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**The effects of experimental diabetes mellitus on the
adrenergic functions of the rat uterus**

Summary of Ph.D. thesis

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INTRODUCTION

Pregnancy is a kind of diabetogenic condition that is characterized by insulin resistance and a compensatory hyperinsulinemia caused by β -cell hyperactivity. These changes in carbohydrate and lipid metabolism serve to ensure the continuous supply of nutrients for the growing fetus despite intermittent maternal food intake. Gestational diabetes mellitus (GDM) occurs if the maternal β -cell functions are not able to provide enough insulin to compensate the elevated insulin resistance. GDM is a carbohydrate intolerance, with onset or first recognition during pregnancy, that also disappears after delivery. It is a heterogeneous disorder in which age, obesity and genetic background also contribute to the severity of the disease, complicating about 7% of pregnancies.

GDM is associated with both maternal and fetal risk factors. The most important maternal risk factors are complications during delivery, such as pre-eclampsia, postpartum hemorrhage or reparative distress syndrome. There is also a long-term risk of developing type-II diabetes. Macrosomia a birth traumas are typical fetal complications. Children of GDM mothers also suffer a long-time risk of obesity and developing abnormal glucose tolerance resulting in type-II diabetes later in life.

The first aim of the treatment is maintaining normoglycemia and avoiding ketosis. If medical nutrition therapy fails to maintain the desired blood glucose levels, insulin therapy follows. Insulin analogues aspart and lispro proved to have a great safety profile. The use of oral hypoglycemic agents is currently not recommended, however some studies already shown promising results in oral anti-diabetic therapy during GDM.

The effects of GDM on uterine contractility have not yet been completely investigated. Contractility is defined by two major components, the hormonal system and the neural enervation and both of these components change significantly during pregnancy. The elevated progesterone concentration keeps the uterine blood flow on a constant level, while adrenergic denervation serves to prevent spontaneous contractions. The balance of this sensitive system can be largely affected by GDM. The adrenergic system is also an important target of tocolytic therapy, because of that adrenergic changes occurring during GDM can lead to unexpected side effects which seriously need to be considered during therapy.

AIMS

A bevezetésben összefoglaltak alapján a következő kísérleti célokat határoztuk meg:

- Creating an experimental model for GDM studies.
- Functional analysis of the presynaptic side of noradrenergic neurotransmission in the uterus during GDM by conducting superfusion experiments. Observing the transmitter uptake and also the transmitter release capacity of the uterus elicited by electric stimuli.
- Functional analysis of the postsynaptic side of neurotransmission in the uterus during GDM using selective α - and β -adrenergic agonists and also electric stimuli under the conditions of isolated organ experiments.
- Analysis of contractions elicited by oxytocin in GDM under the conditions of isolated organ experiments.
- Explaining the results of the isolated organ experiments conducted on the postsynaptic side of neurotransmission and also using oxytocin, with the help of RT-PCR. Analyzing the oxytocin and α - and β -adrenergic receptors' mRNA expressions and deciding if the changes in mRNA levels correlate to the changes registered in contractility earlier.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats (200-250g) were used in the experiments. Copulation was determined by the presence of a copulation plug or sperm in a native vaginal smear. The day of conception was considered to be the first day of pregnancy.

Streptozotocin is a diabetogenic agent that accumulates in the β -cells of the pancreas and causes DNA fragmentation by alkylation and generating reactive oxygen species. Diabetes was induced with 60 mg/kg iv injection of streptozotocin. The non-pregnant control animals were sacrificed on the 12th day after diabetes induction. Blood glucose levels were monitored using an enzymatic colorimetric kit. Pregnant animals were sacrificed on the 7th, 14th, and 21st days of pregnancy. Diabetes was induced on the 5th day of pregnancy except in the case of the 7day group. They received the streptozotocin treatment on the 2nd day of pregnancy.

