca^{2+} channels has a crucial influence on smooth muscle contraction. One possible mechanism of action of $MgSO_4$ is the blockade of the voltage-dependent Ca^{2+} channel, which ability can be blocked in Ca^{2+}-poor environment [26]. Hence, the co-administration of the two agents was also investigated in a buffer containing Ca^{2+} reduced by 90% in order to verify the above-mentioned hypothesis. In a Ca^{2+}-poor buffer, $MgSO_4$ was not able to enhance the effect of terbutaline. These results led us to conclude that $MgSO_4$ potentiates the uterorelaxant effect of terbutaline via the inhibition of voltage-dependent Ca^{2+} channels.

In the second series of our isolated organ bath studies, we found that the PDE5 inhibitor sildenafil has a concentration-dependent uterus relaxant effect on each investigated gestational day (5/15/18/20/22) and on non-pregnant uteri. No significant changes in EC_{50} values were found, and we confirmed previous findings that there is no significant difference in PDE5 activity during pregnancy in rat [43]. However, the maximum relaxing effect of sildenafil slightly decreased towards the end of the gestational period, although it remained still high on the last day of pregnancy (over 80% relaxation). The drop in the relaxing effect from gestational day 18 might due to the increased uterine NO metabolism at the end of pregnancy [46].

In isolated organ bath studies we also investigated the uterus relaxant effect of co-administered sildenafil and terbutaline. As demonstrated in the previous part of this study, terbutaline elicited a significant uterine relaxing effect in 22-day pregnant rats. When terbutaline was added in a single and low concentration with cumulatively administered sildenafil, we observed a significant potentiating effect at lower concentrations of sildenafil, but the maximal inhibitory effect of sildenafil did not increase. In the opposite case, sildenafil treatment followed by increasing doses of terbutaline, the presence of sildenafil was not able to enhance the effect of terbutaline. A previous study already confirmed the biphasic effect of sildenafil in adipocytes and hepatocytes. According to these results, a higher (micro molar) concentration of sildenafil allosterically activates PDE2 and reduces the level of cAMP [47]. A similar mechanism in the uterus might explain the inefficacy of sildenafil to potentiate the effect of terbutaline.

During our isolated organ bath study with sildenafil, compared to non-pregnant uteri, on day 20 of pregnancy, we measured a stronger uterine relaxing effect in lower concentrations of our used concentration range. Therefore, the cAMP and cGMP measurements were taken on gestational days 20 (highest sildenafil efficacy in low concentration) and 22 (last day of pregnancy). We proved that the levels of cGMP are elevated with the concentration of sildenafil on days 20 and 22, however, sildenafil has a greater potential to increase cGMP levels on day 20. The presence of terbutaline significantly enhanced the levels of cGMP on the last day of pregnancy, especially in the lower concentration range. In the case of cAMP measurements, sildenafil was not able to elevate the levels and there was no concentration-dependent relation either on day 20 or on day 22. Moreover, the low concentration of terbutaline used in our experiments could not affect cAMP levels significantly on day 22. This phenomenon suggests that the potentiating effect observed in the in vitro and in vivo results at low doses is mainly due to the increase in the level of cGMP. Previous studies already proved that β_2-mimetics are coupled to the NO/cGMP signalling pathway in the vascular system indicating the possible

cGMP-enhancing role of terbutaline in the mechanism of action in sildenafil – terbutaline co-administration [48]. However, earlier studies with human found low efficacy of elevation in myometrial cGMP levels for relaxation [46], that may suggest potential limited applicability our rat results in the clinical practice.

In SMEMG measurements, we proved that the synergistic combination of $MgSO_4$ with terbutaline, and sildenafil with terbutalin also work *in vivo*. At first, in the case of $MgSO_4$ and terbutaline, we saw that the relaxing effect of the combination was maintained successfully with the applied repeated administrations. Although $MgSO_4$ could not increase the maximal effect of terbutaline again, it shifted the dose-response curve to the left, which means that a beneficial effect can be expected when using the combination in the lower dose range of terbutaline. It is known that β_2-adrenergic receptor desensitization could worsen the efficacy of β_2-mimetics during tocolytic therapy [38]. Consequently, a lower dose of terbutaline with the potentiation of $MgSO_4$ could reduce the desensitization of β_2-adrenergic receptors, additionally contributing to the potential advantages of this combination during tocolytic therapy.

It is known that the cardiac effects of $MgSO_4$ and terbutaline are the opposite. Due to the unfavourable tachycardia-inducing effect of terbutaline, we also wanted to examine how this kind of combination affects the heart rate. Low and high doses of terbutaline increased cardiac frequency in rats, but in both cases the tachycardia-causing effect of terbutaline was minimized or reduced by the co-administration of $MgSO_4$. This means that besides the improved uterorelaxant activity, $MgSO_4$ may significantly reduce the main cardiovascular side effect of terbutaline during tocolysis.

In case of the sildenafil – terbutaline combination SMEMG measurements, this study also revealed a similar potentiating effect, as it was demonstrated in our in vitro experiments. The cumulative administration of sildenafil in the presence of low-dose terbutaline elicited an enhanced relaxing effect, especially in the lower dose range, which also points to the potential benefits of this combination in tocolysis, since by reducing the dosage of each tocolytic agent, the occurrence of possible maternal and foetal side effects can also be reduced. The dose of terbutaline applied during in vivo studies was almost 40-fold lower than that of its concentration eliciting the maximum effect. Both compounds have an anti-inflammatory action, which suggests an extra benefit of this combination in preterm birth with an inflammatory background

[49][50]. High doses of β_2-mimetics induce receptor desensitization [38] and reduce efficacy, supporting the benefit of a combined therapy using a single low dose of terbutaline.

Co-administration with lower doses may have the advantage of reducing both the hypotonic effect of PDE5-Is and the tachycardic effect of β_2-mimetics, which is beneficial in terms of maternal cardiac side effects. It is known that terbutaline raises maternal glucose level during pregnancy. Therefore, the improving effect of β-cell function of PDE5-Is could also be beneficial [51].

As it is well-known, the main limitation of this study is that the rat model is quite different from the human model, therefore further studies are required before the clinical trials. An other weakness of this study is that the experiments do not provide data on the maternal and foetal side effects of the combined drug regimen. Unfortunately, the side effects of terbutaline, such as foetal tachycardia or nausea, could not be investigated as the rat model is unsuitable for that purpose. Another limitation of this study is that the heart rate modulating effects of the combination were not measured in case of the sildenafil – terbutaline combination. Moreover, in the highest concentration the PDE4 inhibitory effect of sildenafil cannot be excluded [52].

6. Conclusion

Based on our results, we conclude that the combination of β_2-agonist terbutaline with $MgSO_4$, and PDE5-inhibitor sildenafil with terbutaline may be of clinical importance in tocolytic therapy. We found that terbutaline and sildenafil alone have a significant uterine relaxing effect both in vitro and in vivo. In the case of the combination of $MgSO_4$ and terbutaline, the administration of a small dose of $MgSO_4$ significantly enhanced the relaxant effect of terbutaline, especially in the lower range. However, in Ca^{2+}-poor environment, $MgSO_4$ was not able to increase the effect of terbutaline, indicating the role of $MgSO_4$ as a Ca^{2+} channel blocker. In the cardiovascular studies, $MgSO_4$ significantly decreased the tachycardia-inducing effect of terbutaline in late-pregnant rats. In the case of the sildenafil – terbutaline combination, low-dose terbutaline further enhanced the effect of sildenafil, especially in the lower concentration range. We have also demonstrated that the potentiating effect of terbutaline is due to its ability to increase cGMP levels. We hypothesize that combination therapy may reduce the unfavourable side effects of each compound. Nevertheless, the applicability and pharmacokinetic properties of the combinations must be demonstrated in well-designed clinical studies.

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ORIGINAL RESEARCH ARTICLE

Combined uterorelaxant effect of magnesium sulfate and terbutaline: Studies on late pregnant rat uteri in vitro and in vivo

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Abstract

Introduction: Preterm delivery and its complications are among the biggest challenges and health risks in obstetrical practice. Several tocolytic agents are used in clinical practice, although the efficacy and side effect profiles of these drugs are not satisfying. The aim of this study was to investigate the uterus relaxant effect of the coadministration of β_2-mimetic terbutaline and magnesium sulfate ($MgSO_4$) in an isolated organ bath and to perform in vivo smooth muscle electromyographic (SMEMG) studies in pregnant rats. In addition, we also investigated whether the tachycardia-inducing effect of terbutaline can be reduced by the presence of magnesium, due to the opposite heart rate modifying effects of the two agents.

Material and methods: In the isolated organ bath studies, rhythmic contractions of 22-day- pregnant Sprague–Dawley rats were stimulated with KCl, and cumulative dose–response curves were constructed in the presence of $MgSO_4$ or terbutaline. The uterus-relaxing effects of terbutaline were also investigated in the presence of $MgSO_4$ in both normal buffer and Ca^{2+}-poor buffer. The in vivo SMEMG studies were carried out under anesthesia with the subcutaneous implantation of an electrode pair. The animals were treated with $MgSO_4$ or terbutaline alone or in combination in a cumulative bolus injection. The implanted electrode pair also detected the heart rate.

Results: Both $MgSO_4$ and terbutaline reduced uterine contractions in vitro and in vivo, furthermore, the administration of a small dose of $MgSO_4$ significantly enhanced the relaxant effect of terbutaline, especially in the lower range. However, in Ca^{2+}-poor environment, $MgSO_4$ was not able to increase the effect of terbutaline, indicating the role of $MgSO_4$ as a Ca^{2+} channel blocker. In the cardiovascular studies, $MgSO_4$ significantly decreased the tachycardia-inducing effect of terbutaline in late pregnant rats.

Abbreviations: AUC, area under the curve; EC_{50}, half maximal effective concentration; E_{max}, maximal inhibitory effect; SMEMG, smooth muscle electromyography.

Conclusions: The combined application of $MgSO_4$ and terbutaline may have clinical significance in tocolysis, which must be confirmed in clinical trials. Furthermore, $MgSO_4$ could substantially reduce the tachycardia-inducing side effect of terbutaline.

