THE EFFECTS OF EXPERIMENTAL DIABETES MELLITUS ON THE ADRENERGIC FUNCTIONS OF THE RAT UTERUS

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Ph.D. dissertation

2010

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Publications directly related to the subject of the dissertation:

I. 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) IF_{2007}: 2.975

II. 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)

III. 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) IF_{2008}: 2.196

TABLE OF CONTENTS

Abbreviations

ADA	American Diabetes Association
AGE	Advanced glycation endproducts
AKAP	A-kinase anchoring protein
ANOVA	Analysis of variance
AR	Adrenergic receptor
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
DAG	Diacyl-glycerol
DM	Diabetes mellitus
DNA	Dezoxy-ribonucleic-acid
EFS	Electrical field stimulation
eNOS	Endothelial nitric oxide synthase
FFA	Free fatty acid
GDM	Gestational diabetes mellitus
GLUT2	Glucose transporter 2
HDL	High density lipoprotein
IP3	Inositol 1,4,5-triphosphate
LDL	Low density lipoprotein
MNT	Medical nutrition therapy
mRNA	Messenger ribonucleic-acid
NA	Noradrenaline
NADPH	Nicotinamide adenine dinucleotide phosphate
NDDG	National diabetes data group
NO	Nitric oxide
OGTT	Oral glucose tolerance test
ONOO	Peroxynitrate
OT	Oxytocin
OTR	Oxytocin receptor
PAI-1	Plasminogen activator inhibitor-1
PCOS	Polycystic ovary syndrome

PG	Prostaglandin
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGI_2	Prostacyclin
PK-A	Protein kinase-A
PK-C	Protein kinase-C
RAGE	Advanced glycation endproducts receptors
RNA	Ribonucleic-acid
ROS	Reactive oxygen species
RT-PCR	Real time polymerase chain reaction
SPTD	Spontaneous preterm delivery
SR	Sarcoplasmic reticulum
STZ	Streptozotocin
VLDL	Very low density lipoprotein
WHO	World Health Organization

Introduction

Pregnancy and diabetes

Before the advent of insulin in 1922, pregnancies complicated with diabetes mellitus (DM) were associated with a >90% infant mortality and a 30% maternal mortality rate. As late as 1980, physicians were still counseling diabetic women to avoid pregnancy. Later treatment strategies started to stress better control of plasma glucose levels, and infant mortality rates started to improve.

Pregnancy is a diabetogenic condition characterized by insulin resistance with a compensatory increase in β-cell response and hyperinsulinemia. The changes in carbohydrate and lipid metabolism during pregnancy serve to ensure the continuous and steady supply of nutrients for the growing fetus despite intermittent maternal food intake.

The physiological role of maternal insulin resistance is to provide better nutrition for the fetus, however in some cases the glucose intolerance can access levels that approximate the insulin resistance seen in type-2 diabetes.In women with a suboptimal β-cell function, the increase in insulin secretion may not be sufficient to compensate the increased insulin resistance, resulting in gestational diabetes mellitus (GDM) [1].

GDM is defined as carbohydrate intolerance of varying degrees of severity with onset or first recognition during pregnancy and disappearance after pregnancy. It is a controversial clinical entity believed to be the unmasking of a compensated metabolic abnormality characterized by relative insulin deficiency and increased insulin resistance. DM during pregnancy can occur in two forms. With the pregnancy of a previously diabetic patient, or DM caused by the pregnancy itself. GDM is one of the most frequent complications in pregnancy. It is a heterogeneous disorder, in which age, obesity and genetic background also contribute to the severity of the disease, complicating ~7% of all pregnancies.

GDM is associated with an increased maternal risk for other pregnancy-related complications, such as pre-eclampsia, postpartum hemorrhage, and indicates an increased risk for developing type 2 diabetes later after pregnancy [2]. It also puts the infant at risk, since gestational diabetes is associated with an increased risk for macrosomia, jaundice and birth trauma. Later

in life, children of gestational diabetic mothers are at a more increased risk of developing obesity, abnormal glucose tolerance, and type-2 diabetes

Carbohydrate metabolism during physiological pregnancy

Functional control of the smooth muscle in the uterus is of vital importance during pregnancy and parturition. Changes in the contractile uterine functions during pregnancy serve to prevent preterm labor, and hypo- or hypertonic term labors; however the original contractile activity of the non-pregnant uterus plays an important role in the human reproduction process. Significant alterations in uterine contractility occur during the menstrual cycle, and perhaps physiological function can be attributed to these changes. During the follicular phase, stimulation of uterine contractions by estrogens fosters sperm transport toward the fertilization site. After ovulation, contractility decreases in response to progesterone, a phenomenon that is probably involved in the embryo implantation porcess.

During the start of a physiological pregnancy, glucose tolerance is normal or slightly improved, peripheral sensitivity to insulin and hepatic basal glucose production is normal [3]. An increased sensitivity to the blood glucose lowering effect of exogenously administered insulin develops in the first trimester, and insulin responses to oral glucose are also getting elevated. This metabolic milieu favors lipogenesis and fat storage. Insulin resistance usually begins in the second trimester and progresses throughout the remainder of the pregnancy. Insulin action in late normal pregnancy is 50-70%, lower than that of normal non-pregnant women [4]. A progressive increase in basal and postprandial insulin concentrations is seen with advancing pregnancy, but the developing insulin resistance serves to shunt ingested nutrients to the fetus after maternal food intake.

Mechanisms of this insulin resistance occurring in late pregnancy are still not fully understood. Insulin resistance in GDM is not likely to be caused by a defective insulin receptor tyrosine kinase, but decreased insulin receptor binding might have some pathogenic importance to this condition. Thus most cellular insulin resistance could be attributed to effects that lie distal to receptor binding and tyrosine kinase activity in the insulin action pathway [5].

In the pathogenesis of GDM, the β-cells of the pancreas also play a crucial role. During normal pregnancy, the marked reduction of insulin sensitivity could be compensated by a

reciprocal increase in β-cell production. In contrast the β-cell adaptation to insulin resistance is impaired in women with GDM [4].

Placental secretion of hormones, such as progesterone, cortisol, placental lactogen, prolactin, and growth hormone, is also a major contributor to the insulin resistant state occurring during pregnancy. The insulin resistance likely plays a role in ensuring adequate supply of glucose to the fetus by changing the maternal energy metabolism from carbohydrates to lipids.

Pronounced changes in lipid metabolism occur during physiological pregnancy. In early and mid pregnancy accumulation of maternal fat stores is promoted, but in late pregnancy fat mobilization becomes enhanced. This shift from anabolic to catabolic state promotes the use of lipids as a maternal energy source, while preserving glucose and amino acids for the fetus [3]. After an initial decrease, from the 8^{th} week of pregnancy, there is a steady increase in the concentration of fatty acids, cholesterol, phospholipids, and lipoproteins. Higher concentration of estrogen and also insulin resistance leads to hypertrigliceridemia.

Changes in total cholesterol concentration reflect changes in the various lipoprotein fractions. High density lipoprotein (HDL) levels increase by 12 weeks of gestation in response to estrogen and remain elevated throughout the pregnancy. Total and low density lipoprotein (LDL) concentrations decrease initially, but then increase in the second and third trimesters. Very low density lipoprotein (VLDL) and triglycerides decrease in the first 8 weeks of gestation and then continuously increase until term.

GDM induces a state of dyslipidemia, meaning that during pregnancy women with GDM do have higher serum tryacilglycerol concentrations, but lower LDL cholesterol concentrations [3].

Decreased carbohydrate tolerance during GDM

Like other forms of hyperglycemia, GDM is also characterized by pancreatic β-cell function that is insufficient to meet the body's insulin needs. Available evidence suggests that β-cell defects in GDM result from the same spectrum of causes that underlie hyperglycemia in general, including autoimmune disease, monogenic causes, and most importantly insulin resistance. GDM is defined as any degree of glucose intolerance with onset or first recognition during pregnancy [2]. This definition applies regardless whether the condition is treated with insulin or only with diet modification. GDM is also associated with an increased

long-term risk of developing type-2 diabetes. A small percentage of women with a history of GDM might even develop type-1 diabetes postpartum.

Glucose intolerance in GDM may occur due to both reduced insulin sensitivity and an impaired ability to increase insulin secretion in response to glucose. Women with GDM have a more severe insulin resistance compared to the insulin resistance seen in normal pregnancies. They also suffer impairment at the increase of compensatory insulin secretion, particularly first-phase insulin secretion. This decrease in first phase insulin release may be a marker for deterioration of β-cell function.

The pathophysiology of GDM still remains controversial; GDM may reflect a predisposition to type-2 diabetes expressed under the metabolic conditions of pregnancy, or it may represent the extreme manifestation of metabolic alterations that normally occur during pregnancy. The insulin resistance in pregnancy may result from a combination of increased maternal adiposity and the insulin-desensitizing effects of hormones secreted by the placenta. Rapid abatement of insulin resistance after delivery suggests a major contribution of placental hormones.

GDM is more related to pronounced peripheral resistance to insulin, meaning that the main reason is not the defective insulin secretion, or disproportionate secretion of proinsulin, or glucagon.

Screening

Deterioration of glucose tolerance occurs normally during pregnancy. The original diagnostic criteria for GDM were established by O'Sullivan and Mahan in 1964 [6]. Since then several additional diagnostic criteria were developed (Table I.) and they all differ according to the American Diabetes Association (ADA) or the World Health Organization (WHO). By ADA, GDM is diagnosed if two or more plasma glucose levels meet or exceed the following thresholds during 100-g oral glucose tolerance test (OGTT): fasting glucose concentration of 5.3 mM/l, 1-hour glucose concentration of 10 mM/l, 2-hour glucose concentration of 8.6 mM/l, or 3-hour glucose concentration of 7.8 mM/l. The WHO diagnostic criteria are based on a 2-hour 75-g OGTT. GDM is diagnosed if either fasting glucose exceed 7 mM/l or the 2-hour glucose is greater than 7.7 mM/l [7].

Time point	Criteria (mM/L)		
	ADA	NDDG	WHO
Fasting	≥5.3	≥5.8	≥7.0
1h	≥10.0	≥10.5	Not applicable
2h	≥8.6	≥9.1	≥7.8
3h	≥7.8	≥8.0	Not applicable

Table I. Diagnostic criteria for GDM

Abbreviations: ADA, American Diabetes Association; NDDG, National Diabetes Data Group; WHO, World Health Organization;

ADA works with a glucose tolerance test of 100g of oral glucose. Two or more abnormal values are indicatives of GDM. The 75g 2h test can also be used with the same thresholds. NDDG uses a glucose tolerance test of 100g oral glucose. Two or more abnormal values are indicatives of GDM. WHO uses glucose tolerance test with 75g oral glucose. Either an abnormal fasting glucose or 2 h glucose is indicative of GDM.