[³H]noradrenalin release

Animals were sacrificed in a carbon-dioxide chamber and uterus samples were taken (20-30 mg). In case of pregnant animals it was made a distinction between samples of implantation and interimplantation sites and they both were processed separately. Samples were cleared of connective tissue and endometrium, minced and incubated in 10^{-7} M [³H]noradrenalin at 37 °C for 60 min. After that, they were washed three times with de Jongh buffer and placed into superfusion chambers; a continuous flow rate of 1 ml/min was maintained for 60 min with de Jongh buffer containing the monoamine oxidase inhibitor pargyline, the noradrenalin-reuptake inhibitor desipramine and the extra neuronal reuptake inhibitor deoxycorticosterone. After a 60-min wash-out period 3-min fractions were collected, a total of 22. At the end of the experiment, the tissue samples were solubilized in 1 ml Solvable for 3 h at 60 °C. The [³H] content in each 3-min fraction and in the tissue solution was determined with a liquid scintillation spectrometer.

Isolated organ experiments

Animals were sacrificed in a carbon-dioxide chamber and uterus samples were immediately taken. The uterine rings were mounted vertically in a chamber containing oxygenated tissue bath at 37°C. The tissue samples were equilibrated with an initial tension of 1,5g for 90 minutes. Cumulative dose-response curves were constructed for the α -adrenergic agonist noradrenalin and the β -adrenergic agonist terbutaline and non-cumulative dose-response curves for oxytocin.

The α -adrenergic functions were analyzed by constructing cumulative dose-response curves for noradrenalin in the concentration range 1×10^{-10} – 1×10^{-5} M (a total of 11 doses) in the presence of propranolol (10^{-6} M) to block the effects of noradrenalin on the β -adrenergic receptors. At the end of each experiment, KCl (70 mM) was added to the chamber and the evoked contractions were considered maximal and recorded for 5 min. The contractions induced by noradrenalin were expressed as a percentage of the KCl evoked contractions.

The β -adrenergic functions were analyzed by constructing cumulative dose-response curves for terbutaline. The experimental design was similar to the previous one, but the tissue chamber did not contain propranolol, instead KCl (50 mM) was added at the start of the experiment in order to elicit an initial tension of the uterine rings which was regarded as 100% of the motor activity. The terbutaline concentration range was 10^{-8} – 10^{-5} M (altogether 7 concentrations). The contraction elicited by KCl was considered 100% of the possible motoric activity.

Non-cumulative dose response curves were constructed for oxytocin in the concentration range of 10^{-9} – 10^{-6} M. The maximal effect and the EC_{50} values of the curves were calculated using the software Graphpad Prism 4.

During the experiments using electric field stimulation we administered the same stimulation 3 times with the following parameters: voltage: 40 V, duration of stimulation: 3 minutes, pulse width: 0.6 ms, period time: 50 ms. At the end of the experiment, KCl (70 mM) was added to the chamber to induce maximal contraction, that was recorded for 5 min. For every 3 minutes of stimulation, 3 minutes of poise was also registered. The electrical excitabilities of the tissues were expressed by the ratio of the area under the curve of these two periods.

RT-PCR

During the RT-PCR studies only those days of pregnancy were investigated, when the isolated tissue studies showed the most significant differences between the uterine functions of diabetic and control animals. Accordingly for α_1 and β_2 adrenergic receptor RT-PCR studies we used the tissue samples of non-pregnant animals. For oxytocin receptor RT-PCR studies samples obtained from 15-day pregnant animals were used.