KEYWORDS
magnesium sulfate, pregnancy, tocolysis, uterine contractility, β_2-adrenoreceptor agonists

1 | INTRODUCTION

Preterm birth is defined by the World Health Organization as childbirth between 20 and 37 weeks of gestation, which is one of the biggest challenges and health risks in obstetrical practice. Preterm delivery and its complications are the leading cause of mortality and a variety of health and developmental problems, such as cerebral palsy, intellectual disabilities, and vision or hearing impairments all over the world.[1,2] Several mechanisms of diseases implicated in spontaneous preterm labor are known nowadays. Infections, vascular or cervical diseases, decidual senescence, uterine overdistension, breakdown of maternal-fetal tolerance and other genetic or environmental factors may contribute to the process.[3] A wide range of drugs (tocolytics) are applied to inhibit myometrial contractions, including β-adrenergic receptor agonists (betamimetics), calcium channel blockers, prostaglandin inhibitors, oxytocin receptor antagonists, nitric oxide donors and magnesium sulfate ($MgSO_4$). The main goal of using these tocolytic drugs is to delay delivery long enough to allow for the administration of prophylactic corticosteroids to decrease the severity of lung disease of prematurity, prolong gestation to achieve fetal maturation and allow time to transfer the mother to a high-level health care facility.[4] Although the currently available tocolytic agents are used in clinical practice, the efficacy and side effect profiles of these drugs are not satisfying.[5]

It is known that β_2-agonist terbutaline had been considered for first-line clinical use; however, the applied high doses and the presence of β-adrenergic receptors in the cardiovascular system cause a number of severe maternal side effects, such as reflex tachycardia, which compensate for the hypotension caused by vasodilation. Moreover, other adverse effects such as headache, tremor, dyspnea or pulmonary oedema are responsible for its limited use.[6] Its smooth muscle relaxant effect is linked to the increased intracellular level of cyclic adenosine monophosphate (cAMP) as myosin light-chain kinase activity is inhibited.[7]

$MgSO_4$ was first described as a tocolytic agent, but nowadays it is mostly used in the treatment of eclampsia and for fetal neuroprotection.[8,9] However, the exact mechanism of action of $MgSO_4$ has not been completely defined. It is suggested that magnesium acts both intra- and extracellularly. It is able to inhibit the transport of Ca^{2+} through the voltage-gated calcium channels and to decrease Ca^{2+} release from the sarcoplasmic reticulum.[10] The therapeutic use of $MgSO_4$ is controversial regarding the benefits and maternal and fetal adverse effects. Common maternal side effects such as bradycardia, flushing, nausea, headache, and hypothermia, as well as fetal side effects like lethargy, hypotonia, hypocalcemia, and respiratory depression limit the applicability of $MgSO_4$.[7,11] In spite of these unfavorable side effects, magnesium can be used successfully in cardiac arrhythmias due to its important role in cardiac metabolism and its electrophysiological properties.[12]

Key message

$MgSO_4$ significantly enhanced the uterorelaxant effect of terbutaline, while the tachycardia-inducing adverse effect of terbutaline was reduced. The combined application of $MgSO_4$ and terbutaline may have clinical significance in tocolysis.

There is an increased need for tocolytic therapy with better efficacy, which may involve the combination of already applied drugs. Earlier we proved that the addition of a β_2-agonist to a Ca^{2+} channel blocker enhanced the effect of the Ca^{2+} antagonist on pregnant rat myometrium in vitro and in vivo.[13] As a further combination with terbutaline, our aims were to investigate the uterus relaxant effect of the coadministration of β_2-mimetic terbutaline and $MgSO_4$ in an isolated organ bath and to perform in vivo smooth muscle electromyographic (SMEMG) studies in pregnant rats. In addition, we also investigated whether the tachycardia-inducing effect of terbutaline can be reduced by the presence of magnesium, due to the opposite heart rate modifying effects of the two agents.

2 | MATERIAL AND METHODS

2.1 | Housing and handling

The animals were housed in rooms with controlled temperature (22 ± 3°C), humidity (30%–70%), and light (12 h light/dark cycle), with tap water and standard rodent pellet (Animalab Hungary Ltd, Vác, Hungary) available ad libitum.

2.2 | Mating of the animals

Male (240–260 g) and female (180–200 g in the estrus phase) Sprague–Dawley rats were mated in a special mating cage. Copulation was confirmed by the presence of a copulation plug or sperm in the

vaginal smears. After the successful copulation, female rats were separated and were regarded as first-day pregnant animals.

2.3 | Isolated organ studies

2.3.1 | Uterus preparation

The animals were terminated by inhalation of carbon dioxide. Uterine samples were removed from 22-day-pregnant rats (250–350 g). After the horns of uteri were excised, 5-mm-long muscle rings were sliced (4 rings from 1 animal), cleaned of fat and connective tissue, and mounted vertically in an organ bath containing 10 mL of 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 organ bath was maintained at 37°C and carbogen (95% O_2+ 5% CO_2) was bubbled through the buffer. Before carrying out the experiments, the rings were equilibrated for about 1 h, with a solution change every 15 min. The initial tension of the uterus samples was set to about 1.5 g, which was measured with a gauge transducer (SG-02; MDE GmbH.) and recorded and analyzed with a SPEL Advanced ISOSYS Data Acquisition System (MDE GmbH).

2.3.2 | Magnesium sulfate studies

The uteri were placed in the organ bath as described above. Equilibrated contractions were elicited with 25 mM KCl (7–10 min), and cumulative concentration–response curves were constructed in each experiment in the presence of $MgSO_4$ (10^{-8} – 10^{-2} M) (Molar Chemicals Kft.). Recording was performed for 5 min after each concentration of $MgSO_4$. Concentration–response curves were fitted, and the areas under the curves (AUCs) were evaluated and analyzed statistically. From the AUC values, the maximal inhibitory effect (Emax) of $MgSO_4$ and the concentration of $MgSO_4$ eliciting 50% of the maximal inhibition of uterine contraction (EC_{50}) were calculated.

2.3.3 | Magnesium sulfate combination with terbutaline

The drug combination studies were carried out by using the method described above, and the contractions were induced by KCl. The cumulative-concentration response curves were elicited in the presence of $MgSO_4$ in combination with terbutaline (Sigma-Aldrich, Budapest, Hungary). After one dose of $MgSO_4$ (10^{-7} M), terbutaline (10^{-9}–10^{-5} M) was administered every 5 min. The uterus-relaxing effects of terbutaline were also investigated alone.

The uterus relaxant effects of the combination of $MgSO_4$ and terbutaline were also investigated in Ca^{2+}-reduced buffer in vitro. As opposed to the normal 1 mM Ca^{2+}-containing buffer, a solution containing 0.1 mM Ca^{2+} was used to induce a low Ca^{2+} environment. During the incubation time, normal De Jongh solution was used for 1 h, and after this equilibration period, the buffer was changed to the low Ca^{2+}-containing buffer.

2.4 | Smooth muscle electromyographic studies

Twenty-two-day-pregnant rats were used for the SMEMG measurements. Anesthesia was induced by intraperitoneal (i.p.) injection of ketamine (36 mg/kg)-xylazine (4 mg/kg) and maintained by the inhalation of isoflurane (0.5%–1%) (R550 multioutput Animal Anesthesia Machine, Animalab Hungary Ltd.). The jugular vein was cannulated for intravenous drug administration, and a bipolar disk electrode (SEN-15-2; MDE GmbH) was inserted subcutaneously above the uterus to detect the myoelectric signals of contractions in the pregnant uterus (the distance between the two electrodes was 20 mm). Control myoelectric signals were registered for 45 min, and a 1: 100 mixture of heparin-Na and physiological saline was injected into the jugular vein every 15 min to prevent coagulation. The animals were treated with $MgSO_4$ or terbutaline alone in a cumulative way every 15 min. The effect of the coadministration of $MgSO_4$-terbutaline was also registered. In each dose, the animals were treated with constant intravenous $MgSO_4$ (0.3 mg/kg) while increasing doses of terbutaline (from 0.05 to 50 μg/kg every 15 min) were added in a cumulative manner. The myoelectric signals of the pregnant uterus, representing the strength of contractions,[14] were detected by an online computer system (SPEL Advanced ISOSYS Data Acquisition System). Uterine contractility was evaluated by fast Fourier transformation (FFT). The spectrum of the SMEMG activity was characterized in the frequency range of 1–3 cycles per minute, and the power spectrum density maximum (PsD_{max}) was calculated.

2.5 | Heart rate measurements

A bipolar disk electrode (SEN-15-2; MDE GmbH) was placed subcutaneously on the surface of the abdominal wall to detect the heart rate signals of rats on day 22 of pregnancy under anesthesia as described above (Figure 1). After control signals were measured (15 min), terbutaline (50 μg/kg or 500 μg/kg) was injected into the cannulated jugular vein to detect the changes in frequency for 15 min. The combination of terbutaline (50 μg/kg or 500 μg/kg) with $MgSO_4$ (2.1 mg/kg, which is equivalent to 7 doses of $MgSO_4$ used during the SMEMG experiments) was also registered. Heart rate signals were detected with an SMEMG/HR/BT Holter system (MSB-MET Ltd.).

2.6 | Statistical analyses

All data were analyzed using Prism version 5.01 (GraphPad Software) computer program. The values were statistically evaluated with an

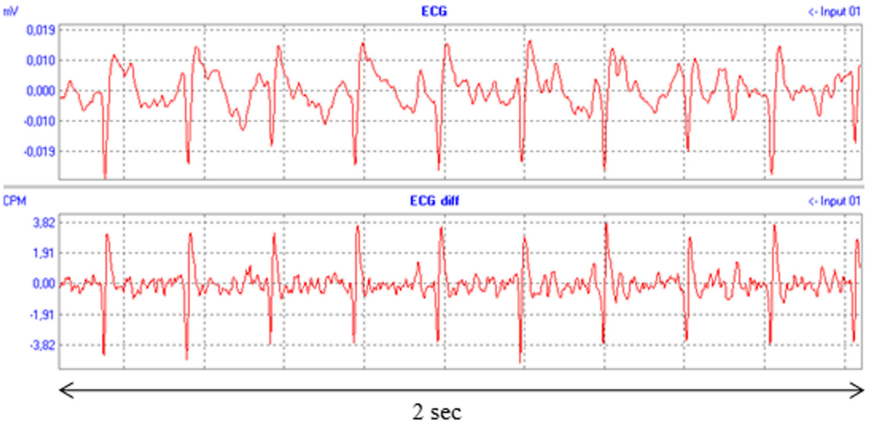

FIGURE 1 Heart rate signals of the pregnant rat detected with a bipolar disk electrode, which was positioned on the abdomen under anesthesia. For easier readability, we used the differential of the electrocardiogram signal (ECG diff) for analysis.