Women at average risk should undergo glucose testing between the 24^{th} and 28^{th} weeks of gestation; however women with high risk of GDM (marked obesity, personal history of GDM, strong family history of diabetes, or being part of certain racial/ethnic groups with high prevalence of diabetes) should be tested as soon as feasible. If the initial testing is negative the patient should also be retested one more time between 24 to 28 weeks of gestation.

Postpartum screening of women with a history of GDM is also recommended. The ADA recommends screening for DM at 6-12 weeks postpartum by either measurement of fasting glucose levels, or with a 75g OGTT. If impaired glucose tolerance or impaired fasting glucose is detected they should be re-evaluated annually, if the results are found to be normal, it is sufficient to screen in every 3 years. The WHO recommends postpartum screening for type-2 DM at 6 weeks or more after delivery, but does not provide recommendations for follow-up screening after this time.

Complications of DM

Uncontrolled diabetes in pregnancy increases the incidence of congenital anomalies from 2-3% to around 7-9%. Fetal complications of GDM also include macrosomia, neonatal hypoglycemia, perinatal mortality, hyperbilirubinemia, hyperinsulinemia, polycythemia, hypocalcemia, and respiratory distress syndrome [5]. Opinions differ whether the rate of spontaneous preterm delivery (SPTD) increases in pregnancies complicated with GDM. Some authors note that hyperglycemia during pregnancy increases the risk of premature delivery [8, 9]; however, more researchers are on the opinion that the rate of SPTD in GDM is not increased in comparison to non-GDM patients, but reaching established levels of glycemic control may reduce the rate of SPTD in GDM [10].

Macrosomia is defined as birth weight greater than 4000 g, it is present in 20-30% of the cases, and often causes further complications, like trauma during delivery and respiratory distress [7, 11, 12]. Maternal factors associated with an increased incidence of macrosomia include hyperglycemia, high BMI, older age and multiparity. Large for gestational age infants often suffer shoulder dystochia or other fetal injury during delivery, and the rate of cesarean deliveries is also higher.

Neonatal hypoglycemia results from maternal hyperglycemia causing fetal hyperinsulinemia, that can delay fetal lung maturation by lowering cortisol levels.

The long term consequences of GDM can also affect both the mother and the offspring. The offspring has an increased risk of developing obesity, glucose intolerance or type-2 diabetes later in life. Maternal complications associated with GDM include hypertension, preeclampsia, and an increased risk of cesarean delivery. In addition, as mentioned before, women with a history of GDM have an increased risk of developing diabetes later after pregnancy.

Treatment

As a first step, adequate nutrition for the mother and fetus should be established by medical nutrition therapy (MNT), maintaining normogylcemia, and avoiding ketosis.

Ketonemia in mothers with diabetes during pregnancy has been associated with lower IQ levels and impaired psychomotor development in the offspring [13].

If MNT fails to maintain blood glucose levels at the desired levels the most commonly administered treatment is insulin therapy. Maternal insulin does not cross the placenta unless it is bound to IgG antibody [14]. The treatment must be designed to normalize maternal blood glucose concentrations, without the use of exogenous insulins that cross the placenta, since diabetic fetopathy is thought to be the result of fetal hyperinslinemia.
The insulin analog lispro (which has the amino acid sequence in the β-chain reversed at position B28 and B29) was shown to be more efficient than regular human insulin to normalize the blood glucose levels in women with GDM. This insulin analog rapidly lowers the postprandial glucose levels, without increasing the anti-insulin antibody levels. The hypoglycemic episodes are also fewer [15].
Insulin aspart is an insulin analog that has been shown to produce a peak blood level at 40 min after administration; it also lowers postprandial glucose levels significantly better than human insulin. The overall safety and effectiveness of insulin aspart was comparable to human insulin in women with GDM.
There are no results available about randomized clinical trials of using long acting insulin analogs in GDM, such as insulin glargine or insulin detemir [16].

Postprandial glucose monitoring, in combination with fasting blood glucose measurements can significantly improve the outcome of pregnancy in women with GDM, who require insulin therapy. A study including 66 women with GDM revealed that postprandial, rather than preprandial glucose monitoring decreased the significance of developing neonatal macrosomia, and the incidence of neonatal hypoglycemia [17].

The use of oral hypoglycemic agents is currently not recommended, however several studies have already shown promising results about these agents being safe and effective in lowering blood glucose levels in GDM. The sulfanyurea glibenclamid was found not to diffuse freely across the placenta. In a randomized, controlled trial by Langer et al, good glycemic control was achieved both in the insulin-treated and glibenclamid-treated groups, but there were less maternal hypoglycemia in the glibenclamid group (2%) as compared to the insulin group (20%) [18]. There were no significant differences between the 2 groups in the incidence of

pre-eclampsia, macrosomia, neonatal hypoglycemia, congenital anomalies, perinatal mortality, and the rate of Cesarean section.

The use of metformin in pregnancy still remains controversial. The advent of its use lies in the treatment of polycystic ovary syndrome (PCOS), by preventing pathogenic mechanisms, such as hyperinsulinemic insulin resistance, hyperandrogenemia, and obesity which may cause miscarriages in women with PCOS. In a retrospective study metformin proved to decrease early pregnancy loss (8.8%) as compared to the control group (41.9%). However, the number of subjects in this study was not very large [19]. There is also a significant reduction in the incidence of gestational diabetes with the use of metformin in pregnancy [20, 21]. Earlier findings showed a high prevalence of pre-eclampsia in pregnant women taking metformin, but Glueck et al later showed that there was no significant difference in the rates of pre-eclampsia between those taking metformin, and the controls.

Acarbose primarily acts in the guts by delaying carbohydrate absorption; it is not absorbed, and does not have any systemic effects. Local side effects include gastrointestinal discomfort, diarrhea and flatulence. This drug has not yet been studied in pregnancy very well, but based on the clinical results of non-pregnant populations it is not expected to be remarkably effective in GDM.

Rosiglitiazone has not been recommended for use in pregnant women, because treatment with the drug during mid to late gestation was associated with fetal death and growth retardation in animal models.

Consequences of DM

The hyperglycemia induced tissue damage is not affecting every cell type the same way. Some cells are more vulnerable for the tissue damaging effect of hyperglycemia, than others. Endothelial cells in the retina and mesangial cells in the renal glomerulus are highly endangered; nevertheless neurons and Schwann cells of peripheral nerves also suffer considerable damage. These cells types can not reduce the transport of glucose inside the cell efficiently enough, when exposed to hyperglycemia [22].

The vascular consequences of DM are well known, macrovascular and microvascular complications are both important. The three major diabetic microvascular consequences are manifested as retinopathy, nephropathy and neuropathy.

The high intracellular glucose level initiates a series of damaging mechanisms. The increased flux throughout the polyol pathway causes the enzyme aldose reductase to reduce glucose to sorbitol, which is later oxidized to fructose. Aldose reductase normally has the function to reduce toxic aldehydes in the cell to inactive alcohols and requires NADPH as a cofactor. By reducing the high amount of glucose, the aldose reductase consumes all the intracellular NADPH. Unfortunately NADPH is also the cofactor of another enzyme called glutathione reductase, which plays an important role in regenerating the intracellular antioxidant, reduced glutathione. Therefore the polyol pathway flux increases intracellular oxidative stress by reducing the amount of reduced glutathione [23].

In the development of diabetic neuropathy advanced glycation endproducts (AGEs) play a crucial role. AGEs are formed with non-enzymatic glycation of proteins, caused by high blood-glucose level. They are irreversible heterogeneous derivates, and by being resistant to proteolytic enzymes, they accumulate in blood and other tissues. Intracellular damaging mechanisms involve modification of proteins involved in the regulation of gene transcription. They can also modify other circulating proteins, such as albumin. The modified proteins act on cell surface AGE receptors (RAGE), and cause perturbation of a variety of homeostatic functions of the vasculature, such as producing inflammatory cytokines and growth factors which in turn, cause vascular pathology [24].

Intracellular hyperglycemia also increases the production of diacyl-glycerol (DAG), which is an activator factor of protein kinase-C (PK-C). PK-C, activated by hyperglycemia exerts a variety of effects on the gene expression. For example the production of the vasodilator endothelial nitric oxide sythase (eNOS) is decreasing, the vasoconstrictor endothelin-1 is increasing, and plasminogen activator inhibitor-1 (PAI-1) is also increasing, reducing fibrinolysis. Elevated levels of NADPH oxydases are in turn responsible for elevated levels of reactive oxygen species (ROS) [25].

Diabetic neuropathy can involve both sensory and autonomic nerves. Peripheral neuropathy manifests as burning pain and numbness that begins at the fingers and toes and progresses up the arms and legs. Patients may experience sensory loss, impaired ability to detect heat and cold and difficulties with walking. Autonomic neuropathy can cause a variety of symptoms such as low blood pressure, slowed digestion or erectile dysfunction.

Macrovascular consequences of DM can be stroke, myocardial infarction and accelerated atherogenesis. Hyperglycemia is not the major determinant factor of diabetic macrovascular disease. Insulin resistance results in a free fatty acid (FFA) flux from adipocytes into arterial

endothelial cells. Increased intracellular FFA oxidation causes mitochondrial overproduction of ROS, which activates the same damaging pathway, AGEs and PK-C pathway [26].

The most carefully investigated consequence of DM involving smooth muscles is gastro paresis, which affects 20-50% of the diabetic population [27]. It represents a part of a generalized autonomic neuropathy, affecting patients with longstanding, often poorly controlled DM. The main symptom is the well recognized delay of gastric emptying without any gastric outlet obstruction. Beside autonomic neuropathy of the enteric nervous system and the micro- and macro angiopathy of the stomach, abnormalities of gut hormones and electrolyte levels, abnormal secretion of insulin and glucagons, and hyperglycemia also play a crucial role in developing diabetic gastro paresis. The mechanisms of diabetic neuropathy are not completely understood, however hyperglycemia, resulting in oxidative stress plays an important role in its development [28, 29]. Diabetic gastro paresis is often part of a generalized autonomic neuropathy that can also include postural hypotension, abnormal heart rate response, bladder dysfunction, and erectile dysfunction. Most smooth muscle functions are influenced by diabetes; however to this day we do not have enough information concerning the occurring changes in uterine functions.

