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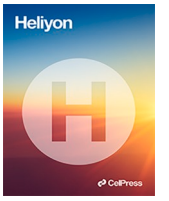

The combined uterorelaxant effect of sildenafil and terbutalin in the rat: The potential benefit of co-administration of low doses

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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

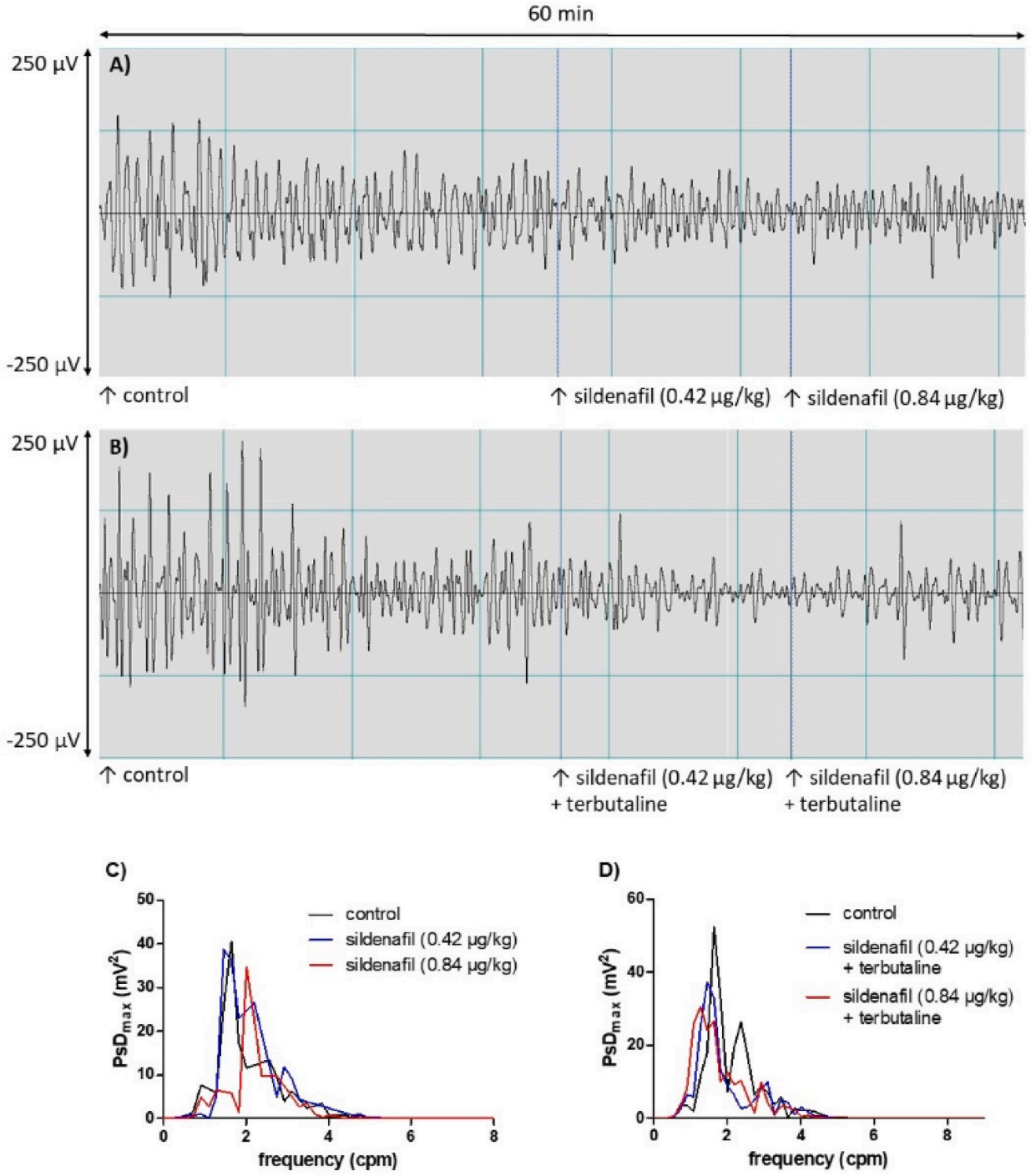

Fig. 1. Primary myoelectric signals of the 22-day pregnant uterus of rat detected with a bipolar disk electrode under anesthesia. (A, B): raw trace of control contractions and contractions in the presence of two doses of sildenafil alone (A) or co-administration with terbutaline (B). The FFT was carried 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