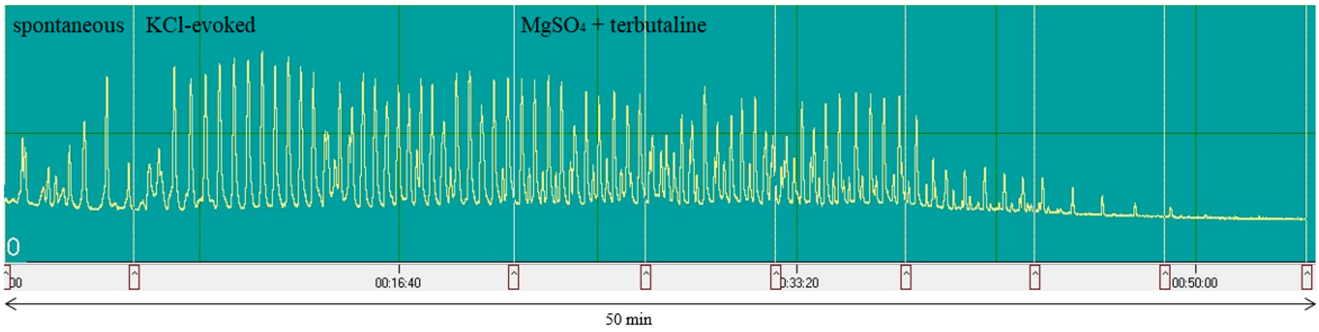

FIGURE 2 Recorded signals of the 22-day-pregnant uterus in vitro: spontaneous contractions, KCl-evoked contractions and contractions in the presence of $MgSO_4$ and terbutaline.

unpaired *t*-test. Shapiro–Wilk test was used to assess normality of distribution (p-value = 0.743).

2.7 | Ethics statement

The animals were treated in accordance with the European Communities Council Directive (2010/63/EU) and the Hungarian Act for the Protection of Animals in Research (Article 32 of Act XXVIII). All experiments involving animal subjects were carried out with the approval of the National Scientific Ethical Committee on Animal Experimentation (registration number: XIII./72/2020, February 25, 2020–February 25, 2023).

3 | RESULTS

3.1 | In vitro contractility studies

Myometrial activity was determined by the AUC of the concentration-response curves of 22-day-pregnant Sprague–Dawley rat uterine strips (Figure 2). Both $MgSO_4$ and terbutaline inhibited the KCl-evoked (25 mM) contractions in a concentration-dependent manner. The maximal inhibitory effect (E_{max}) of $MgSO_4$ reached almost 100% in the range of 10^{-8}–10^{-1} M (Figure 3A; Table 1). The E_{max} of terbutaline alone was also over 90% in the range of 10^{-10}–10^{-4} M. Pre-treatment with $MgSO_4$ (10^{-7} M) significantly enhanced the relaxant effect of terbutaline, especially in the lower range ($p < 0.01$); however, it could not improve the E_{max} of terbutaline (Figure 2, 3B). The maximal uterus relaxant effect (E_{max}) and the EC_{50} of terbutaline compared with the combination ($MgSO_4$ pre-treatment (10^{-7} M + terbutaline)) were not significant in reduced Ca^{2+} buffer (0.1 mM Ca^{2+}) (Figure 3C; Table 1).

3.2 | In vivo contractility studies

Both $MgSO_4$ and terbutaline caused dose-dependent myometrial relaxation on 22-day-pregnant rats in vivo. The E_{max} of $MgSO_4$ was about 70% relaxation in the range of 0.1–30 mg/kg (Figure 5A), while the relaxing E_{max} of terbutaline (94.3% ± 1.8%) was similar to the in vitro results in the range of 0.05–50 μg/kg. The maximal inhibition of the coadministration was not statistically different from the administration of terbutaline alone; however, the curve was shifted to the left (Figure 4, 5B; Table 2).

3.3 | Heart rate studies

Both the lower dose (50 μg/kg) and the higher dose (500 μg/kg) of terbutaline alone increased heart rate, especially at the beginning of the experiment. In the case of the lower dose of terbutaline

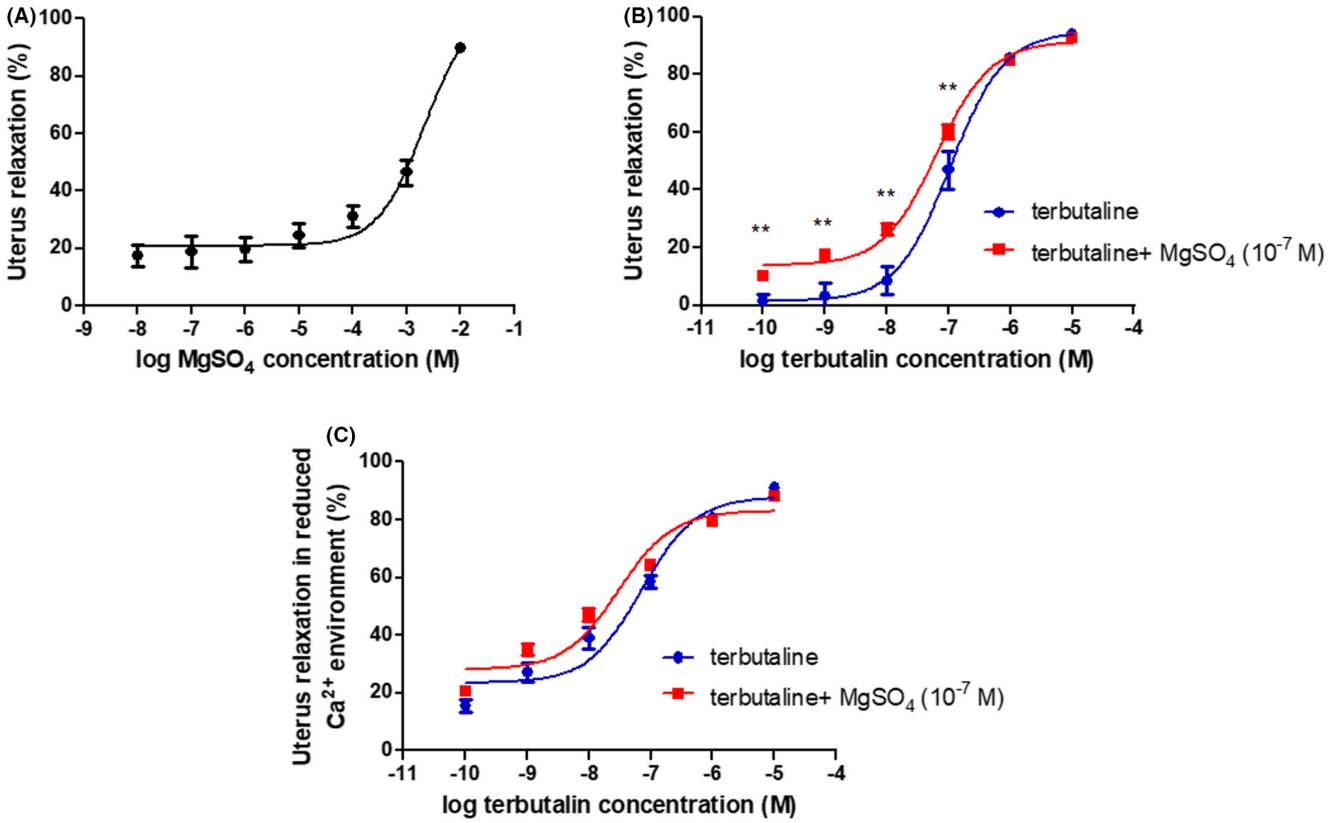

FIGURE 3 Inhibitory effect of $MgSO_4$ alone (A), terbutaline alone (B) and in combination with $MgSO_4$ (10^{-7} M) (B) in normal Ca^{2+}-containing buffer. Also, inhibitory effect of terbutaline alone (C) and in combination with $MgSO_4$ (C) in reduced Ca^{2+} environment on contractions evoked by 25 mM KCl on pregnancy day 22. $^{**}p < 0.01$.

TABLE 1 EC_{50} and E_{max} values of curves of uterine relaxation induced by $MgSO_4$ (A), terbutaline (10^{-10}–10^{-5} M) alone or in the presence of $MgSO_4$ (10^{-7} M) at 1 mM (B) or 0.1 mM (C) Ca^{2+} level-containing buffer. The level of significance is related to the comparison with the values of terbutaline

	EC_{50} (M ± SEM)	*p*-value	E_{max} (% ± SEM)	*p*-value
(A)				
$MgSO_4$	$1.6 \pm 0.7 \times 10^{-3}$	–	89.8 ± 1.1	–
(B)				
terbutaline	$1.3 \pm 0.6 \times 10^{-7}$		95.9 ± 2.3	
terbutaline + $MgSO_4$	$7.2 \pm 3.1 \times 10^{-8}$*	0.022	92.0 ± 4.9	0.089
(C)				
terbutaline	$7.4 \pm 5.4 \times 10^{-8}$		87.5 ± 4.6	
terbutaline + $MgSO_4$	$3.6 \pm 2.7 \times 10^{-8}$*	0.041	83.2 ± 3.3	0.056

Abbreviation: SEM, standard error of the mean.
*$p < 0.05$.

(Figure 6A), the changes in cardiac frequency were below 10% in each minute; however, in the presence of $MgSO_4$ (2.1 mg/kg), the heart rate-increasing effect of terbutaline was significantly decreased between the first and fifth minute ($^{*}p < 0.05$, $^{**}p < 0.01$). Similarly to the lower dose, the higher dose of terbutaline also increased the heart rate (Figure 6B). From the second minute, the changes in cardiac frequency were over 10%. In combination with $MgSO_4$, the heart rate was significantly reduced ($^{*}p < 0.05$, $^{**}p < 0.01$), especially from the third minute. In both cases, there were no changes in cardiac frequency after the fifth minute.