Myometrial functions: Hormonal regulation and adrenoceptors

The uterine smooth muscle is a myogenic organ and its contractility is determined by multiple factors. It is still unclear if the cells in the myometrium have any pacemaker activity, since anatomically distinct pacemaking cells are yet to be identified there. The sarcoplasmic reticulum (SR) only plays a small role in spontaneous uterine contractility in humans [30].
Hormonal input can also modify uterine contractility; however the nervous input is the one to provide the quickest and most characteristic contractility changes. Estrogens during the follicular phase induce uterine hyperemia; progestins maintain uterine blood flow at constant basal levels throughout the luteal phase of the estrus cycle and pregnancy. Ovarian steroid hormones which regulate uterine contractility can also determine the response of the uterus to catecholamines [31].
Estradiol and progesterone affect not only the noradrenaline (NA) content of the adrenergic nerves, but also the turnover of NE, the activity of its synthetic enzyme, and releases of NE from nerve terminals [31]. Treatment with estradiol decreases neuronal and extra neuronal

uptake of NE as well as spontaneous neuronal and tissue release of NE in uterine arteries, leading to increased uterine blood flow seen in the estrus phase of the ovarian cycle [32]. Estradiol treatment increases uterine contractility and oxytocin (OT) response within 4-6 h. The stimulatory actions of estrogens can be rapidly (within 10 minutes) blocked by progesterone, supporting the hypothesis that progesterone can act not just through genomic, but also through non-genomic mechanisms to promote myometrial relaxation.

In the rat progesterone has been shown to increase the β-adrenergic responsiveness of the uterus by two distinct mechanisms; an increase in the coupling of the β-adrenergic receptor (β-AR) with adenyl cyclase, and an increase in the density of β-ARs [33].

Progesterone also augments the activity of the cyclic AMP (cAMP) / protein kinase-A (PK-A) signaling cascade in myometrial cells. PK-A associates with A-kinase anchoring proteins (AKAP) that localize PK-A to specific intracellular compartments. In rats, labor is preceded by a decrease in PK-A association with AKAP complex and progesterone treatment prevents the parturition related PK-A/AKAP decline [34]. At parturition the inhibitory actions of progesterone are substantially decreased and the combined stimulatory actions of estrogens and other factors, such as myometrial distention and immune/inflammatory cytokines transform the myometrium to a highly contractile and excitable state leading to labor and delivery [31]. The role the adrenergic system plays in the regulation of the contractility of the uterine smooth muscle seems to be even more important, if we consider that the most effective tocolytics available in therapy are the β_2 -AR agonists.

The myometrium and the vasculature is innervated with cholinergic, adrenergic and peptidergic fibers. α-ARs exist on peripheral sympathetic nerve terminals, and are divided into two subtypes, α_1-ARs and α_2-ARs. α_1-ARs are mostly located postsynaptically, whilst α_2-ARs are typically sited presynaptically. All α-ARs are members of the heterotrimeric guanine nucleotide-binding regulatory protein (G protein) coupled receptor super family. α_1-ARs are coupled through G_i/G_q mechanism and α_2-ARs are coupled through G_i/G_0. Activation of α_1-ARs initiates the phospholipase C dependent hydrolysis of phosphatydyl inositol 4,5-biphosphate, resulting in the generation of inositol 1,4,5-triphosphate (IP3) and DAG. IP3 enhances the Ca^{2+} concentration in the cytoplasm by opening the Ca^{2+} channels of the SR. DAG activates protein kinase C, resulting in the phosphorylation of specific target proteins.

α_1-ARs are found both in the central and peripheral nervous systems. In the central nervous system they are situated postsynaptically, and they have an excitatory function. Peripherally

they are situated on vascular and on non-vascular smooth muscle, and they are responsible for contractions. α_1-ARs have three subtypes: α_{1A}, α_{1B} and α_{1D}. Most tissues express mixtures of the three different subtypes in different densities and ratios.

α_2-ARs are located presynaptically and regulate the release of the neurotransmitters, but they are also present in postsynaptical locations. The present classification for α_2-ARs stands as follows: α_{2A}-, α_{2B}- and α_{2C}-subtype. α_{2A}- and α_{2B}-ARs control arterial contractions and α_{2C}-AR subtype is responsible for regulating venous vasoconstrictions. α_{2A}-AR subtype is supposed to mediate the most prominent effects of the α_2-AR agonists. The contractions of a large number of smooth muscle containing tissues are mediated by more than one α-AR subtypes. Unfortunately, there are only a few ligands, that are specific to only one α-AR subtype and the lack of specifity in the different drugs, limits their their therapeutic use.

β-ARs are divided into two subgroups, β_1 - and β_2-ARs. β_1-ARs are mostly expressed in heart, the β_2-AR is expressed in the uterus, skeletal muscle and bronchi. Later another subtype was revealed, which was insensitive to typical β-AR antagonists and was classified as the β_3-AR, expressed primarily in brown- and white adipose tissue. It is likely that β_3-ARs mediate thermogenesis and lipolysis, leading to increased energy expenditure and decreased fat stores [35]. β-ARs are G_s protein coupled receptors, and their intracellular signaling starts with adenylyl cyclase. Activation of the enzyme adenylyl cyclase forms cAMP from ATP. cAMP activates PK-A, which phosphorilates and inactivates myosin light chain kinase, the contractile machinery of smooth muscle, causing smooth muscle relaxation.

β_2-ARs are mainly postsynaptic, and are located on a number of tissues, including blood vessels, bronchi, the gastrointestinal tract, the skeletal muscle, liver and mast cells, where they mediate a number of tissue responses. Activation of β_2-ARs dilates the bronchial smooth muscle and uterine smooth muscle responds in a similar way, thus β_2-AR agonists are frequently used to delay premature labor, although the long-term efficacy and therapeutic usefulness of this approach have been questioned. Agonist stimulation of β-ARs causes receptor desensitization in many cells and tissues, including the bronchi and myometrium [36]. β-adrenergic desensitization can occur by multiple biochemical mechanisms. The two most important mechanisms are the rapid uncoupling between receptors and their G-proteins caused by phosphorylation of the receptors by β-adrenergic receptor kinases [37], followed by binding of the inhibitor protein β-arrestin, and the much slower reduction of the receptor number (down–regulation) that evolves over several hours. When different isoforms of β-ARs are expressed in identical cell lines, these desensitization mechanisms are most pronounced

for the β_2-AR subtype, less pronounced for the β_1-AR subtype, and largely nonexistent for the β_3-AR subtype [38]. Other than β_2-ARs, the calcium-activated potassium channels, and the long lasting (L) type calcium channels are also involved in the regulation of uterine contraction-relaxation process. The tocolytic effects of the calcium channel blocker nifedipine and the β_2-AR agonist ritodrine were compared by a long-term follow-up research of newborns after 2 years of age in the Netherlands. No significant differences were found in neonatal outcome and long-term development after 2 years of age; however ritodrine caused significantly more maternal side-effects [39].

Uterine spontaneous contractility was thought to be stimulated by α-adrenergic agonists, and inhibited by β-adrenergic agonists, [40] however more careful investigations revealed that there is a very close correlation between the α_1/β-AR density ratio and the spontaneous contractility of the uterus [41]. In the pregnant human myometrium, the distribution of α-adrenergic receptors is approximately 70% α_2- and 30% α_1 [42]. Both β_1- and β_2-ARs are present, with large β_2-AR predominance (80-85%) [43, 44]. During pregnancy the uterus undergoes a profound denervation [45]. Adrenergic, cholinergic and peptidergic nerve distributions and actions are enhanced during estrus, diminished during pregnancy and reappear in the postpartum period, thereby adapting myometrial sensitivity and blood flow to gestational requirements [46, 47]. This remodeling involves a profound denervation procedure in which the histologically visualized density of these nerves decreases substantially, as the pregnancy continues. According to our previous superfusion studies, adrenergic denervation regarding the uterine contractile functions can be detected, as early as at the 7^{th} day of pregnancy in the rat [48]. However the morphological degeneration of the axons only becomes evident at day 15 of pregnancy, detected by hystochemical characterization [49]. Electric field stimuli applied to uterine tissue in vitro showed impaired nerve function already on the 5^{th} day of pregnancy, despite of intact nerve- and axon morphology [44]. Superfusion technique was shown to be able to clearly demonstrate that particular period of pregnancy, when the adrenergic fibers still appear to be morphologically intact, but they have already started to lose their function. This kind of uterine denervation is of physiological importance, because it largely contributes to the quiescence of the uterus during pregnancy. Activation of the sympathetic system would result in an increase of myometrial contractility and vasoconstriction, mediated by α-ARs. The loss of the adrenergic fibers may serve to prevent all of these potentially harmful consequences [50, 51].

The changes during denervation are also related to the position of the fetuses in the uterus, meaning that in myometrial tissue of horns distended by fetuses denervation starts earlier. Tissue samples of uterine horns devoid of fetuses (in the case of unilateral pregnancy in guinea-pigs), and from the cervix also show a loss in adrenergic nerves, but this phenomenon only occurs at a much more subsequent period of pregnancy [52].
The denervation of the uterus is speculated to serve two functions: (1) myometrial denervation prevents the autonomic nervous system from either precipitating or preventing the onset of labor and (2) uterine vascular denervation permits local regulation of the placental circulation. Uterine reinnervation in the rat begins rapidly. The partial reinnervation at two days postpartum is followed by a more pronounced reinnervation within a few weeks postpartum, however it will never reach the level of innervation of the previous virgo state [28].

Specific aims

The main aim of the present set of studies was to investigate the effects of experimentally induced DM on the neural functions and motor activity of the myometrium as a function of the gestational age. As mentioned before, the hyperglycemia induced tissue damage largely affects the smooth muscles and because uterine smooth muscle undergoes such a profound transformation during pregnancy, we wanted to investigate how these changes can be influenced by DM.
For the functional analysis of the presynaptic side of noradrenergic neurotransmission in the myometrium, experiments were conducted using superfusion technique. Other than pharmacological stimulation, we also analyzed contractility elicited by neuronal stimulation, using electric field stimuli.
For the determination of the postsynaptic effects of GDM, dose-response curves were generated via isolated organ experiments, using selective adrenergic agonists. Beside the adrenergic system, oxytocin is also a crucial determining factor of myometrial activity, therefore investigating its uterotonic effects in GDM bears great importance. In the present work, not only the adrenergic system was analyzed, but isolated organ experiments were also conducted to determine the changes in the uterine reactivity for oxytocin via DM.

The receptor expressions were also characterized. To do so, we used the data of our isolated organ experiments to stipulate the days of pregnancy worth analyzing. We only conducted

real time polymerase chain reaction (RT-PCR) experiments with tissue samples of the days of pregnancy, which showed the most significant differences during isolated organ experiments between DM and control state.

Materials and methods

Animals and chemicals

Female Sprague-Dawley rats (200-250 g) were mated in a special cage in the early morning; 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. All experimental animal protocols satisfied the Guidelines for Animal Experimentation approved by the Animal Experimentation Committee of the University of Szeged. Substances were purchased, if otherwise not specified, from Sigma-Aldrich, Budapest, Hungary.

Streptozotocin treatment

Streptozotocin (STZ) and alloxan are widely used to induce experimental diabetes in animals. Both are toxic hexose analogs that preferentially accumulate in pancreatic β cells. The cytotoxic effect of both these diabetogenic agents is mediated by reactive oxygen species; however, the source of their generation is different. Our agent of choice to induce a diabetic metabolic state was streptozotocin, due to the greater stability and the wider range of effective dose compared to alloxan [53].