Total RNA was extracted from all collected tissues with acid guanidinium thiocyanate-phenol-cholorophorm by the procedure of Chomczynski and Sacchi. The RNA was denaturated at 70 °C for 5 minutes in a reaction mixture containing 20 μ M oligo(dT), 20 U RNase inhibitor, 200 μ M dNTP in 50 mM Tris-HCl, pH 8.3, 75 mM KCl and 5mM $MgCl_2$ in a final reaction volume of 20 μ l. After the mixture had been cooled to 4°C, 20 U M-MLV reverse transcriptase and ribonuclease inhibitor were added and the mixture was incubated at 37 °C for 60 min. The RT-PCR was carried out with 5 μ l cDNA, 25 μ l ReadyMix REDTaq PCR reaction mix, 2 μ l 50 pM sense and antisense primer of the α - and β -ARs and OTR and 16 μ l DNase and RNase-free distilled water. A rat β -actin probe (GeneID: 81822) was used as internal control in all samples.

The RT-PCR products were separated on 2% agarose gels, stained with ethidium bromide and photographed under a UV transilluminator. The amount of RT-PCR products in each sample was measured by fluorometric assay. Semi-quantitative analysis was performed by densitometric scanning of the gel.

RESULTS

Blood and urine glucose levels

Significant postprandial hyperglycemia, defined as plasma glucose > 12 mM, was detected in non-pregnant rats on post-STZ day 4 and the condition became stable from day 7 after STZ treatment.

Results of tissue radioactivity determination

The tissue activity (expressed in dpm/mg/tissue) was used to describe the uptake capacity of the sample for [³H]noradrenaline. This parameter of the uterus of virgo animals was significantly decreased by experimental DM. During the course of pregnancy, a gradual decrease in the uptake capacity and site-dependent differences were found between the implantation and interimplantation areas (figure 1).

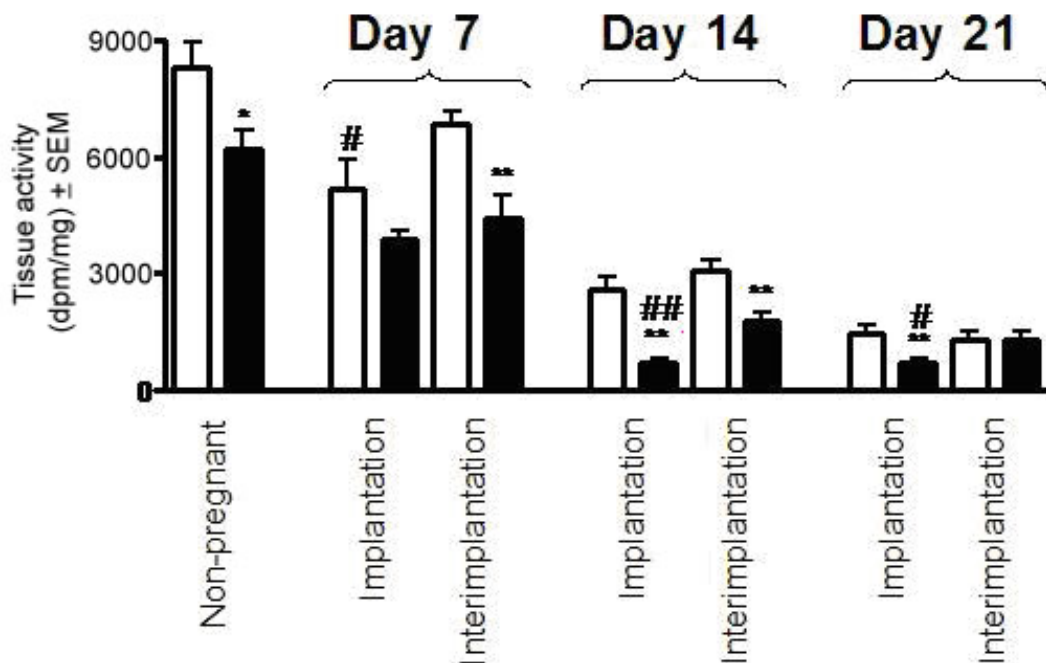


Figure 1. [³H]noradrenaline uptake capacity of myometrial samples as a function of pregnancy. Open and filled columns denote control and STZ-treated values, respectively. * p<0.05 and ** p<0.01 as compared with the non-diabetic value respectively. # p<0.05 and ## p<0.01 as compared with the different sites of the same treatment, respectively.