4 | DISCUSSION

In view of the efficiency of the currently used tocolytic therapy, combinations of different smooth muscle relaxant agents should be considered as an option to decrease adverse maternal and fetal events and enhance efficacy. Progestogen treatment was previously found to enhance the tocolytic effect of salmeterol in hormone-induced preterm labor in rats in vivo, and the addition of terbutaline to nifedipin also increased the uterorelaxant effect of nifedipin on pregnant rat myometrium in vitro and in vivo.[13,15]

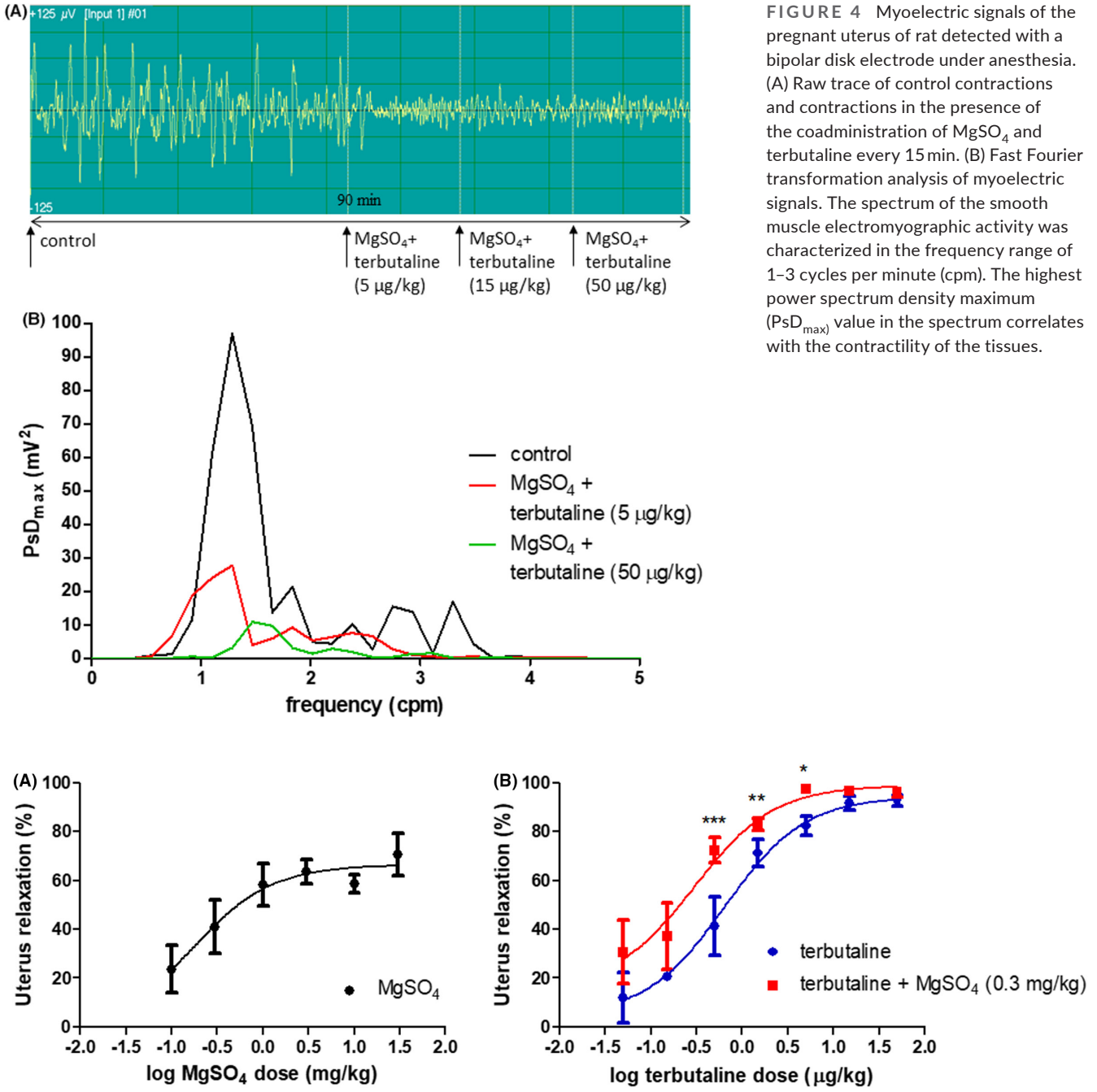

FIGURE 4 Myoelectric signals of the pregnant uterus of rat detected with a bipolar disk electrode under anesthesia. (A) Raw trace of control contractions and contractions in the presence of the coadministration of $MgSO_4$ and terbutaline every 15 min. (B) Fast Fourier transformation analysis of myoelectric signals. The spectrum of the smooth muscle electromyographic activity was characterized in the frequency range of 1–3 cycles per minute (cpm). The highest power spectrum density maximum (PsD_{max}) value in the spectrum correlates with the contractility of the tissues.

FIGURE 5 Inhibitory effect of $MgSO_4$ alone (A), terbutaline alone (B) and in combination with $MgSO_4$ (0.3 mg/kg) (B) on pregnancy day 22 in vivo. $^*p<0.05$; $^{**}p<0.01$, $^{***}p<0.001$

Since terbutaline and $MgSO_4$ are widely used tocolytic agents, even though their efficacy and side effect profiles are not completely satisfying, our main goal was to investigate the uterus relaxant effect of the combination of these drugs. In our study, we have successfully demonstrated that both the in vitro and in vivo combinations of the two compounds elicited a uterorelaxant effect stronger than that of the compounds alone.

In the isolated organ bath studies, we found synergism in the uterorelaxant effect of terbutaline and $MgSO_4$, although $MgSO_4$ was not able to enhance the maximal inhibitory effect of terbutaline. However, we observed a significant potentiating effect at lower concentrations, which may have importance in the reduction of adverse effects. We also proved that Ca^{2+} plays a significant role in this process. It is known that the entry of Ca^{2+} into the cells through the voltage-dependent Ca^{2+} channels has a crucial influence on smooth muscle contraction. One possible mechanism of action of $MgSO_4$ is the blockade of the voltage-dependent Ca^{2+} channel, which ability can be blocked in Ca^{2+}-poor environment.[10] Hence, the coadministration of the two agents was also investigated in a buffer containing Ca^{2+} reduced by 90% in order to verify the above-mentioned hypothesis. In a Ca^{2+}-poor buffer, $MgSO_4$ was not able to enhance the effect of terbutaline. These results led us to conclude that $MgSO_4$

TABLE 2 ED_{50} and E_{max} values of curves of uterine relaxation induced by $MgSO_4$, terbutaline (10^{-10}–10^{-5} M) alone or in the presence of $MgSO_4$ (0.3 mg/kg) in vivo. The level of significance is related to the comparison with the values for terbutaline

	ED_{50} (± SEM)	*p*-value	E_{max} (% ± SEM)	*p*-value
(A) $MgSO_4$	0.20 ± 0.08 mg/kg	—	69.9 ± 12.2	—
(B) terbutaline	0.65 ± 0.19 μg/kg		94.3 ± 1.8	
terbutaline + $MgSO_4$	0.30 ± 0.10* μg/kg	0.036	98.7 ± 2.2	0.106

Abbreviation: SEM, standard error of the mean.

*$p < 0.05$.

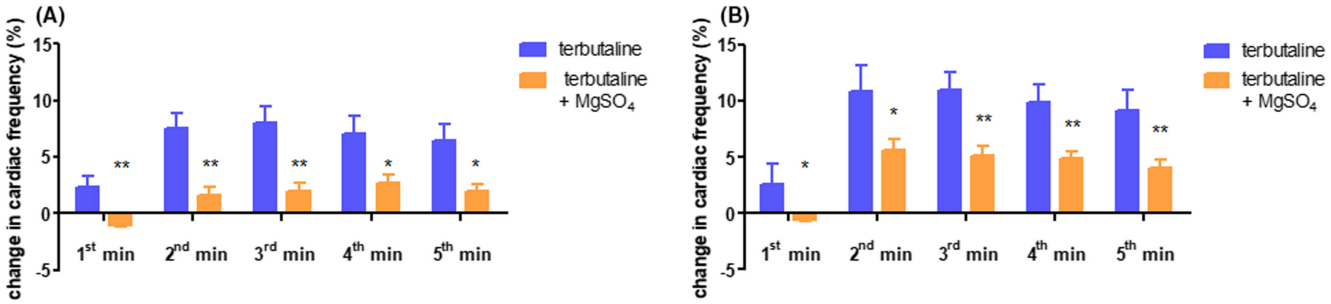

FIGURE 6 Changes in cardiac frequency during treatment with 50 μg/kg (A) or 500 μg/kg (B) terbutaline alone or in combination with $MgSO_4$ on pregnancy day 22. *$p < 0.05$

potentiates the uterorelaxant effect of terbutaline via the inhibition of voltage-dependent Ca^{2+} channels.

In the second series of our experiments, we proved that the synergistic combination of $MgSO_4$ and terbutaline also works in vivo. The relaxing effect of the combination was maintained successfully with the applied repeated administrations. Although $MgSO_4$ did not increase the maximal effect of terbutaline again, it shifted the dose–response curve to the left, which means that a beneficial effect can be expected when using the combination in the lower dose range of terbutaline. It is known that β_2-adrenergic receptor desensitization could worsen the efficacy of β_2-mimetics during tocolytic therapy.[16] Consequently, a lower dose of terbutaline with the potentiation of $MgSO_4$ could reduce the desensitization of β_2-adrenergic receptors, additionally contributing to the potential advantages of this combination during tocolytic therapy.

It is known that the cardiac effects of $MgSO_4$ and terbutaline are the opposite. Due to the unfavorable tachycardia-inducing effect of terbutaline, we also wanted to examine how this kind of combination affects the heart rate. Low and high doses of terbutaline increased cardiac frequency in rats, but in both cases the tachycardia-causing effect of terbutaline was minimized or reduced by the coadministration of $MgSO_4$. This means that in addition to the improved uterorelaxant activity, $MgSO_4$ may significantly reduce the main cardiovascular side effect of terbutaline during tocolysis.

As to the strengths of this study, we successfully proved the potentiating effect of low concentration of $MgSO_4$ on terbutaline effect both in vitro and in vivo. $MgSO_4$ was also able to decrease the heart rate enhancing effect of terbutaline. The applied dose of $MgSO_4$ during our in vivo studies was hundredfold less than that of its concentration eliciting the maximum effect, therefore we have a high chance to reduce or even eliminate the potential side effects and to maintain the cardiac benefit. Nevertheless, the study also had some limitations. As it is known, $MgSO_4$ has several maternal and fetal side effects. Unfortunately, a rat model was not suitable to check the side effects of hot flushes or burning sensations, thus only a clinical trial can clarify this question. The fetal cardiovascular effect of the combination has also not been measured. Another limitation of this study is that the blood pressure modulating effect of the combination was not nvestigated.

It is known that β-mimetic drugs cause hypertension in high doses; however, $MgSO_4$ also has the potential to decrease the enhanced blood pressure as it was seen in the heart rate. Therefore, one of our aims in the future is also to investigate the blood pressure modifying effect of the $MgSO_4$ + terbutaline combination. Additionally, among future research, the in vitro examination of the combination in cases of full-term and preterm human uteri tissues from cesarean section also should be considered.

5 | CONCLUSION

In the light of our results, we can conclude that the combined administration of β_2-agonist terbutaline with $MgSO_4$ has potential therapeutic importance in the inhibition of uterine contractions, especially in the lower concentration range. Moreover, this combination may reduce the unfavorable tachycardia-inducing effect of terbutaline. We hypothesize that if the dose of $MgSO_4$ is substantially reduced, the side effect both of terbutaline and $MgSO_4$ should be more tolerable with an enhanced clinical effect, that may give the clinical importance of this combination. The coadministration of these compounds can be efficacious and safe for tocolysis, but its applicability should be confirmed in well-designed clinical trials.