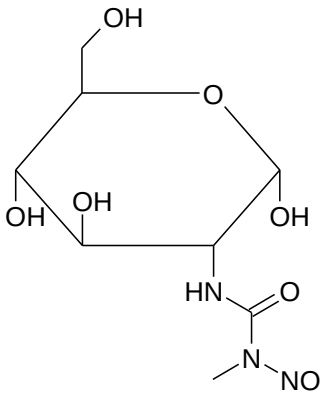

Figure 1. Streptozotocin molecule

STZ (2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) (Figure 1.) is synthesized by *Streptomycetes achromogenes*, and is used to induce both insulin-dependent and non-insulin-dependent diabetes mellitus. Circulating STZ is taken up by pancreatic β cells via glucose transporter 2 (GLUT2). Intracellular action of STZ results in changes of DNA in pancreatic β cells comprising its fragmentation [54]. The main reason for the STZ-induced β cell death is alkylation of DNA, as well as STZ being also a nitric-oxide (NO) donor, this molecule contributes to STZ-induced DNA damage. However, NO is only liberated when STZ is metabolized inside cells. NO is not the only molecule responsible for the cytotoxic effect of STZ, as it generates ROS, which also contribute to DNA fragmentation. NO and ROS can act separately or form the highly toxic peroxynitrate (ONOO), therefore intracellular antioxidants or NO scavengers substantially attenuate STZ toxicity [55].

DM was induced in the rats with 60 mg/kg intravenous STZ injection, and non-pregnant, control animals were sacrificed 12 days after the treatment. In the event of pregnancy, the induction of DM occurred on day 5 of gestation, except for the animals sacrificed on day 7 of pregnancy, which were treated on day 2. Control animals were treated with physiological saline. Hyperglycemia was verified by measuring the plasma glucose level with a colorimetric enzymatic kit (Reanal, Budapest, Hungary).

After administration of STZ to the animals, a significantly higher blood glucose level stabilized at around days 5-6 (Figure 2.). Our main goal was to sacrifice the GDM animals after at least 5 days of SZT treatment. The experiments were conducted at days 7, 14 and 21 of pregnancy. Every group, including the control group, consisted of at least 5 animals.

Experiments with 7 days pregnant and diabetic rats were complicated. If the animals were treated with STZ before the mating, the pregnancy did not occur. The reason behind this could be the lost willingness for mating and also the spontaneous abortion occurring early in the pregnancy. If the day of STZ treatment was the 1st day of pregnancy, a high rate of spontaneous abortion was observed. The number of abortions decreased if the animals were treated on the 2nd day of pregnancy, but the abortion rate was still higher than those treated on the 5th day of pregnancy. This way the time period for developing diabetes grew shorter (2 days), but this showed to be the only possibility to conduct experiments on 7 days pregnant DM animals. In the case of animals, which were used for experiments on days 14 or 21, the 5th day of pregnancy was decided to be the day of STZ treatment.

Release of [^{3}H]noradrenaline

All diabetic and non-diabetic virgo and pregnant rats were sacrificed in a CO_2 chamber. Virgo animals were in an estrous state, as proved by a native vaginal smear. Samples of the uterine tissue were dissected (20-30 mg) and, in the case of pregnant rats, tissues from the implantation and interimplantation sites were processed separately. 4 samples were collected from every animal, 2 from both uterine horns. Samples were cleared of connective tissue and endometrium, minced and incubated in 10^{-7} M [^{3}H]noradrenaline (Perkin Elmer Life Sciences, Boston, Ma, USA; specific activity: 7.94 Ci/mmol) at 37 °C for 60 min. After that, they were washed three times with de Jongh buffer and placed into superfusion chambers (Experimetria, Budapest, Hungary); a continuous flow rate of 1 ml/min was maintained for 60 min with de Jongh buffer containing the monoamine oxidase inhibitor pargyline, the noradrenaline-reuptake inhibitor desipramine and the extra neuronal reuptake inhibitor deoxycorticosterone (10 μM each). The composition of the buffer was 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM Na_2HPO_4 and 6 mM glucose and pH 7.4. The buffer was kept at 37 °C and equilibrated throughout the experiment with O_2 containing 5% (v/v) CO_2. 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 (Canberra-Packard, Budapest, Hungary) for 3 h at 60 °C. The [^{3}H] contents in each 3-min fractions and in the tissue solutions were determined with a liquid scintillation spectrometer. Electrical field stimulation (EFS) consisting of square wave pulses was applied to the tissues, using a programmable stimulator (Experimetria, Budapest, Hungary) during fractions 5 and 15. Each period of stimulation consisted of 360 pulses (voltage, 40 V; pulse width, 2 ms; frequency, 2 Hz; these parameters were suitable for neuronal stimulation). The [^{3}H]noradrenaline contents in the fractions were expressed as the fractional release, which is the amount of labeled transmitter liberated during a 3-min fraction as a percentage of the actual radioactivity content in the tissue at the time of sampling. Peak releases were calculated by subtraction of the radioactivity of fractions 4 and 14 from that of fractions 5 and 15, respectively.

Isolated tissue studies

In every isolated tissue study group there were at least 5 animals. Diabetes was induced as discussed previously. The experiments were also conducted at days 7, 14 and 21 of pregnancy.

Preparation of the tissues:

Both diabetic and non-diabetic animals were killed in a CO_2 chamber. Uterine rings were immediately taken from the uterine horns. Two muscle rings were sliced from both horns of the uterus and mounted vertically in a tissue bath containing 10 ml de Jongh buffer. The temperature of the tissue bath was set to and maintained at 37 °C, and O_2 containing 5% (v/v) CO_2 was perfused continuously through the bath. Tissue samples were equilibrated under these conditions for 90 min before the experiments were started. The tension of the myometrial rings was measured with a strain gauge transducer (SG-02, Experimetria, Budapest, Hungary) and recorded with Isosys Data Acquisition System (Experimetria, Budapest, Hungary). The initial tension of the uterus rings was set to 1.5 g, which dropped to approximately 0.5 g by the end of the equilibration period. During equilibration period (90 minutes), buffer was changed every 15 minutes, totally 6 times, in the chambers. The areas under the curves were analyzed for a 5-min period after each administration of the tested substances.

Determination of contractility changes:

To analyze the changes in the α-adrenergic functions during diabetes, cumulative dose-response curves were constructed for noradrenaline in the concentration range $1x10^{-10}$ – $1x10^{-5}$ M (a total of 11 doses). The chamber contained propranolol (10^{-6} M) to block the effects of NA 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 NA were expressed as a percentage of the KCl evoked contractions.

To characterize the effects of diabetes on the β-adrenergic receptor-mediated myometrial relaxation, cumulative dose-response curves were additionally constructed 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).
A sigmoid curve was fitted individually to all dose-response curves (both noradrenaline and terbutaline) and the maximal effect and EC_{50} values were calculated by means of GraphPad Prism 4 (Graphpad Software, San Diego, CA, USA).

Oxytocin

Non-cumulative dose response curves were constructed for oxytocin (Gedeon Richter, Budapest, Hungary) in the concentration range of 10^{-9} – 10^{-6} M (altogether 7 doses). At the end of the experiment, KCl (70 mM) was added to the chamber and the evoked contractions were recorded for 5 min. The contractions induced by oxytocin were expressed as a percentage of the KCl evoked contractions and the effects of oxytocin were only analyzed at the end of pregnancy, when the oxytocin sensitivity is highest in the myometrium.

Electric field stimulation

Uterine rings were taken, and mounted in a tissue bath the same way as in the case of the other isolated tissue studies. The tissues were mounted vertically to the same strain gauge transducer. The initial tension of the uterus rings was set to 1.5 g. Electric field stimuli was applied on the mounted tissue 3 times, with the following parameters: Voltage: 40 V, duration of stimulation: 3 minutes, pulse width: 0.6 ms, period time: 50 ms. These parameters fit the requirements for selective neuronal stimuli [56, 57]. Before every 3 minutes of stimuli 3 minutes of poise was also recorded. 3 stimuli were used in order to obtain information on the exhaustion of the tissue. At the end of the experiment, KCl (70 mM) was added to the chamber and the evoked contractions were recorded for 5 min. The contractions induced by electric field stimuli were expressed as a percentage of the KCl evoked contractions. The electrical excitabilities of the tissues were expressed by the ratio of the area under the curve of the stimulated period and the area under the curve of their respective poise period.

RT-PCR studies

Animals were killed in a CO_2 chamber, and uterine samples were collected within 10 minutes. Samples were quickly cleared from connecting tissue, frozen and maintained at -70 °C until RNA isolation. The conditions of RT-PCR studies were chosen considering the results of experiments on isolated uterine rings. 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, the tissue samples of non-pregnant animals were used (diabetic and control n = 4 each). 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 [58]. After precipitation with isopropranol, the RNA was washed three times with ice-cold 75% ethanol and then dried. The pellet was resuspended in 100 µl DNase and RNase-free distilled water. The RNA concentrations of the samples were determined from their absorbance at 260 nm.

The RNA was denaturated at 70 °C for 5 minutes in a reaction mixture containing 20 µM oligo(dT) (Invitrogen, Carlsbad, CA, USA), 20 U RNase inhibitor (Invitrogen), 200 µM dNTP in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 5mM $MgCl_2$ in a final reaction volume of 20 µl. 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 µl cDNA, 25 µl ReadyMix REDTaq PCR reaction mix, 2 µl 50 pM sense and antisense primer of the α- and β-ARs and OTR and 16 µl DNase and RNase-free distilled water. The primer sequences used to amplify the α_{1A}-AR, the α_{1B}-AR, the α_{1D}-AR, the β_2-AR and OTR, as well as RT-PCR conditions are shown on Table II. A rat β-actin probe (GeneID: 81822) was used as internal control in all samples.

The RT-PCR was performed with a PCR Sprint thermal cycler (Hybaid, Middlesex, UK) with the following cycle parameters as shown in Table II. The applied RT-PCR protocol furnished optimized conditions and linear phase amplification for each of the primer sets employed. The optimum number of cycles for each set of primers was determined by performing kinetic analyses.

Receptor	Primer sequence	GeneID	Product size (bp)	Coupling temperature (°C)	Number of cycles
α_{1A}-AR	5'-GTA GCC AAG AGA GAA AGC CG-3' 5'-CAA CCC ACC ACG ATG CCC AG-3'	29412	212	50	35
α_{1B}-AR	5'-GCT CCT TCT ACA TCC CGC TCG-3' 5'-AGG GGA GCC AAC ATA AGA TGA-3'	24173	301	54	35
α_{1D}-AR	5'-CGT GTG CTC CTT CTA CCT ACC-3' 5'-GCA CAG GAC GAA GAC ACC CAC-3'	29413	304	53	35
β_2-AR	5'-TCT TCG AAA ACC TAT GGG AAC GGC-3' 5'-GGA TGT GCC CCT TCT GCA AAA TCT-3'	24176	343	54	25
OTR	5'-GGG ACG TCA ATG CGC CCA AGG AA-3' 5'-ACC AAT AGA CAC CTA ATG CA-3'	25342	375	56	35

Table II. Primer pairs used for RT-PCR of α- and β-ARs and OTRs, the length of RT-PCR products and the parameters of the experiment's GeneID numbers.