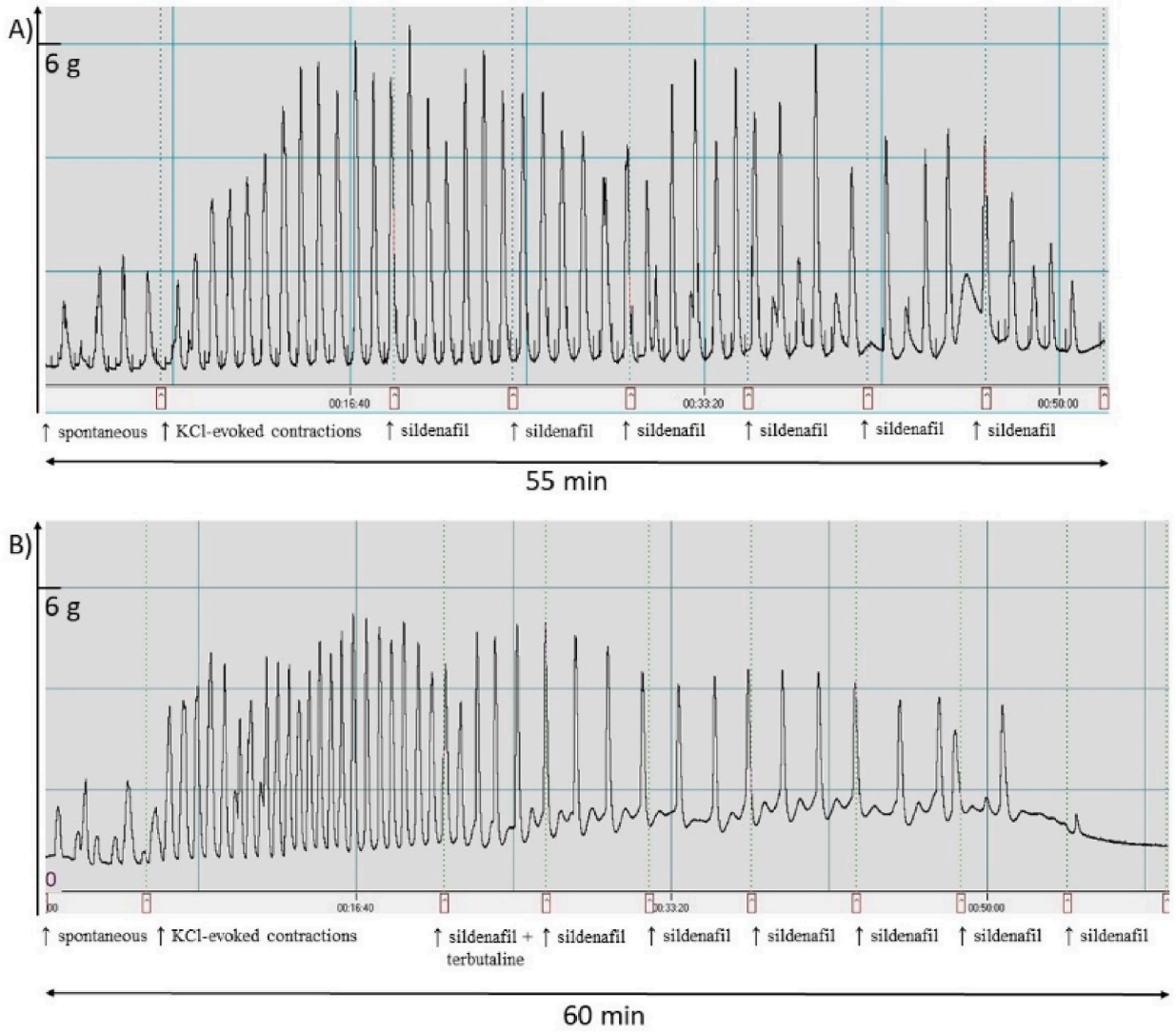

Fig. 2. Representative raw traces of the 22-day pregnant uterine contractions in vitro: spontaneous contractions, KCl-evoked contractions and contractions in the presence of sildenafil alone (A) or in combination with terbutaline (B).