AUTHOR CONTRIBUTIONS

TB investigation: performed in vitro and in vivo studies, contributed to sample preparation; formal analysis of the results of contractility measurements; writing - original draft. KFS investigation: designed and performed in vivo contractility studies; formal analysis of the results. AS investigation: performed in vitro contractility studies; formal analysis: aided in interpreting the results. MM investigation: performed in vitro contractility studies. JH-T conceptualization; investigation: performed in vitro contractility studies; formal analysis: aided in interpreting the results. RG conceptualization; methodology; writing - review & editing: provided critical feedback; supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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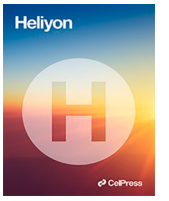

The combined uterorelaxant effect of sildenafil and terbutalin in the rat: The potential benefit of co-administration of low doses

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ABSTRACT

Aims: Our aims were to investigate the uterus relaxant effect of sildenafil alone and co-administered with β_2-mimetic terbutaline in an isolated organ bath and to perform in vivo smooth muscle electromyographic studies in pregnant rats. The modifications in uterine cAMP/cGMP levels were also detected.

Main methods: Contractions of non-pregnant and 5/15/18/20/22-day pregnant uterine rings were measured in an isolated organ bath system in the presence of sildenafil alone or with terbutaline. The uterine levels of cAMP and cGMP were determined by commercial ELISA assays. The in vivo efficacy of the combination was measured by smooth muscle electromyography.

Key findings: Sildenafil reduced uterine contractions in vitro and in vivo; additionally, terbutaline significantly increased the uterorelaxant effect of sildenafil in the lower concentration or dose ranges. Terbutaline enhanced the cGMP level increasing effect of sildenafil.

Significance: The co-administration of sildenafil and terbutaline could be a promising tocolytic combination to reduce maternal and foetal adverse events and increase efficacy.

1. Introduction

Preterm birth prevention and treatment have always been a difficult challenge in obstetrical practice. Despite the percentage of premature birth decreasing over the last decade in the U.S., its global incidence is over 15 million each year, and the rates of neonatal morbidity remain high, which also means an economic burden for the families due to medical costs and events that arise later [1]. Our current knowledge of the mechanisms underlying preterm labor is still restricted, despite significant advancements in our understanding of myometrial physiology. Contributing factors include infections, stress, uteroplacental thrombosis and intrauterine vascular lesions associated with fetal stress or decidual hemorrhage, uterine overdistension, and cervical insufficiency [2]. Unfortunately, despite the well-known smooth muscle relaxant effects of many different classes of drugs (e.g., β-adrenergic receptor agonists, calcium channel blockers, prostaglandin inhibitors, oxytocin receptor antagonists, nitric oxide donors and magnesium sulfate), no new medications have achieved widespread clinical use for the treatment preterm labor in recent decades. This treatment gap is likely related to dose restrictions that are necessary because of unacceptable effects of smooth muscle relaxants on vascular tone or that directly affect the heart. One possible approach to maximizing tocolytic effects while minimizing cardiovascular effects would be to identify combinations of drugs that function synergistically in the uterus.

Sildenafil citrate is a selective inhibitor of phosphodiesterase type 5 (PDE5-Is) and is known for being efficacious as a treatment for

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erectile dysfunction. On the other hand, there is limited clinical knowledge on the effect of sildenafil in pregnancy [3]. One study suggests that sildenafil-nifedipine combination may be beneficial in preterm birth [4]. PDE-5 is the main enzyme responsible for the degradation of cyclic guanosine monophosphate (cGMP) following the release of nitric oxide (NO) from nitrergic nerves or vascular endothelial cells, leading to smooth muscle relaxation [5]. Although the presence of PDE5 has been proven in the uterus, only few studies have been carried out to investigate the effect of PDE-Is on uterine contractility [6]. It was found that sildenafil dose-dependently reduces the contractions of isolated pregnant human myometrium, which action is associated with the putative role of BK_{Ca} channels [7]. Moreover, the relaxant effect of sildenafil also appears to be related to the H_2S pathway in mouse uteri [8]. These findings imply that the potential tocolytic effect of sildenafil and other PDE5-Is may be mediated through various pathways.

Unfortunately, there is insufficient evidence to suggest that the recently used drugs substantially improve neonatal outcome during threatened preterm birth [9]. Medical therapies for acute tocolysis (maximum 48 h) appear to be beneficial. However, ACOG has not recommended medical therapy for maintenance tocolysis (treatment beyond 48 h) and the US FDA has not approved any medication for this indication. Despite the lack of sanctioning, treatments are often employed on an individual basis in clinical practice [10]. Therefore, it is necessary to either conduct research on new active compounds or investigate existing drugs, such as sildenafil citrate, as potential tocolytic agents. As noted above, combination therapy may offer benefit, although a wide range of possible cardiovascular side effects require scrutiny. For example, combining Ca-channel blockers and beta-adrenergic receptor agonists are associated with pulmonary hypertension [11]. Some evidence exists for synergistic effects with sildenafil and nifedipine [4]. Additionally, we have previously demonstrated in the pregnant rat, that synergistic tocolytic effects are found when combining terbutaline and magnesium sulfate or terbutaline and nifedipine [12,13]. Based on these, the importance of terbutaline in combination therapy is confirmed.

β-Adrenergic receptor agonists (β-mimetics), such as terbutaline, were among the most potent inhibitors of uterine contractility. They are able to enhance the level of cyclic adenosine monophosphate (cAMP), which reduces intracellular calcium levels by inhibiting myosin light-chain kinase activity [14]. Due to their numerous maternal and foetal adverse effects, like palpitations, tremor, nausea, pulmonary oedema and fetal tachycardia, β-mimetics have been found to be ineffective for maintenance tocolysis [15] and are not currently widely used.

Therefore, our aims were to investigate the uterus relaxant effect of sildenafil alone and in the presence in combination with β_2-mimetic terbutaline in an isolated organ bath in vitro and to perform in vivo smooth muscle electromyographic (SMEMG) studies in pregnant rats. Moreover, the changes of uterine cAMP and cGMP associated with these treatments were measured.

2. Materials and methods

2.1. Housing and handling of the animals

The animals were housed in rooms with controlled temperature (22 ± 3 °C), humidity (30%–70 %), and light (12 h light/dark cycle), with tap water and standard rodent pellet (Animalab Hungary Ltd, Vác, Hungary) available *ad libitum*.

2.2. Mating of the animals

Male (240–260 g) and female (180–200 g in the estrus phase) Sprague-Dawley rats (Animalab Hungary Ltd, Vác, Hungary) were mated in a special mating cage in the early morning hours. The estrus cycle was measured by an Estrus Cycle Monitor (IM-01, MSB-MET Ltd., Balatonfüred, Hungary). Rats with vaginal impedance values between 5.0 and 8.0 kΩ are in the proestrus phase and were chosen for the mating process. Intercourse was confirmed by the presence of a copulation plug or sperm in the vaginal smears. Positive cases were separated and were regarded as first-day pregnant animals.

2.3. Ethics statement

The animals were treated in accordance with the European Communities Council Directive (2010/63/EU) and the Hungarian Act for the Protection of Animals in Research (Article 32 of Act XXVIII). All experiments involving animal subjects were carried out with the approval of the National Scientific Ethical Committee on Animal Experimentation (registration number: XIII./735/2023.).

2.4. Isolated organ bath studies

On days 5, 15, 18, 20 and 22 of pregnancy or in the estrus phase of non-pregnant rats, the animals were sacrificed by inhalation of carbon dioxide. The uterine horns were removed from the rats, muscle rings with length of 5 mm (diameters varied according gestational age between 1 and 3 mm) were sliced and cleaned of connective tissue and fat, then immediately placed in an organ bath filled with 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 bubbled through it. After the tissue rings were mounted vertically, a 1-h equilibrium incubation period started with a solution change every 15 min. During the 60 min incubation time the spontaneous uterine contraction were stable. The initial tension of the uterus samples was set to about 1.5 g which remained unchanged during the experiment, which tensions were measured with a gauge transducer (SG-02; MDE GmbH., Walldorf, Germany) and recorded and analysed with a SPEL Advanced ISOSYS Data Acquisition System (MDE GmbH, Walldorf, Germany).

Rhythmic contractions were elicited by exposure to 25 mM KCl for 7–10 min, and concentration–response curves were constructed