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 using the Qubit fluorometer (Csertex, Budapest, Hungary).

Semi-quantitative analysis was performed by densitometric scanning of the gel with KODAK EDAS290 (Csertex, Budapest, Hungary).

Statistical analysis

All the presented data on tissue organ contractility are the averages of the result of at least 5 independent experiments, while RT-PCR experiments were carried out in quadruplicate. The contractions elicited by the electric field stimuli were evaluated by one-way ANOVA, followed by Dunnett's multiple comparison tests. To calculate the DM-related differences between the main values of EC_{50} and the maximum effects in the concentration-response curves and the DM related difference in the RT-PCR products, unpaired t-tests were carried out. All statistical analyses were performed by means of GraphPad Prism. 4.

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 (Figure 2.). During the experiments the excessive daily water intake and the increase in urinary volume also confirmed the diabetic state. All included animals were hyperglycemic at the days of the experiments.

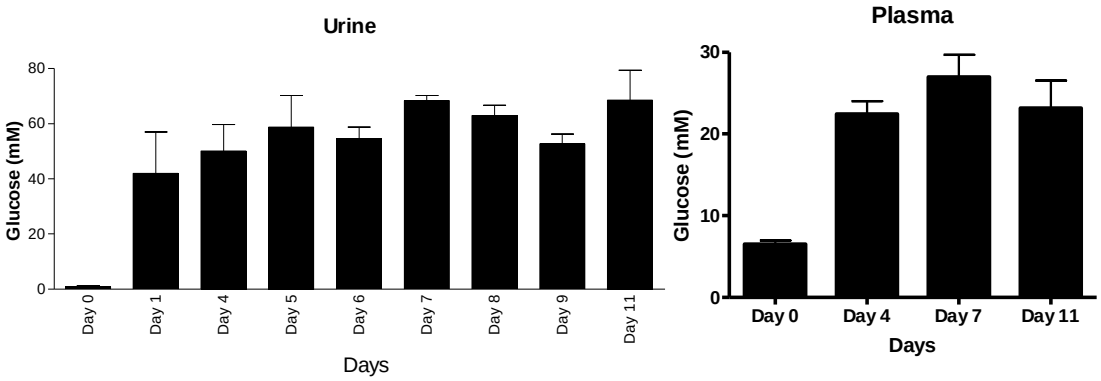

Figure 2. Glucose levels (mM) in urine and blood plasma of non-pregnant rats after STZ treatment on day 0.

Results of tissue radioactivity determination

The tissue activity (expressed in dpm/mg/tissue) was used to describe the uptake capacity of the sample for [^{3}H]noradrenaline. This parameter of the uterus of virgo animals was significantly decreased by experimental DM (Figure 3.). During the course of pregnancy, a gradual decrease in the uptake capacity and site-dependent differences were found between the implantation and interimplantation areas. The STZ-induced DM caused a decline in the tissue activity at the interimplantation sites in early pregnancy (day 7), at both sites in mid-pregnancy (day 14) and in the implantation regions at term (day 21). As regards interregional differences, the implantation sites were found to have a significantly lower uptake capacity in the early-pregnant control rats, and later in the DM animals.

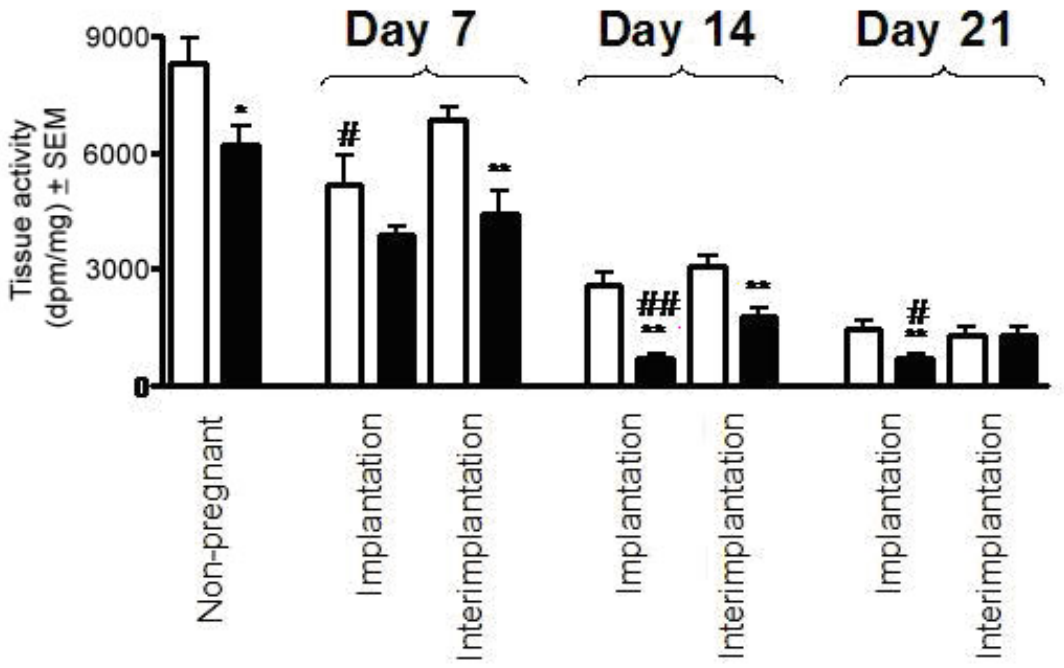

Figure 3. [^{3}H]noradrenaline uptake capacity of myometrial samples as a function of pregnancy. Open and filled columns denote control and STZ-treated values, respectively. * $p<0.05$ and ** $p<0.01$ as compared with the non-diabetic value respectively. # $p<0.05$ and ## $p<0.01$ as compared with the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

Results of electrically stimulated [^{3}H]noradrenaline release

EFS evoked a substantial [^{3}H]noradrenaline release in the non-pregnant and non-diabetic uterus. (Figure 4.) Two EFSs 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. The second peak was practically abolished (Figure 4.). On days 7, 14 and 21 of pregnancy, the [^{3}H]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 5.). In the implantation sites in mid- and late-pregnancy, the transmitter release from the DM animals seemed to exceed that from the control rats, but this does not mean an actual higher amount of [^{3}H]noradrenaline liberation. Under these conditions, the tissue activity is extremely low (Figure 3.) and therefore the minimal amount of electrically released transmitter or even the basal liberation represents a higher percentage of the total amount.

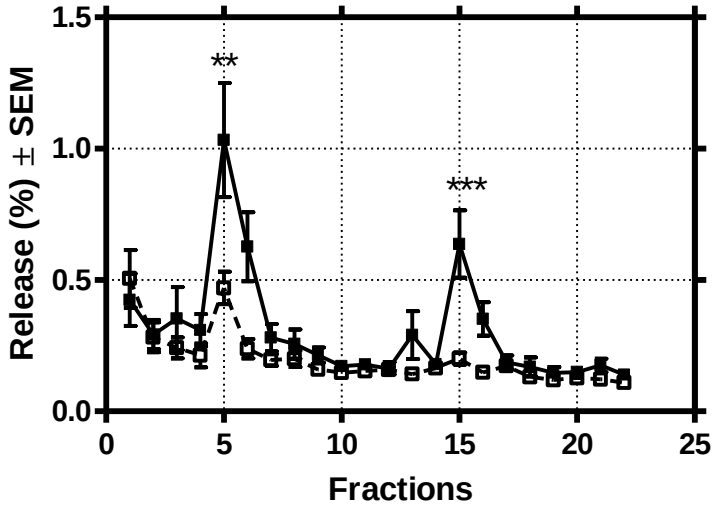

Figure 4. EFS-evoked fractional [^{3}H]noradrenaline release from myometrial samples at estrus from control (■) and diabetic (□) rats. **p<0.01 and ***p<0.001 as compared with the control values respectively.

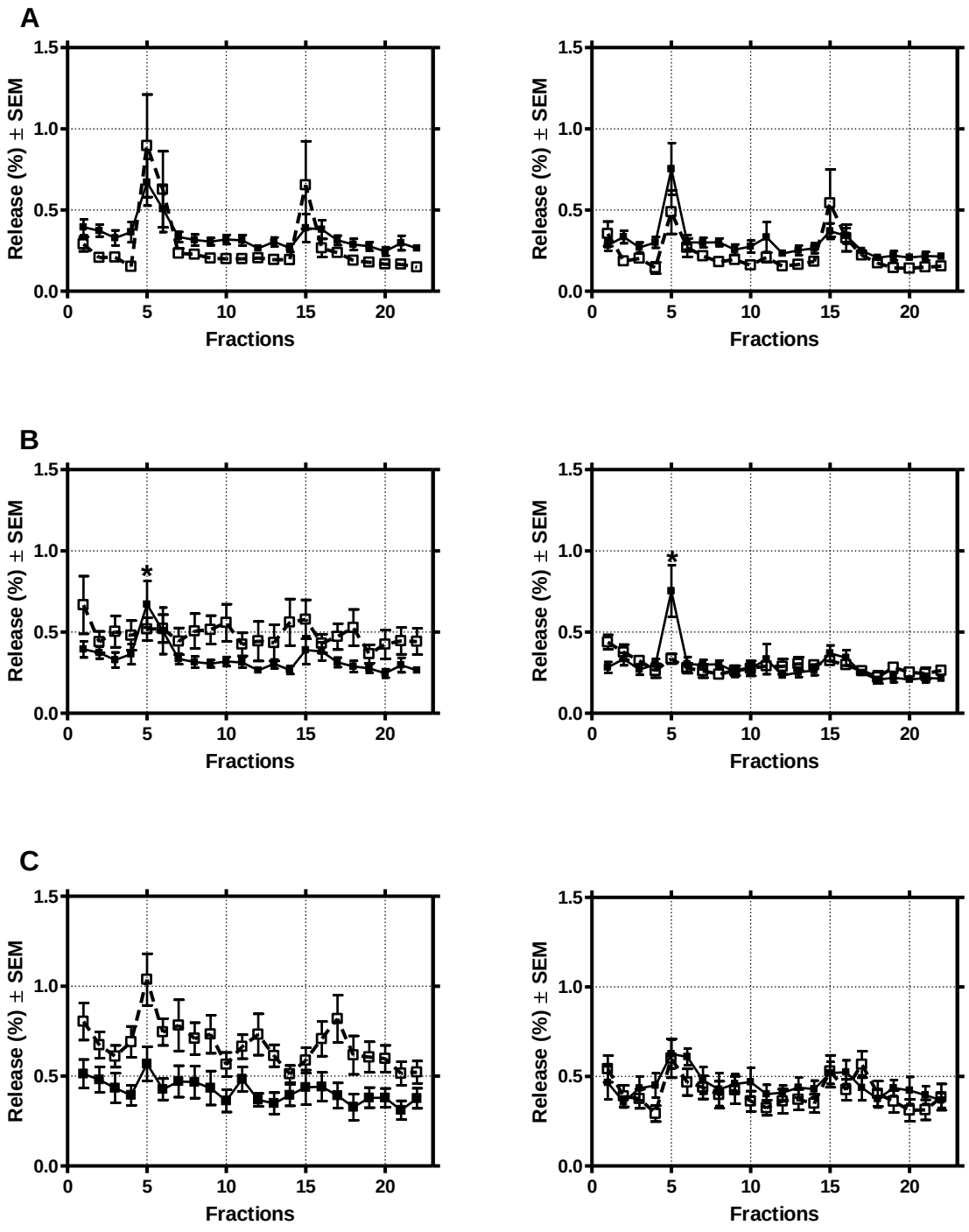

Figure 5. EFS evoked fractional [^{3}H]noradrenaline release from myometrial samples from control (■) and diabetic (□) rats on day 7 (A), 14 (B) and 21 (C). Left and right panels denote implantation and interimplantation areas, respectively. *$p<0.05$ as compared with the control value.