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

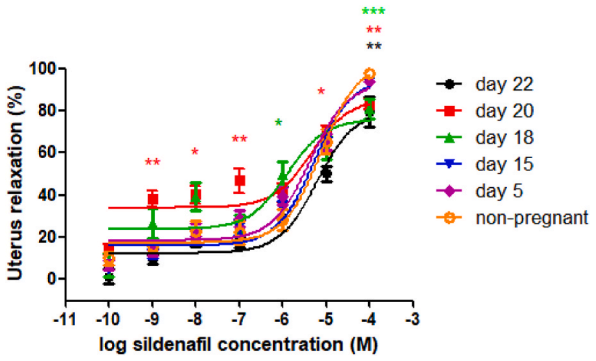

Fig. 3. Inhibitory effect of sildenafil (10^{-10} – 10^{-4} M) on pregnant (days 22/20/18/15/5) and non-pregnant uterine contractions stimulated with KCl (25 mM) (n = 8 rats/day). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ as compared with the values of non-pregnant uteri.

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 **

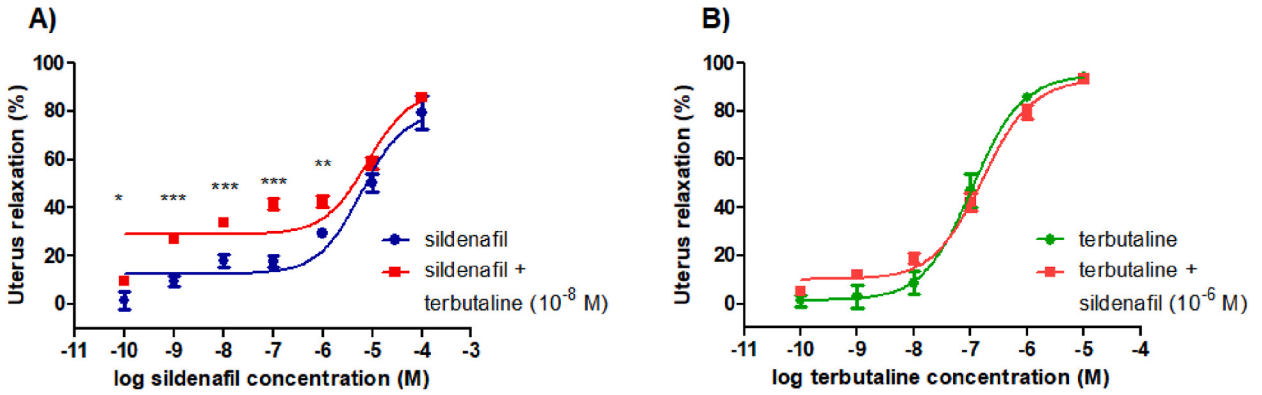

Fig. 4. A): Inhibitory effect of sildenafil (10^{-10} – 10^{-4} M) alone or in combination with terbutaline (10^{-8} M) on 22-day pregnant uterine contractions stimulated with KCl (25 mM). **: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$* as compared with the values of sildenafil alone. B): Inhibitory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

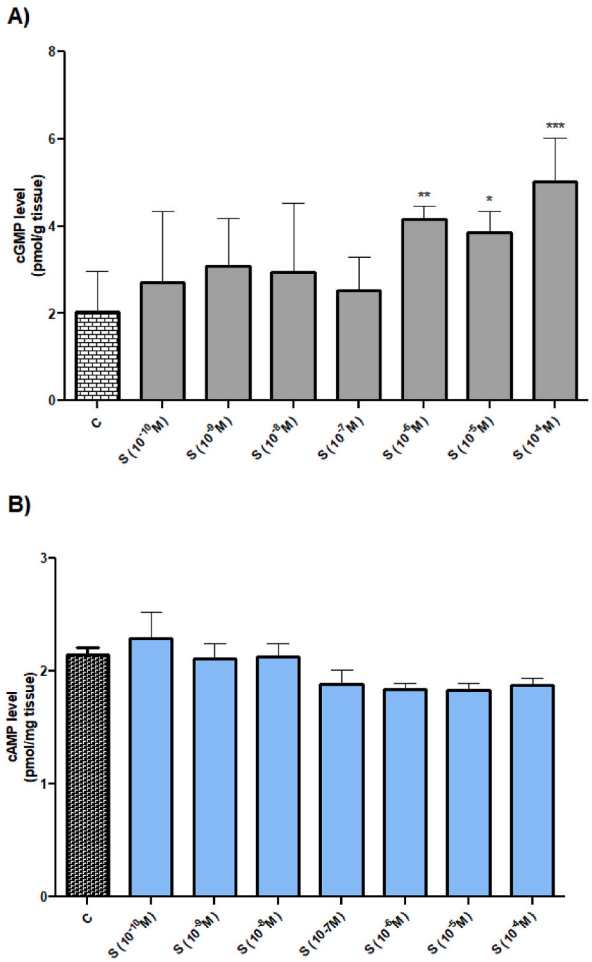

Fig. 5. The levels of cGMP (A) and cAMP (B) in the presence of different concentrations of sildenafil (S) on day 20 (n = 8 rats/group). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ as compared with the level of non-treated control uterus (C).