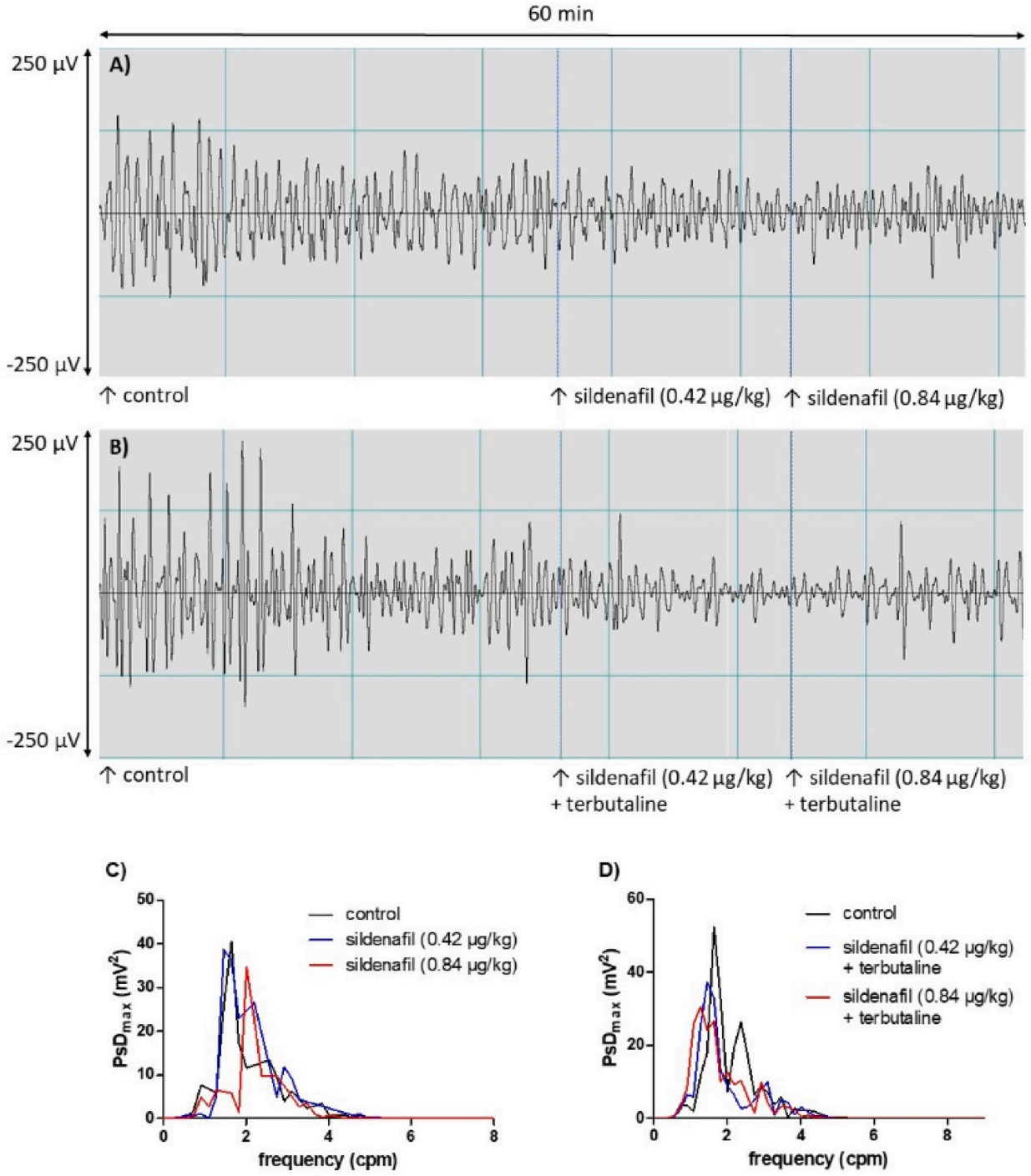

Fig. 1. Primary myoelectric signals of the 22-day pregnant uterus of rat detected with a bipolar disk electrode under anesthesia. (A, B): raw trace of control contractions and contractions in the presence of two doses of sildenafil alone (A) or co-administration with terbutaline (B). The FFT was carried on in period of 15 min after each dose filtering to frequency range of 1–3 cpm that is characteristic for pregnant uterus during the last day of gestational period. The biggest peaks called power spectrum density maximum (PsD_{max}) in the frequency range reflect the contraction force of the uterus [17]. As the dose was increased, the height of the peaks were reduced corresponding to the uterine relaxation (C, D). The uterine relaxant effect of the drugs was calculated as a percentage compared to the control PsD_{max} value.

in each experiment. For single drug studies, sildenafil-citrate (10^{-10} – 10^{-4} M) (Sigma-Aldrich, Budapest, Hungary) and terbutaline (10^{-10} – 10^{-5} M) (Sigma-Aldrich, Budapest, Hungary) were sequentially added every 5 min to final concentrations. (The concentration of terbutaline was determined based on previous literature data [13], while the concentration of sildenafil was determined by our preliminary, unpublished pilot study.). In the case of the terbutaline – sildenafil combination studies, sildenafil was administered every 5 min after one dose of terbutaline (10^{-8} M). In the opposite case, after a single dose of sildenafil (10^{-6} M), terbutaline was administered by using the method described above. Concentration–response curves were fitted, and the areas under the curves (AUCs) were evaluated and analysed. The maximal inhibitory effect (E_{max}) of sildenafil or the combination and the concentration of sildenafil eliciting 50 % of the maximal inhibition of uterine contraction (EC_{50}) were obtained from the AUC values.

2.5. Measurement of uterine cAMP and cGMP accumulation

Uterine tissue samples from 20- and 22-day pregnant rats were incubated in de Jongh solution, under the same conditions as detailed above. The tissues were incubated for 5 min in KCl (25 mM), then isobutylmethylxanthine (10^{-4} M) and different concentrations of sildenafil (from 10^{-10} until 10^{-4} M) without or with terbutaline (10^{-8} M) were added for a further 20 min. At the end of the incubation period, forskolin (10^{-5} M) was added for 10 min. The samples were immediately frozen in liquid nitrogen after stimulation and stored at −70 °C until cAMP and cGMP extraction. Frozen tissue samples were then ground, weighed, homogenized in 10 vol of ice-cold 5 % trichloroacetic acid and centrifuged at 600 *g* for 10 min. The supernatants were extracted with 3 vol of water-saturated diethyl ether, repeated 3 times, while the ether supernatants were removed. After drying, the extracts were stored at – 70 °C until the

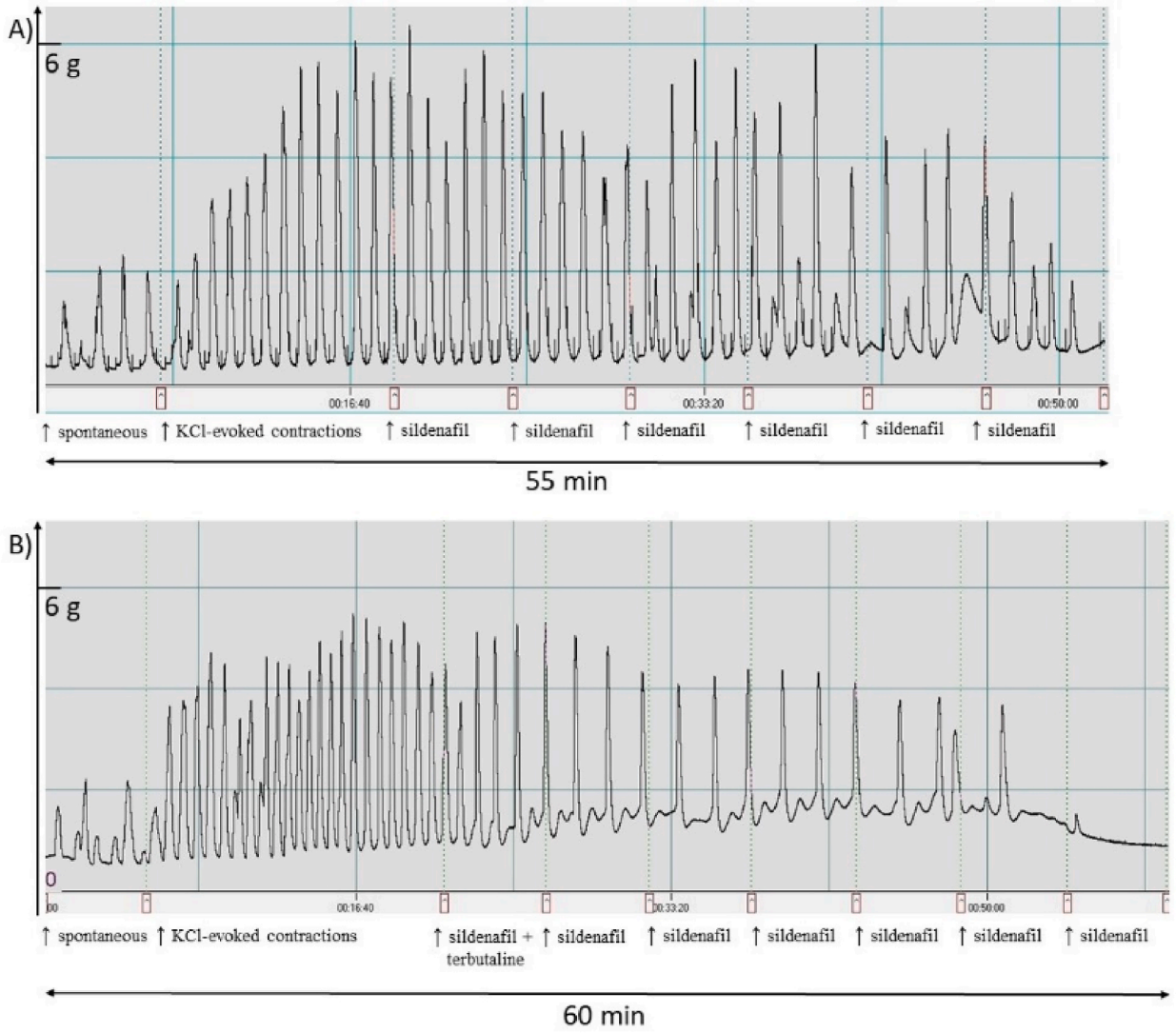

Fig. 2. Representative raw traces of the 22-day pregnant uterine contractions in vitro: spontaneous contractions, KCl-evoked contractions and contractions in the presence of sildenafil alone (A) or in combination with terbutaline (B).

cAMP/cGMP assays. The accumulation of uterine second messengers was measured with commercial cAMP Enzyme Immunoassay Kit (Enzo Life Sciences, USA) and cGMP Enzyme Immunoassay Kit (DRG Instruments GmbH, Germany). The levels of cAMP and cGMP were expressed in pmol/mg and pmol/g tissue.

2.6. In vivo uterine studies

In vivo uterine studies were performed by smooth muscle electromyography (SMEMG) on day 22 of pregnancy under anesthesia induced by the i. p. Injection of ketamine (36 mg/kg)-xylazine (4 mg/kg) and maintained by the inhalation of isoflurane (0.5–1%) using a small animal anesthesia device (R550 Multi-output Animal Anesthesia Machine, Animalab Hungary Ltd, Vác, Hungary). A bipolar disk electrode pair (SEN-15-2; MDE GmbH, Walldorf, Germany) was placed subcutaneously on the surface of the abdominal wall, while the jugular vein was cannulated for later intravenous (i.v.) drug administration. Control myoelectric signals of the uterus were registered for 45 min with heparin-Na and physiological saline (1: 100 mixture) injection every 15 min to prevent coagulation. Following this, the animals were treated with sildenafil and terbutaline alone in a cumulative way every 15 min. The effect of co-administered sildenafil and terbutaline was also monitored as the animals were treated with repeated i. v. Single doses of terbutaline (0.15 μg/kg) along with increasing doses of sildenafil (from 0.42 μg/kg to 4.2 mg/kg, sequential additions of 0.42 μg/kg, 1.26 μg/kg, 4.2 μg/kg, 12.6 μg/kg, 42 μg/kg, 0.126 mg/kg, 0.42 mg/kg, 1.26 mg/kg, 4.2 mg/kg) (The concentration of terbutaline was determined based on previous literature data [12,16], while the concentration of sildenafil was determined by our preliminary, unpublished pilot study.) every 15 min in a cumulative manner. A computer system (SPEL Advanced ISOSYS Data Acquisition System) monitored the myoelectric signals of the pregnant uterus, which represented the relative intensity of contractions. Electromyographic signals were amplified by using a custom-made amplifier. The custom made-amplifier functions with 4 channels, the gain is 1500-fold, the input impedance is 100 M Ohm, input type is symmetric, output impedance is 1 Ohm, output type is asymmetric, the amplification bandwidth is 0.01–1 Hz. All analogue signals were filtered with a first-order bandpass Bessel-type filter with a frequency of 1–3 cycles per minute (cpm) and were digitized at a sample rate of 2 Hz. Uterine contractility was evaluated by fast Fourier transformation (FFT), then the power spectrum density maximum (PsD_{max}, the highest peak of PsD) of the SMEMG activity was determined as previously described [17] (Fig. 1.). The FFT analysis were carried out every dosing interval (15 min). When more than one peak was found in the spectrum, only the highest peak was considered in the evaluation. The uterine relaxant effect of the drugs was expressed as a percentage compared to the control PsD_{max} value.