Results of the isolated tissue studies

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 NA, as opposed to the non-diabetic state, which showed to be nearly unresponsive for NA in the presence of propranolol. As the maximum contractility of the myometrium of the DM animals could not be approached up to 10 μM NA, therefore no EC_{50} values were calculated. Instead all pairs of points were compared: the samples from the DM rats exhibited a significantly higher basal activity at low concentrations and a higher reactivity at micro molar amounts of NA. During the middle of the pregnancy and at term, the uteri responded to NA with greater efficacy than in early gestation. In contrast with the non-pregnant state, no GDM-related differences were found in the calculated parameters of the concentration-response curves at these time points (Figure 7. Table III.).

Similarly to the α-adrenergic stimulation, only in the non-pregnant state was a significantly lower relaxing effect detected, using β-AR agonists. Gestation itself, on the other hand, induced a more pronounced relaxation effect in early- and mid-pregnancy, while this relaxation became similar to that of the non-pregnant state in term.

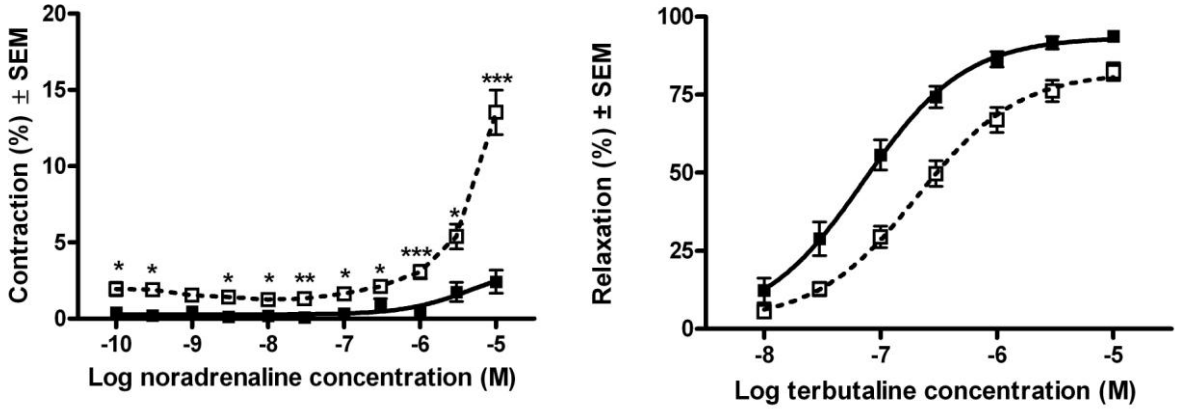

Figure 6. Effects of experimental DM on the noradrenaline- and terbutaline-stimulated myometrial responses of control and diabetic non pregnant rats. (■) and (□) indicate values from control and diabetic animals respectively. *, ** and *** denote significance levels of $p<0.05$, $p<0.01$ and $p<0.001$ as compared with the non-DM value.

In pregnancy, contractility evoked by NA did not change in a significant manner. At 7 days of pregnancy only a slight elevation in contractility was found. On days 14 and 21 much more profound contractions were observed, however the diabetic animals showed no significant difference compared to the control ones regarding uterine contractility evoked by NA (Figure 7., Table III.).

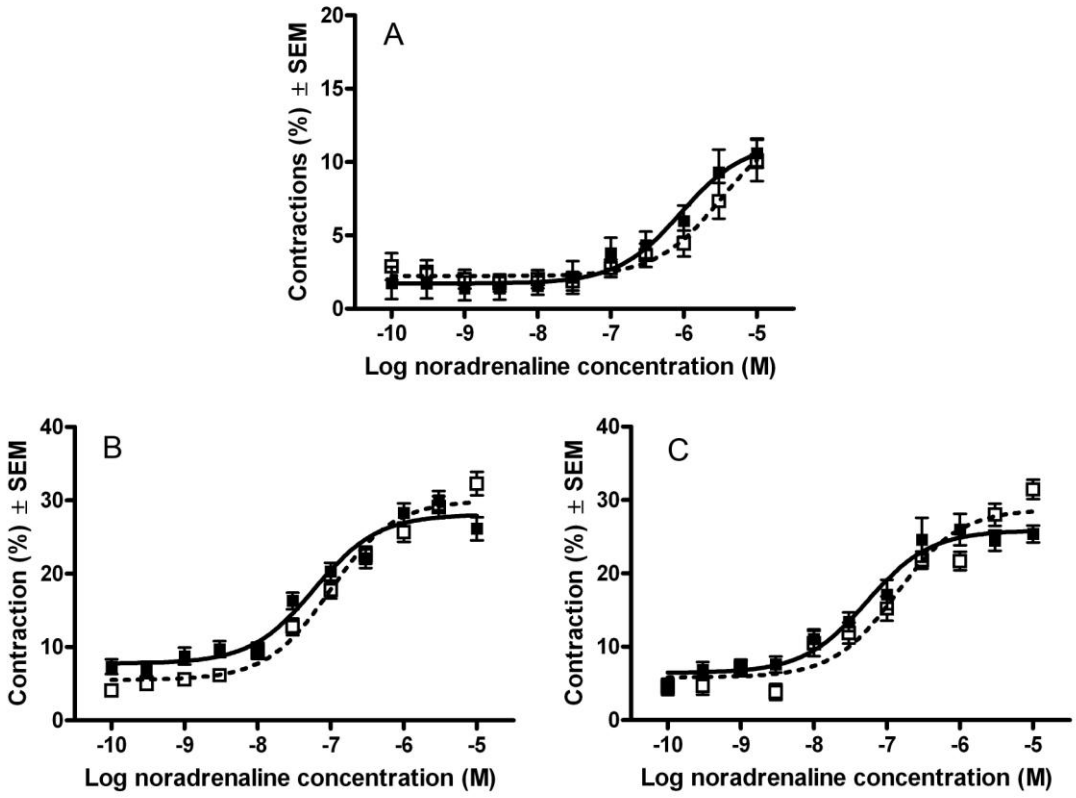

Figure 7. Effects of experimental DM on noradrenaline-stimulated myometrial contractility of the rat on day 7 (panel A), day 14 (panel B), and day 21 (panel C) of pregnancy. (■) and (□) indicate values from control and diabetic animals respectively. No significant differences were detected between the maximal effects or the EC_{50} values of the control and diabetic groups.

GDM did not result in any significant changes in the effects of terbutaline during gestation. At early pregnancy a trend still existed towards lower relaxation rates evoked by terbutaline, the same way as it was observed in tissue samples of non-pregnant rats; but this phenomenon disappeared later during pregnancy (Figure 8., Table III.).

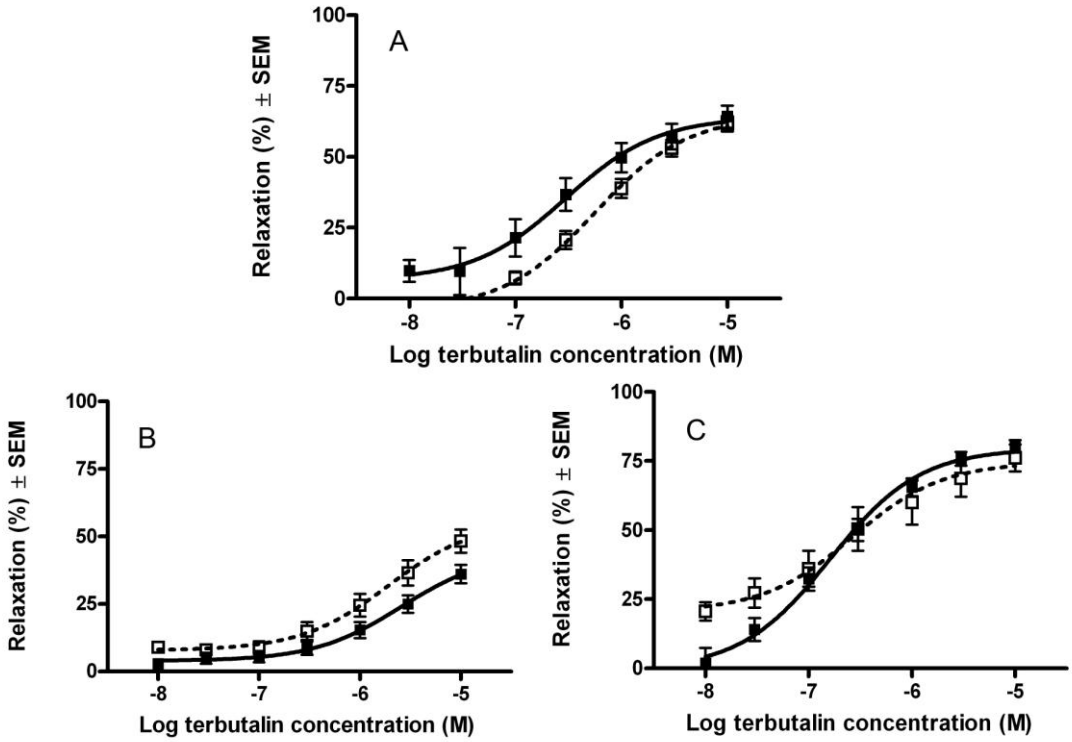

Figure 8. The effects of DM on the terbutaline-induced myometrial relaxation of the rat on day 7 (panel A), day 14 (panel B) and day 21 (panel C) of pregnancy. (■) and (□) indicate values from control and diabetic animals respectively. No significant differences were detected between the maximal effects or the EC_{50} values of the control and diabetic groups.