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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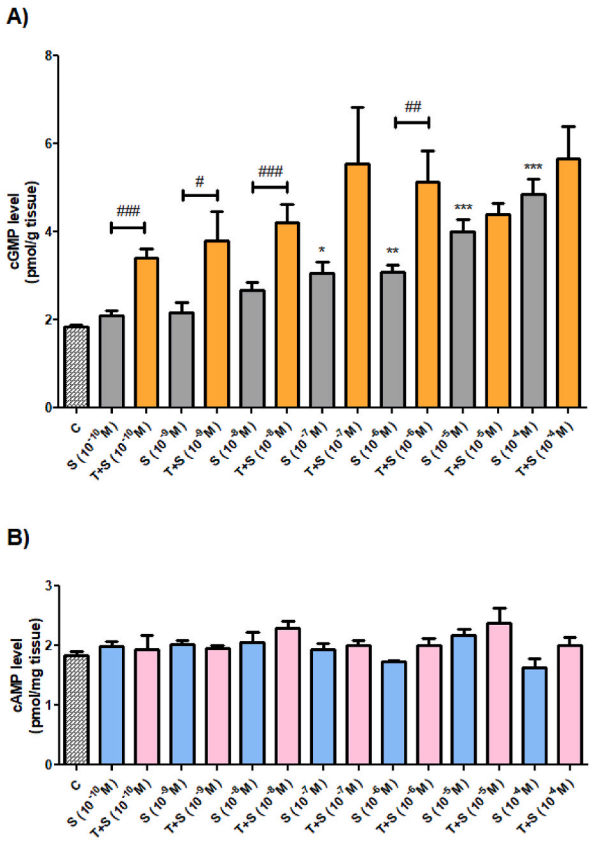

Fig. 6. The levels of cGMP (A) and cAMP (B) in the presence of different concentrations of sildenafil alone (S) or in combination with terbutaline (T + S) on day 22 (n = 8 rats/group). **: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$* as compared with the level of non-treated control uterus (C), *#: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

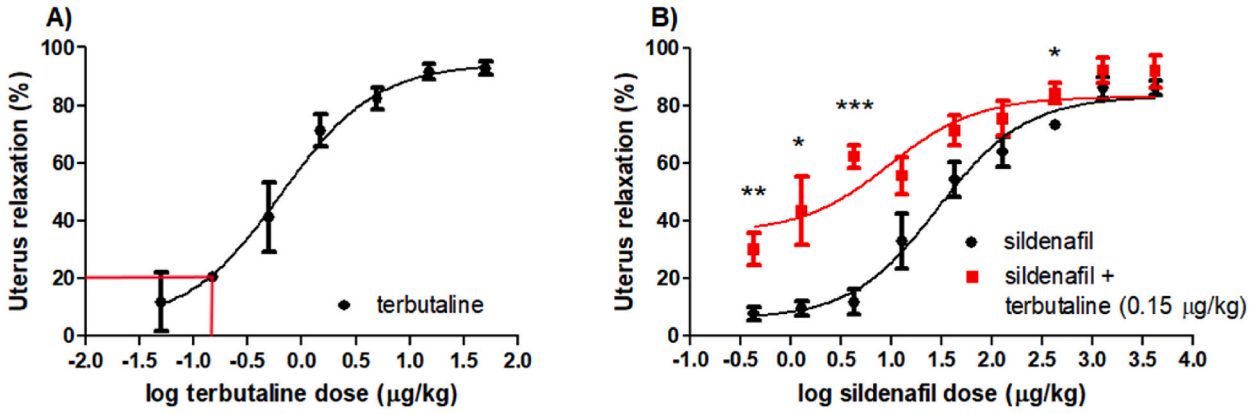

Fig. 7. Inhibitory effect of terbutaline (0.05–50 μg/kg) alone (A), sildenafil (0.42 μg/kg – 4.2 mg/kg) alone (B) or in combination with terbutaline (0.15 μg/kg, log (0.15) is indicated by the red line) on 22-day pregnant uterine contractions in vivo. **: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$*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