2.7. Statistical analysis

All data were analysed using Prism version 5.01 (GraphPad Software, San Diego, CA) computer program. The values of the isolated organ bath combination studies and the in vivo uterine studies were statistically evaluated with unpaired *t*-test (two-tailed) and the isolated organ bath studies on different days of pregnancy and the values of the cAMP/cGMP studies were evaluated with one-way ANOVA-test (Dunett's post hoc test). Shapiro-Wilk test was used to assess normality of distribution.

3. Results

3.1. Isolated organ bath studies

Contractions of uterine strips were determined based on the AUC values of the raw traces (Fig. 2.). The uterine relaxant effect of the drugs was expressed as a percentage compared to the KCl-evoked equilibrium activity. Sildenafil inhibited the KCl-evoked contractions in a concentration-dependent manner on each investigated gestational day and on non-pregnant uterine strips (Fig. 3.). There were significant differences in the lower concentration range (10^{-9}-10^{-7} M) on day 20 as compared with non-pregnant uteri. The maximal relaxing effect (E_{max}) of sildenafil was greater at the beginning of pregnancy and on non-pregnant uteri than on gestational days 18, 20

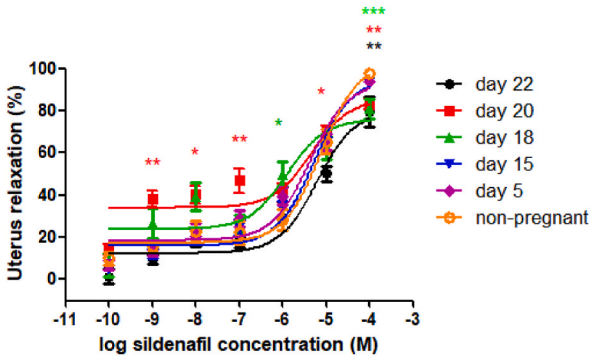

Fig. 3. Inhibitory effect of sildenafil (10^{-10} – 10^{-4} M) on pregnant (days 22/20/18/15/5) and non-pregnant uterine contractions stimulated with KCl (25 mM) (n = 8 rats/day). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ as compared with the values of non-pregnant uteri.

and 22 (Table 1.).

As sildenafil, terbutaline also caused dose-dependent myometrial relaxation (Fig. 4. A; B) in 22-day pregnant rats. The maximal inhibitory effect (E_{max}) of terbutalin alone was over 90 % at 10^{-5} M. The presence of terbutaline (10^{-8} M) significantly enhanced the cumulative relaxant effect of sildenafil, especially in the lower concentration range, however, it could not improve E_{max} (Table 2.). In the opposite case, sildenafil treatment followed by increasing doses of terbutaline, the presence of sildenafil (10^{-6} M) did not affect the cumulative uterine relaxing effect of terbutaline (Fig. 4. B). Since this combination had no benefit in the potentiating effect, no further in vivo testing was done with this kind of arrangement.

3.2. The effects of sildenafil and terbutaline on uterine cAMP and cGMP levels

The cGMP and cAMP levels of 20-day pregnant uterine tissue samples were measured in the presence of 10^{-10} – 10^{-4} M sildenafil (Fig. 5.). A concentration-dependent increase in cGMP levels was induced by sildenafil, however, it could not affect the levels of cAMP substantially. The levels of the second messengers were also measured on day 22 in the presence of 10^{-10} – 10^{-4} M sildenafil alone or in combination with 10^{-8} M terbutaline (Fig. 6.). Sildenafil also increased the cGMP levels in a concentration-dependent manner similarly to day 20. However, the effect of the lowest concentration of sildenafil on raising cGMP on day 20 is equivalent to the effect of 10^{-6} M sildenafil on day 22, which means that on day 20, compared to day 22, sildenafil has a greater ability to enhance the level of cGMP. Nevertheless, terbutaline significantly enhanced the levels of cGMP in the case of lower concentrations (10^{-10} – 10^{-6} M) of sildenafil, but it was not able to modify the levels of cAMP.

3.3. In vivo contractility studies

Similarly, to the organ bath contractility studies, uterus contractions were inhibited by both compounds in a dose-dependent manner in 22-day pregnant rats (Fig. 7.). The maximal relaxing effects (E_{max}) of terbutaline and sildenafil were around 95 % in the range of 0.5–50 μg/kg, and around 80 % in the range of 0.42 μg/kg – 4.2 mg/kg, respectively (Fig. 7.; Table 3.). The action of sildenafil was potentiated by terbutaline significantly at lower doses, however, the maximal inhibitions achieved with terbutaline + sildenafil were not statistically different, similarly to in vitro results.

4. Discussion

Due to its smooth muscle relaxant effect, sildenafil citrate, the selective inhibitor of PDE5 might be considered as a new, tocolytic agent. In the present study, in addition to the uterus relaxant effect of sildenafil, we also investigated the efficacy of its combination with terbutaline. As these two agents act through different pathways in uterine relaxation, we hypothesized that they would demonstrate a synergistic tocolytic effect on the uterus. Furthermore, the synergistic effect may be unique to the uterus, which would then allow reduced dosing of either or both drugs and provide a mechanism for reducing maternal and foetal side effects. The possibility of combination therapy for tocolysis, such as β_2-agonists with gestagens or sildenafil with Ca^{2+}-channel blockers, has been previously considered [18].

In isolated organ bath studies we investigated uterine ring contractions that involved both longitudinal and transverse muscle layers responses. We found that sildenafil has a concentration-dependent uterus relaxant effect on each investigated gestational day (5/15/18/20/22) and on non-pregnant uteri. No significant changes in EC_{50} values were found, and we confirmed previous findings that there is no significant difference in PDE5 activity during pregnancy in rat [19]. However, the maximum relaxing effect of sildenafil slightly decreased towards the end of the gestational period, although it remained still high on the last day of pregnancy (over 80 % relaxation). The drop in the relaxing effect from gestational day 18 might due to the increased uterine NO metabolism at the end of pregnancy [20].

In isolated organ bath studies we also investigated the uterus relaxant effect of co-administered sildenafil and terbutaline. As in our previous study [12], terbutaline elicited a significant uterine relaxing effect in 22-day pregnant rats. When terbutaline was added in a single and low concentration with cumulatively administered sildenafil, we observed a significant potentiating effect at lower concentrations of sildenafil, but the maximal inhibitory effect of sildenafil did not increase. In the opposite case, sildenafil treatment followed by increasing doses of terbutaline, the presence of sildenafil was not able to enhance the effect of terbutaline. A previous study already confirmed the biphasic effect of sildenafil in adipocytes and hepatocytes. According to these results, a higher (micro molar)

Table 1
EC_{50} and E_{max} values of curves of uterine relaxation induced by sildenafil on days 5/15/18/20/22 or on non-pregnant animals. *Ns: non-significant* **: $p < 0.01$; ***: $p < 0.001$ as compared with the values of non-pregnant uteri.

	EC_{50} (M± S.E.M.)	E_{max} (%± S.E.M.)
non-pregnant	$7.6 \times 10^{-6} \pm 3.9$	98.9 ± 0.9
day 5	$4.2 \times 10^{-6} \pm 2.5$	93.5 ± 3.7 ns
day 15	$5.2 \times 10^{-6} \pm 1.9$	95.3 ± 4.3 ns
day 18	$1.2 \times 10^{-6} \pm 3.4$	75.8 ± 11.2 ***
day 20	$4.5 \times 10^{-6} \pm 1.8$	85.4 ± 8.9 **
day 22	$4.1 \times 10^{-6} \pm 4.3$	82.3 ± 16.7 **

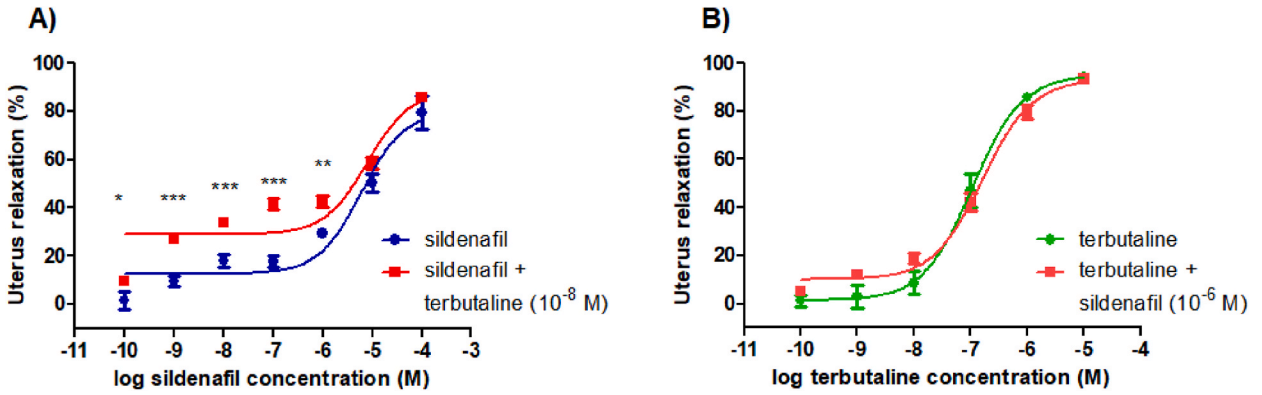

Fig. 4. A): Inhibitory effect of sildenafil (10^{-10} – 10^{-4} M) alone or in combination with terbutaline (10^{-8} M) on 22-day pregnant uterine contractions stimulated with KCl (25 mM). **: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$* as compared with the values of sildenafil alone. B): Inhibitory effect of terbutaline (10^{-10} – 10^{-5} M) alone or in combination with sildenafil (10^{-6} M) on 22-day pregnant uterine contractions stimulated with KCl (25 mM).

Table 2
EC_{50} and E_{max} values of curves of uterine relaxation induced by sildenafil alone and in combination with terbutaline, terbutaline alone and in combination with sildenafil on gestational day 22 (n = 8 rats/group*). Ns: non-significant* as compared with sildenafil or terbutaline alone.

	EC_{50} (M± S.E.M.)	E_{max} (%± S.E.M.)
sildenafil	$4.1 \times 10^{-6} \pm 4.3$	82.3 ± 16.7
sildenafil + terbutaline (10^{-8} M)	$8.8 \times 10^{-6\ ns} \pm 6.3$	$86.5 \pm 10.3^{\ ns}$
terbutaline	$1.3 \times 10^{-7} \pm 0.6$	95.9 ± 2.3
terbutaline + sildenafil (10^{-6} M)	$2.3 \times 10^{-7\ ns} \pm 2.0$	$94.5 \pm 4.2^{\ ns}$

concentration of sildenafil allosterically activates PDE2 and reduces the level of cAMP [21]. A similar mechanism in the uterus might explain the inefficacy of sildenafil to potentiate the effect of terbutaline.