Results of electrically stimulated [³H]noradrenaline release

Two electric field stimuli were applied (in fractions 5 and 15) to obtain information on the release capacity of the tested tissues. The second stimulus resulted in a smaller transmitter peak than the first one. In the non-pregnant myometrial tissues, a 12-days history of DM caused a marked and statistically significant decrease in the transmitter release evoked by EFS (figure 2.). On days 7, 14 and 21 of pregnancy, the [³H]noradrenaline release evoked by EFS in the DM animals exhibited a tendency to be lower than that in the control rats. However, these differences proved to be significant only in mid-pregnancy.

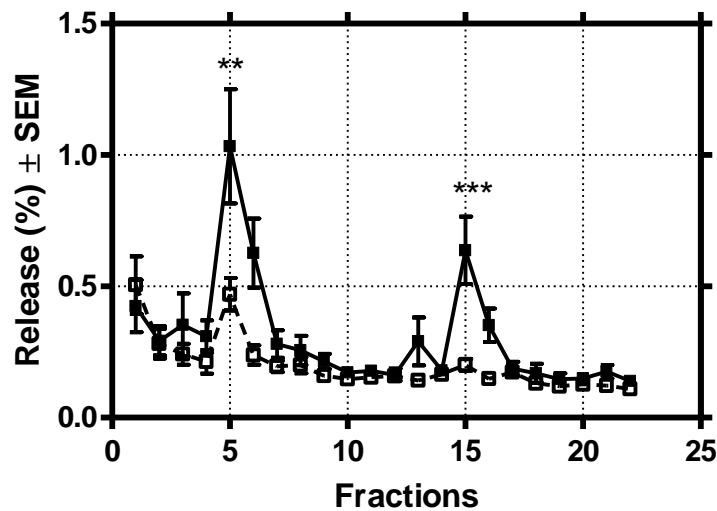


Figure 2. EFS-evoked fractional [³H]noradrenaline release from myometrial samples at estrus from control (■) and diabetic (□) rats. **p<0.01 and ***p<0.001 as compared with the control values respectively.

Contractions and relaxations induced by agonists

Cumulative dose-response curves were generated using sympathomimetics acting on the α - and β -ARs in order to investigate agonist-induced changes in motor activity. In non-pregnant, diabetic animals the contractility increased for noradrenalin, as opposed to the non-diabetic state, which showed to be nearly unresponsive for NA in the presence of propranolol. Similarly to the α -adrenergic stimulation, only in the non-pregnant state was a significantly lower relaxing effect detected, using β -adrenoceptor agonists (figure 3.). However pregnancy did not evoke any significant differences between the contractility of diabetic and non diabetic uterus samples.