Agonist	Gestational state	Treatment (number of animals)	Maximum effect (%, 95% confidence limits)	Significance	EC_{50} (μM, 95% confidence limits)	Significance
NA	Non-pregnant	Control (6)	3.46 (1.12 – 5.79)	—	4.56 (0.81 – 25.74)	—
		Diabetic (5)	Not determined		Not determined	
TB	Non-pregnant	Control (5)	94.11 (88.84 – 97.99)	$p < 0.05$	0.072 (0.047 – 0.140)	n.s.
		Diabetic (5)	82.37 (77 – 87.11)		0.32 (0.140 – 0.303)	
NA	7-day pregnant	Control (6)	11.33 (8.92 – 13.73)	n.s.	0.91 (0.35 – 2.38)	n.s.
		Diabetic (5)	12.58 (8.41 – 16.75)		3.13 (1.07 – 9.18)	
TB	7-day pregnant	Control (5)	64.02 (54.66 – 73.37)	n.s.	0.30 (0.12 – 0.74)	n.s.
		Diabetic (5)	64.59 (59.42 – 69.77)		0.56 (0.39 – 0.81)	
NA	14-day pregnant	Control (5)	28.04 (26.57 – 29.51)	n.s.	0.059 (0.038 – 0.092)	n.s.
		Diabetic (5)	29.95 (28.51 – 31.40)		0.097 (0.071 – 0.14)	
TB	14-day pregnant	Control (7)	43.95 (32.91 – 55.00)	n.s.	2.55 (1.11 – 5.83)	n.s.
		Diabetic (6)	55.79 (43.84 – 67.73)		1.92 (0.85 – 4.36)	
NA	21-day pregnant	Control (5)	25.84 (23.98 – 27.70)	n.s.	0.053 (0.030 – 0.095)	n.s.
		Diabetic (5)	28.74 (26.28 – 30.66)		0.136 (0.087 – 0.213)	
TB	21-day pregnant	Control (6)	79.56 (74.07 – 85.06)	n.s.	0.16 (0.10 – 0.25)	n.s.
		Diabetic (5)	74.62 (64.51 – 84.74)		0.27 (0.092 – 0.78)	

Table III. Calculated parameters of concentration-response curves of noradrenaline (NA), terbutaline (TB); n.s., not significant

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 [59].

On days 15 and 21 of pregnancy, the maximal effect of oxytocin showed a significant increase in diabetes ($p<0.005$ and $p<0.05$). Oxytocin exerted a more pronounced uterotonic effect at the start of the 3rd trimester, however at term, no GDM related differences were observed (Figure 9., Table IV.).

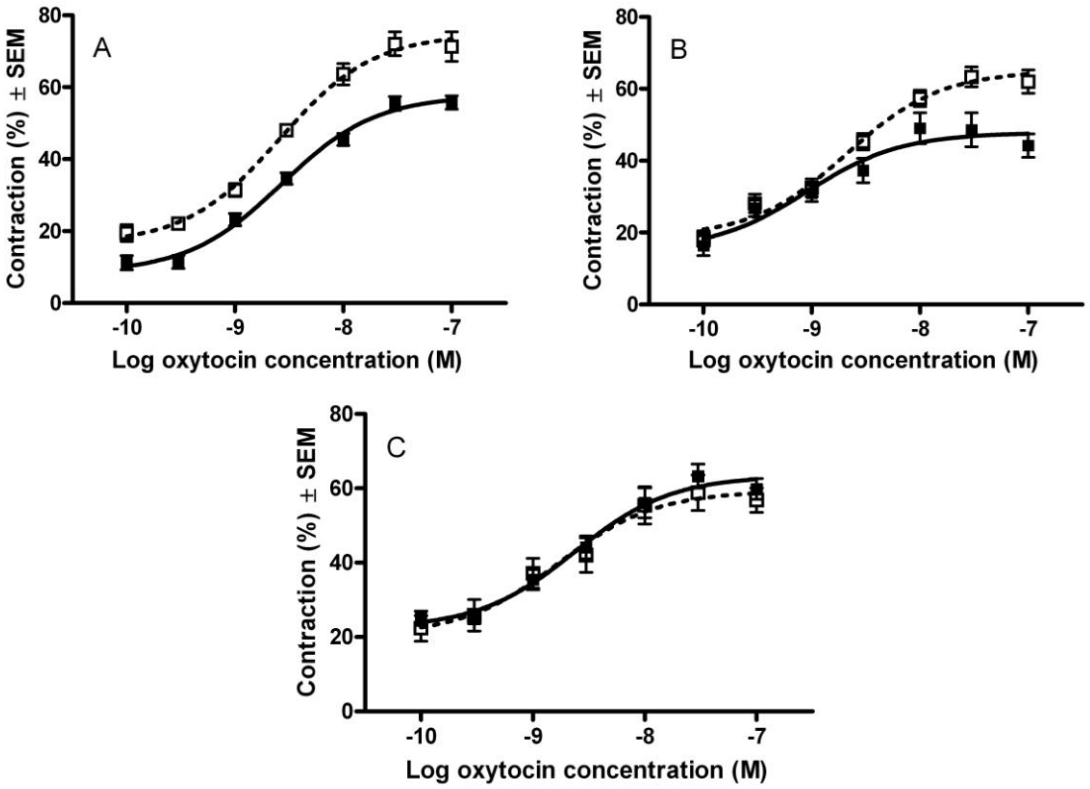

Figure 9. Effects of oxytocin on uterine contractility on day 15 (panel A), day 21 (panel B), and day 22 (panel C) of pregnancy of control (■) and GDM (□) rats during pregnancy. Significant differences were found in the maximal effect of oxytocin on uterine contractility of diabetic and non-diabetic rats in the 3rd trimester.

Agonist	Gestational state	Treatment (number of animals)	Maximum effect (%, 95% confidence limits)	Significance	EC_{50} (nM, 95% confidence limits)	Significance
OT	15-day pregnant	Control (6)	57.69 (54.70 – 60.69)	$p < 0.005$	2.69 (1.90 – 3.82)	n.s.
		Diabetic (5)	74.85 (70.53 – 79.16)		2.58 (1.68 – 3.96)	
OT	21-day pregnant	Control (6)	47.78 (43.30 – 52.25)	$p < 0.05$	0.91 (031 – 2.68)	n.s.
		Diabetic (6)	65.01 (60.79 – 69.22)		2.11 (1.25 – 3.55)	
OT	22-day pregnant	Control (6)	63.42 (58.89 – 67.96)	n.s.	2.37 (1.23 – 4.55)	n.s.
		Diabetic (5)	59.28 (53.12 – 65.44)		1.69 (0.61 – 4.75)	

Table IV. Calculated parameters of concentration-response curves of oxytocin; n.s. = not significant.

Effects of electric field stimuli

During the experiments the same electric field stimulus (EFS) was administered 3 times on the same tissue sample, in order to evaluate if the tissue shows any fatigue during the experiment. We did not observe any significant differences between the excitabilities evoked by the 3 stimuli, but there was a trend towards greater excitability for the third stimulation. This means that in some cases only the third stimulation elicited a significant contractile response. Therefore a substantial contractile response is considered to be higher than 1. Excitability was calculated as a quotient of the area under the curve of the EFS and that of the not stimulated state.

In non-pregnant animals DM decreased the contractions evoked by EFS (Figure 10.). The excitability was found to be around 1, which means that the area under the curve in the case of EFS was about the same as in the case of the unstimulated control, meaning that the EFS was nearly of no effect on the diabetic tissue.

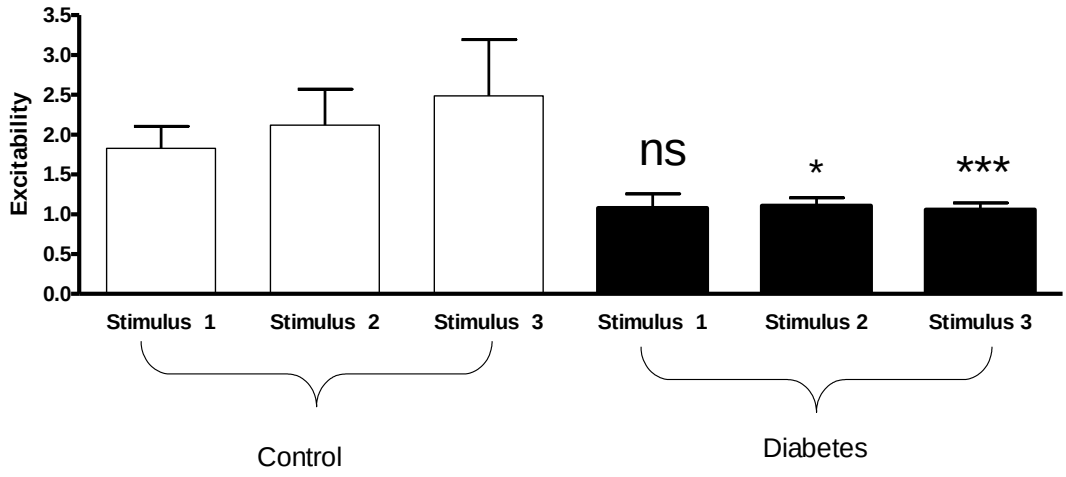

Figure 10. The effects of electrical stimulation on the uterus of non-pregnant control (open column) and diabetic (filled column) animals. * and *** denote $p<0.05$ and $p<0.001$ significance rate as compared with the non-diabetic value of respective stimuli. The excitability is the quotient of the area under the curve of the electrically stimulated period and the rest period.

The uterus in early pregnancy demonstrated a substantially higher excitability compared to the non-pregnant state, which eventually vanished by term. In pregnant animals diabetes also decreased the contractions evoked by electric field stimuli, and did this in a significant manner. 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 (Figure 11.).

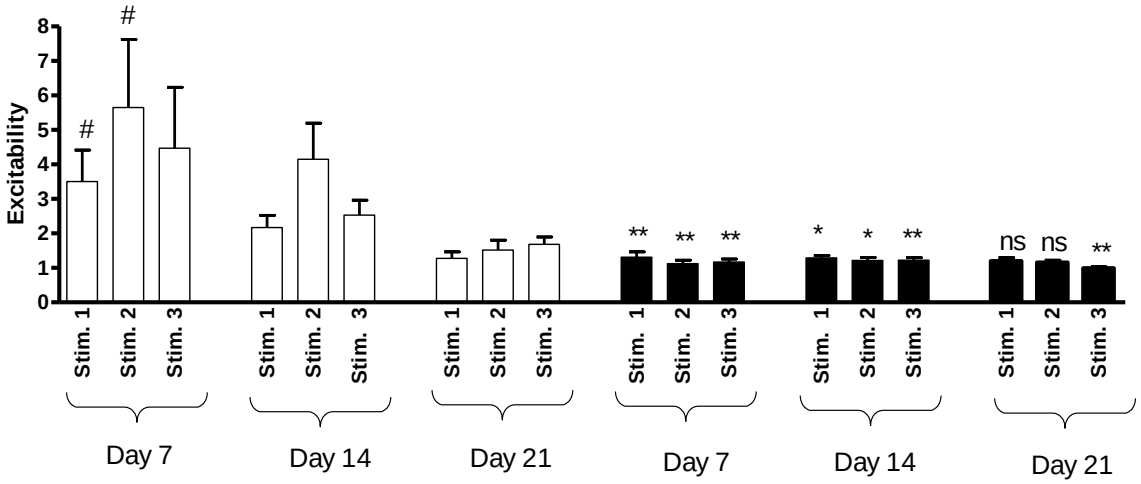

Figure 11. Effects of electric field stimulation on the uterus of control (open column) and diabetic (filled column) rats at days 7, 14, and 21.* and ** denote $p<0.05$ and $p<0.01$ significance-rate as compared with the control value of respective days in pregnancy. # denotes $p<0.05$ as compared with the non-pregnant value.