Compared to non-pregnant uteri, on day 20 of pregnancy, we measured a stronger uterine relaxing effect at the lower concentrations of the concentration range we used. Therefore, the cAMP and cGMP measurements were taken on gestational days 20 (highest sildenafil efficacy in low concentration) and 22 (last day of pregnancy). We proved that the levels of cGMP are elevated with the concentration of sildenafil on days 20 and 22, however, sildenafil has a greater potential to increase cGMP levels on day 20. The presence of terbutaline significantly enhanced the levels of cGMP on the last day of pregnancy, especially in the lower concentration range. In the case of cAMP measurements, sildenafil was not able to elevate the levels and there was no concentration-dependent relation either on day 20 or on day 22. Moreover, the low concentration of terbutaline used in our experiments could not affect cAMP levels significantly on day 22. This phenomenon suggests that the potentiating effect observed in the in vitro and in vivo results at low doses is mainly due to the increase in the level of cGMP. Previous studies already proved that β_2-mimetics are coupled to the NO/cGMP signalling pathway in the vascular system indicating the, possible cGMP-enhancing role of terbutaline in the mechanism of action in sildenafil – terbutaline co-administration [22]. However, earlier studies with human found low efficacy of elevation in myometrial cGMP levels for relaxation [20], that may suggest potential limited applicability our rat results in the clinical practice.

In SMEMG measurements we found that the synergistic combination of sildenafil and terbutaline also works in vivo. This study also revealed a similar potentiating effect, as it was demonstrated in our in vitro experiments. The cumulative administration of sildenafil in the presence of low-dose terbutaline elicited an enhanced relaxing effect, especially in the lower dose range, which also points to the potential benefits of this combination in tocolysis, since by reducing the dosage of each tocolytic agent, the occurrence of possible maternal and foetal side effects can also be reduced. Co-administration with lower doses may have the advantage of reducing both the hypotonic effect of PDE5-Is and the tachycardic effect of β_2-mimetics, which is beneficial in terms of maternal cardiac side effects. It is known that terbutaline raises maternal glucose level during pregnancy. Therefore, the effect of PDE5-Is in improving β-cell function may also be beneficial [23].

The main limitation of this study is that the rat model is quite different from the human model, therefore further studies are required before the clinical trials. An other weakness of this study is that the experiments do not provide data on the maternal and foetal side effects of the combined drug regimen. Unfortunately, the side effects of terbutaline, such as foetal tachycardia or nausea, could not be investigated as the rat model is unsuitable for that purpose. Another limitation of this study is that the blood pressure and the heart rate modulating effects of the combination were not measured. In the highest concentration the PDE4 inhibitory effect of sildenafil cannot be excluded [24].

Despite these weaknesses, we successfully proved the potentiating effect of low-dose terbutaline on the uterine relaxing action of sildenafil both in vitro and in vivo. The dose of terbutaline applied during in vivo studies was almost 40-fold lower than that of its

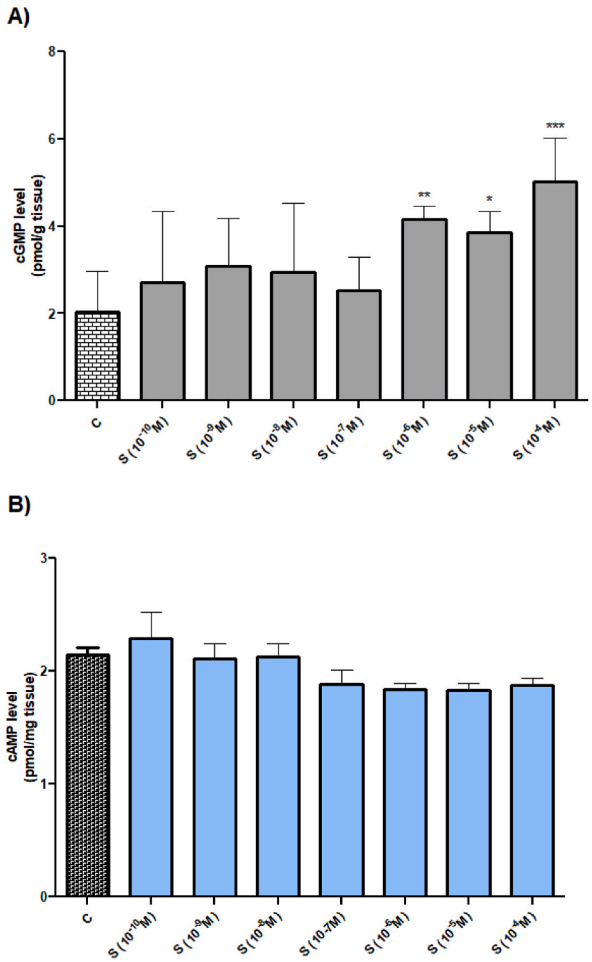

Fig. 5. The levels of cGMP (A) and cAMP (B) in the presence of different concentrations of sildenafil (S) on day 20 (n = 8 rats/group). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ as compared with the level of non-treated control uterus (C).

concentration eliciting the maximum effect. Both compounds have an anti-inflammatory action, which suggests an extra benefit of this combination in preterm birth with an inflammatory background [25,26]. High doses of β_2-mimetics induce receptor desensitization [27] and reduce efficacy, supporting the benefit of a combined therapy using a single low dose of terbutaline.

5. Conclusion

Based on our results, we conclude that the combination of PDE5-inhibitor sildenafil and β_2-agonist terbutaline may be of clinical importance in tocolytic therapy. We found that sildenafil alone has a significant uterine relaxing effect both in vitro and in vivo, however, its co-administration with low-dose terbutaline further enhanced its effect, especially in lower concentration ranges. We hypothesize that the combination may reduce the unfavourable side effects of both PDE5-inhibitor and β_2-agonist.We have demonstrated that the potentiating effect of terbutaline is due to its ability to increase cGMP levels. Nevertheless, the applicability and pharmacokinetic properties of the combination must be demonstrated in well-designed clinical studies.

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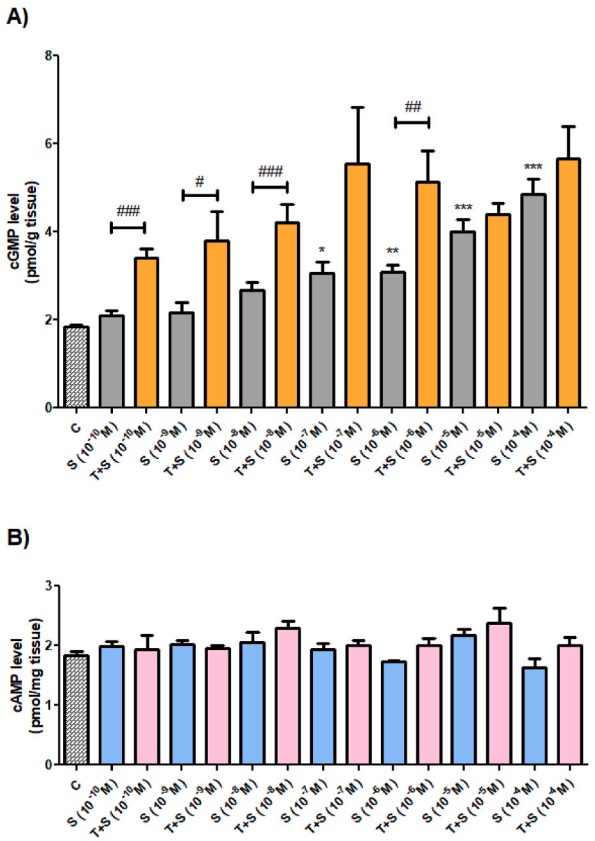

Fig. 6. The levels of cGMP (A) and cAMP (B) in the presence of different concentrations of sildenafil alone (S) or in combination with terbutaline (T + S) on day 22 (n = 8 rats/group). **: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$* as compared with the level of non-treated control uterus (C), *#: $p < 0.05$; ##: $p < 0.01$; ###: $p < 0.001$* as compared with the levels of sildenafil alone.

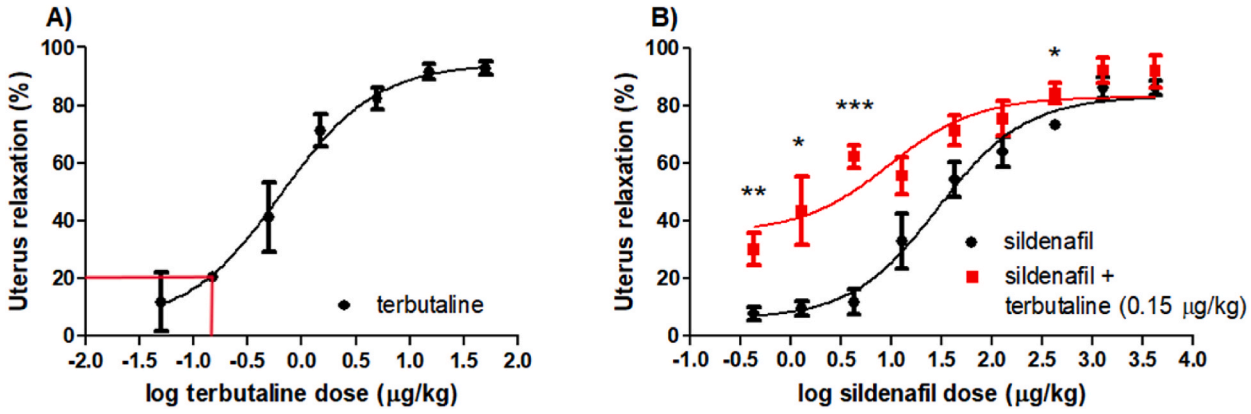

Fig. 7. Inhibitory effect of terbutaline (0.05–50 μg/kg) alone (A), sildenafil (0.42 μg/kg – 4.2 mg/kg) alone (B) or in combination with terbutaline (0.15 μg/kg, log (0.15) is indicated by the red line) on 22-day pregnant uterine contractions in vivo. **: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$* as compared with sildenafil values.

CRediT authorship contribution statement

Tamara Barna: Conceptualization, Formal analysis, Investigation, Writing – original draft. **Kalman F. Szucs:** Formal analysis, Investigation. **Mohsen Mirdamadi:** Investigation. **Robert Gaspar:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Table 3
ED_{50} and E_{max} values of curves of uterine relaxation induced by terbutaline, sildenafil alone or in the presence of terbutaline (0.15 μg/kg) in vivo (n = 8 rats/group). *Ns: non-significant, **: $p < 0.01$* as compared with sildenafil values.

	ED_{50} (±S.E.M.)	E_{max} (%± S.E.M.)
terbutaline	0.65 ± 0.19 μg/kg	94.3 ± 1.8
sildenafil	28.7 ± 2.11 μg/kg	83.2 ± 2.3
sildenafil + terbutaline (0.15 μg/kg)	9.5 ± 1.3 μg/kg **	83.6 ± 1.7 ^{ns}

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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