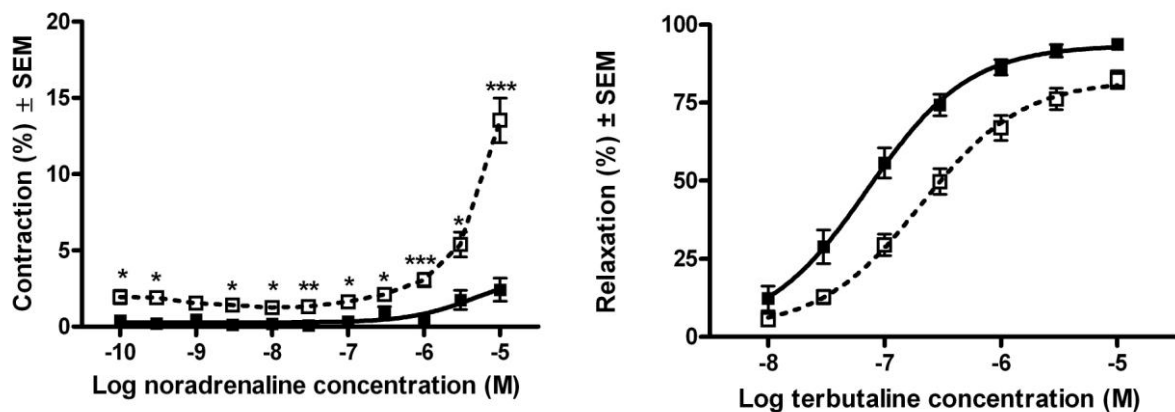


Figure 3. Effects of experimental DM on the noradrenaline- and terbutaline-stimulated myometrial responses of control and diabetic non pregnant rats. (■) and (□) indicate values from control and diabetic animals respectively. *, ** and *** denote significance levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$ as compared with the non-DM value.

Effects of oxytocin

The effects of oxytocin on the pregnant uterus in diabetes were only analyzed in the 3rd trimester of pregnancy, since the rat uterus shows no reactivity to oxytocin in the first two trimesters of pregnancy. Oxytocin exerted a more pronounced uterotonic effect at the start of the 3rd trimester, however at term, no GDM related differences were observed (figure 4).

Effects of electric field stimuli

DM decreased the contractions evoked by electric field stimulation in uterine samples in both the non-pregnant and pregnant state. At the end of pregnancy the decrease became less pronounced, however we must stress, that the uterus was already a denervated organ by that time, so there was a lower limit in the change of contractility .

RT-PCR

The expressions of the involved receptors, such as all types of α_1 - and β_2 -adrenoceptors, and oxytocin receptor were determined at the mRNA level in non-pregnant and late pregnant (day 15) uteri respectively. It was found that the only α_1 -adrenoceptor subtype that showed a significant elevation in mRNA expression as a consequence of diabetes was α_{1B} . The amount of the RT-PCR product for α_{1A} -adrenoceptor did not differ from the controls in diabetes and interestingly, the α_{1D} -adrenoceptor mRNA levels even showed a

decrease in diabetes. The amount of oxytocin RT-PCR products was significantly higher in the diabetic samples collected on day 15 of pregnancy (figure 5).

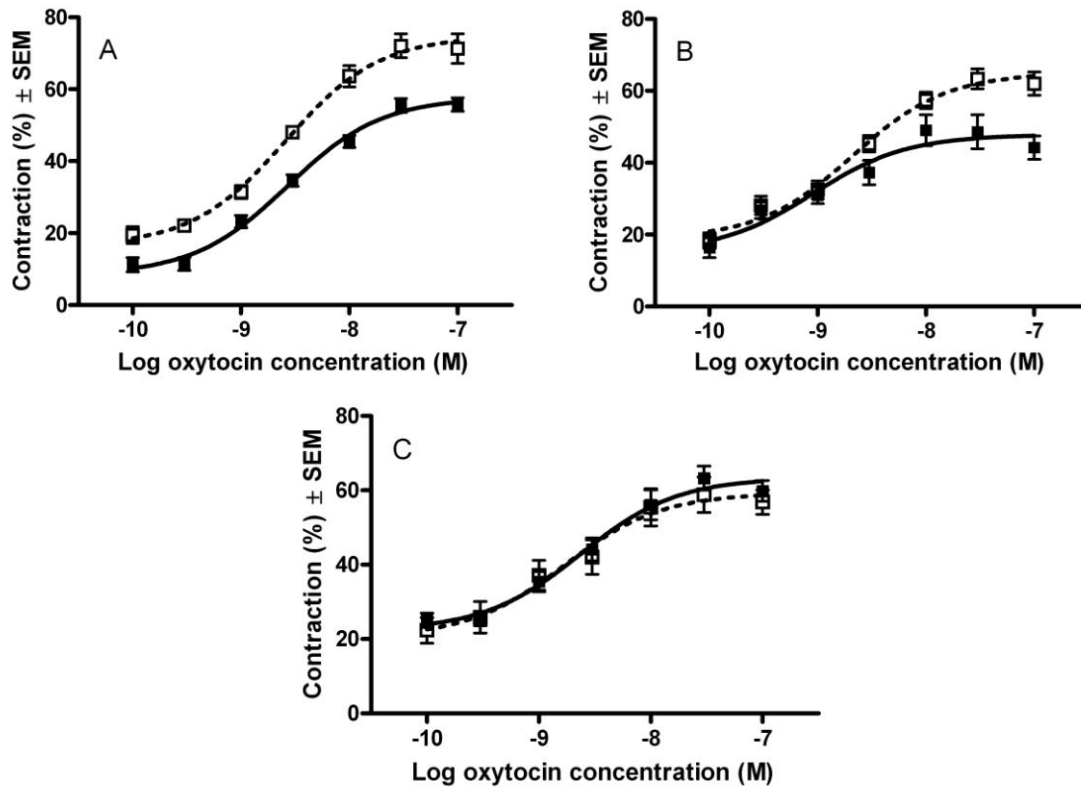


Figure 4. Effects of oxytocin on uterine contractility on day 15 (panel A), day 21 (panel B), and day 22 (panel C) of pregnancy of control (■) and GDM (□) rats during pregnancy. Significant differences were found in the maximal effect of oxytocin on uterine contractility of diabetic and non-diabetic rats in the 3rd trimester.

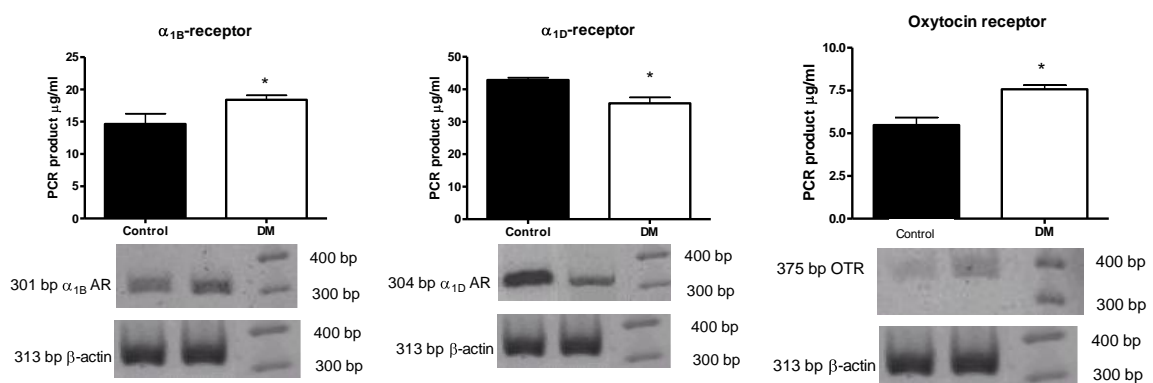


Figure 5. Effects of diabetes on the myometrial α_{1B} -, α_{1D} -adrenergic receptors and on the oxytocin-receptor mRNA levels in rat. * denotes $p < 0,05$ significance compared to control. Panels below graphs are representative gel pictures.

DISCUSSION

The three main factors determining the contractility of the pregnant myometrium are the oxytocin-, the prostaglandin system and the sympathetic nervous system. All of them are important targets of tocolytic agents used in therapy. The changes in the oxytocin and prostaglandin systems during diabetes are well investigated, so I focused on the sympathetic nervous system during my work. During pregnancy the uterus undergoes a profound denervation that affects the neurons innervating both the myometrium and the vasculature. The gestational denervation helps creating a functionally isolated fetoplacental unit that protects the fetus. By term, the uterus is considered a fully denervated organ, that slowly re-innervates after delivery.

The present set of experiments demonstrated, that diabetes remarkably affects the presynaptic side of neurotransmission, i.e. the transmitter uptake and release process. Gestational diabetes creates a more pronounced denervation and by that, the level of denervation reaches the fully denervated state earlier than during the normal process. The methodical significance of this study is the successful use of superfusion technique in the functional analysis of the myometrium. The regression of neuronal response during electric field experiments also points to the fact that neuronal impairment might be present. The functional changes occurring on the postsynaptic side are easily observed through the effects of adrenergic agonists. In non-pregnant animals diabetes increased the contractions evoked by noradrenalin and decreased the relaxing effect of terbutalin, so the resultant changes point towards increased contractility. During pregnancy such remarkable changes in the contractility for adrenergic agonists were not registered. Diabetes increased the sensitivity of the uterus for oxytocin, which was also proved by registering elevated levels of receptor mRNA.

Summarizing these results we can state that diabetes induced by streptozotocin increases the speed of the changes in the myometrial functions that normally occur during pregnancy considering both the adrenergic- and the oxytocin system.

ANNEX

Publications related to the Ph.D. thesis:

1. Falkay G, **Spiegl G**, Csonka D, Zupkó I: Effects of streptozotocin-induced diabetes on the uterine adrenergic nerve function in pregnant rats A superfusion study. *Neurochem Int* **51**: 306-10 (2007) IF₂₀₀₇: 2.975
2. **Spiegl G**, Minorics R, Csík G, Zupkó I, Falkay G: A kísérletes diabetes hatása a patkány uterusának terhesség által indukált adrenerg denervációjára és farmakológiai reaktivitására. *Magy Nőorv L* **71**: 253-60 (2008)
3. **Spiegl G**, Zupkó I, Minorics R, Csík G, Csonka D, Falkay G: Effects of experimentally induced diabetes mellitus on pharmacologically and electrically elicited myometrial contractility. *Clin Exp Pharmacol Physiol* **36**: 884-91 (2009) IF₂₀₀₈: 2.196

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1. **Spiegl G**, Csonka D, Zupkó I, Falkay G: A gesztációs diabetes myometriális hatásainak vizsgálata patkányban. Gyógyszerkutató Szimpózium 2005, Pécs, 2005. XI. 4-5.
2. **Spiegl G**, Zupkó I, Falkay G: Experimental gestational diabetes decreases noradrenaline release in the myometrium of the rat. 15th World Congress of Pharmacology, Beijing, China, 2006. VII. 2-7. [*Acta Pharmacol Sin* **27 Suppl 1**: P280074 (2006)]
3. Zupkó I, **Spiegl G**, Falkay G: Effects of streptozotocin-induced diabetes on the contractility of the uterus in the rat. 8th Congress of the European Association for Clinical Pharmacology and Therapeutics, Amsterdam, The Netherlands, 2007. VIII. 29.-IX. 1. [*Basic Clin Pharmacol Toxicol* **101 Suppl 1**: P408 (2007)]
4. Gáspár R, Zupkó I, Gál A, **Spiegl G**, Csík G, Falkay G: Effects of streptozotocin-induced diabetes on the uterine denervation and contractility in pregnant rats. 5th International Symposium on Diabetes and Pregnancy, Sorrento, Italy, 2009. III. 26-28.
5. Minorics R, Zupkó I, **Spiegl G**, Falkay G: Az uterus adrenerg funkciói kísérletes diabetes modellben
6. Congressus Pharmaceuticus Hungaricus XIV., Budapest, 2009. XI. 13-15. [*Gyógyszerészet* **53 Suppl 1**: S51 (2009)]
7. Zupkó I, Minorics R, **Spiegl G**, Falkay G: Effects of streptozotocin-induced experimental diabetes on pharmacological action of drugs affecting myometrial contractility of the rat. 5th International Symposium on Diabetes and Pregnancy, Sorrento, Italy, 2009. III. 26-28.