RT-PCR

The aim of the RT-PCR studies was to find a possible explanation for the results of our isolated organ experiments. Our goal was to determine any differences in mRNA levels of α-, or β-ARs, according to the changes in contractility noticed at the isolated organ experiments. The expressions of the involved receptors, such as all types of α_1- and β_2-ARs, and oxytocin receptor (OTR) were determined at the mRNA level in non-pregnant and late pregnant (day 15) uteri respectively.

It was found that the only α_1-AR subtype that showed a significant elevation in mRNA expression as a consequence of DM, was α_{1B}. The amount of the RT-PCR product for α_{1A}-AR did not differ from the controls in diabetes and interestingly, the α_{1D}-AR mRNA levels even showed a decrease in diabetes. Our results suggest that the elevated contractility, registered in diabetes at the isolated organ experiments in the non-pregnant state could originate from an elevated level of α_{1B}-ARs (Figure 12.).

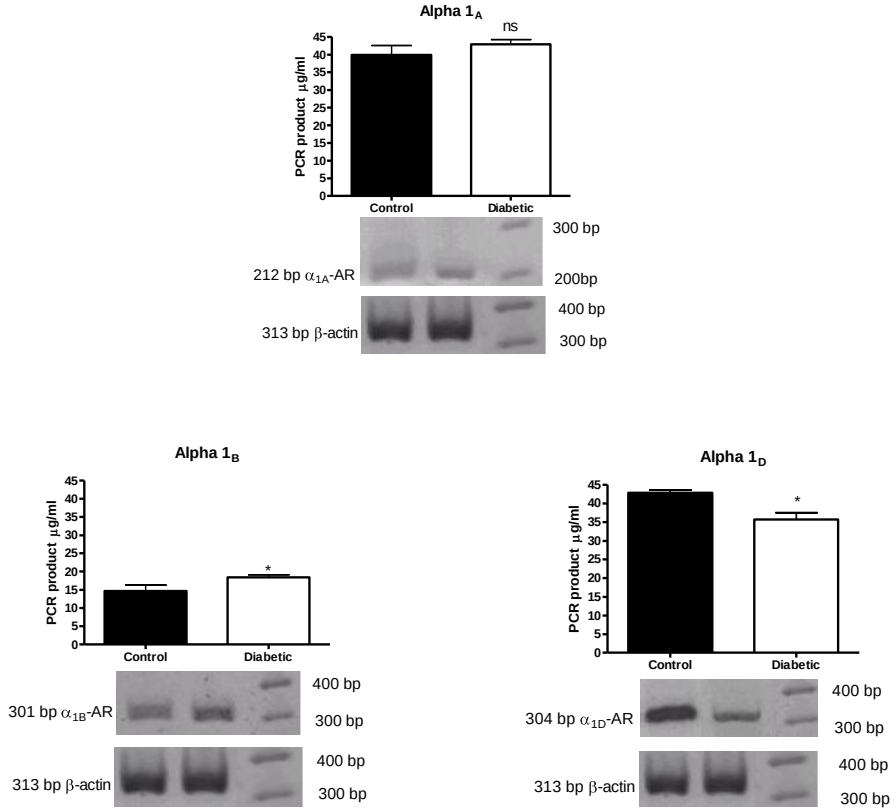

Figure 12. Amount of RT-PCR products in uterus samples of non-pregnant animals. α_{1A}-, α_{1B}-, and α_{1D}-AR mRNA levels were measured individually with RT-PCR. * denote $p<0.05$ significance rate as compared with the control value of respective AR mRNA levels. The β-actin probe was used as internal control. Panels below graphs are representative gel pictures.

Since our isolated organ experiments showed the highest difference in relaxation between diabetic and control values during the non-pregnant state, we investigated the β_2-AR mRNA amounts via RT-PCR in the uterus samples of non-pregnant animals. The β_2-AR mRNA levels of the diabetic and control samples did not differ significantly, though there was a trend towards lower β_2-AR mRNA levels in the diabetic samples (Figure 13.).

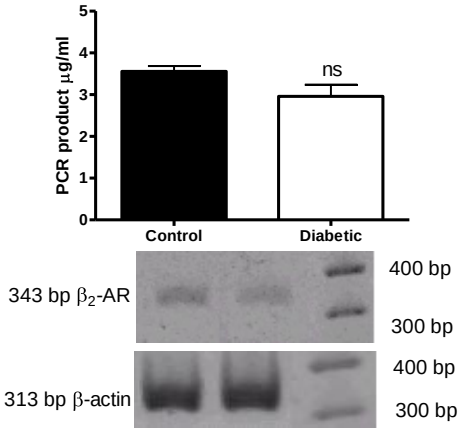

Figure 13. Amounts of RT-PCR products in uterus samples of non-pregnant animals. β_2-AR mRNA levels of diabetic and control samples did not differ in a significant manner. The β-actin probe was used as internal control. n.s., not significant.

To evaluate the differences in the oxytocin receptor mRNA levels we used the uterine samples of 15 days pregnant animals were used for RT-PCR. The isolated organ experiments denoted the most pronounced difference in the contractility caused by oxytocin in diabetic and control animals at day 15 of pregnancy (Figure 14.).

As expected the amounts of RT-PCR products were significantly greater in the samples obtained from diabetic animals compared to control. These data denote a possible elevation in oxytocin receptor mRNA levels in the uterus of diabetic animals, in accordance to our findings during the isolated organ experiments.

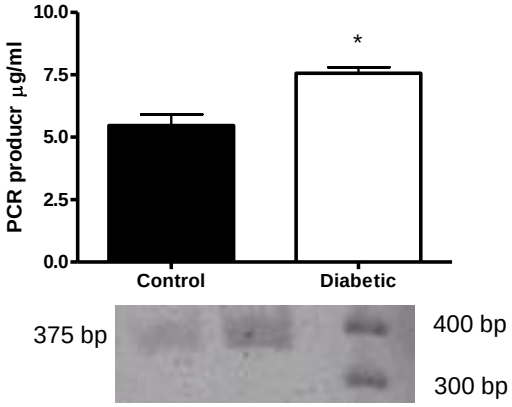

Figure 14. Amount of RT-PCR products in uterus samples of 15 days pregnant animals. * denote $p<0.05$ significance rate as compared with the control value of respective oxytocin mRNA levels. The β-actin probe was used as internal control.

Discussion

In the present set of experiments, the main focused was on the sympathetic nervous system, and OT, two of the three crucial factors to determine uterine contractility, alongside the prostaglandin system [60].

Pregnancy induced, or pregestationally developed DM increases the birth weight, the risk of premature rupture of membranes and preterm birth. These complications are all associated with uterine contractility, so it is understandable, that GDM must have some modulator effect on the determining factors of contractility.

The question may arise: is it possible that the observed changes in myometrial functions are not the result of hyperglycemia, but the direct effect of STZ? The direct deteriorative effect of the STZ treatment on the adrenergic fibers can not be excluded. Rodriguez Filho and Fazan observed morphometric signs of axonal atrophy of the phrenic nerve in rats 12 weeks after STZ treatment [61]. However these changes were preventable with insulin treatment, indicating that hyperglycemia itself was responsible for the neuronal damage.

Uterine PG production increases during DM. In animal experiments DM elevated the levels of uterine PGE_2, $PGF_{2\alpha}$ and also PGI_2. Insulin therapy restored PGE_2 levels, the most potent stimulatory factor of the myometrial fiber at control values, whereas PGI_2 slightly elevated [62]. Because PGE_2 is the most relevant uterotonic PG, it is conceivable, that the elevated levels in DM contribute to elevated uterus contractility, and perhaps to preterm birth.

Pregnancy induced uterine neurodegeneration plays a crucial role in the prevention of a possible preterm birth, by forming a functionally isolated fetoplacental unit. The most commonly used method of accessing the degeneration of the uterine nerves is by immunhistochemistry. Immunhistochemistry is clearly able to reveal the decrease of adrenergic nerves in the pregnant uterus as pregnancy progresses, however in our experiments we chose to focus on the presynaptic and the postsynaptic side of neurotransmission in the uterus to determine the signs of denervation. Using a new and less prevalent method to investigate pregnancy-induced uterine neurodegeneration gave us the opportunity to further amend the hystochemical studies, and also to introduce new results and conclusions, by analyzing the problem from a functional point of view.

The tissue uptake capacity proved to be a more sensitive parameter than the EFS-induced transmitter release. By monitoring the tissue uptake capacity functional changes in the uterus became detectable at such an early level, where the anatomical structure was still intact, but

the physiological functions were already impaired [51]. However, the disadvantage of the method is not being able to differentiate between the changes of innervation in the vasculature and that of in the layers of myometrium. The superfusion experiments showed an "accelerated" rate of the degeneration in the adrenergic nerves of the uterus, meaning that the presynaptic side of the myometrial adrenergic transmission, i. e. NA uptake and stimulated release was substantially affected by DM. The process of denervation started earlier and during the process, the loss of adrenergic nerves was more pronounced [63]. This more pronounced reduction of the adrenergic functions in the uterus could be a sign of a more accelerated denervation process. This may indicate that DM during gestation also causes a more pronounced isolation of the uterus, resulting in post-term delivery. On the contrary, mothers with GDM have an increased risk of preterm delivery, which is most likely caused by macrosomia and hyperglycemia [7, 9]. In this case, these factors easily overweigh the fetus protecting effect of uterine denervation, even if the denervation was also accelerated by the DM. However in the present experimental setting, preterm delivery never occurred. The reason behind this phenomenon could be fetal growth retardation, which is caused by decreasing plasma levels of placental growth factor in GDM [64, 65].

The postsynaptic side of the neurotransmission was investigated with isolated organ experiments using adrenergic agonists, NA, in the presence of propranolol, and terbutaline, for the α-, and β-ARs respectively. These experiments showed the most significant difference between the control and diabetic group in the non-pregnant state. A higher motor activity was observed, as the diabetic uterine tissue samples showed an increase in contractility for NA, and a decrease in the relaxing effect of terbutaline. However the DM evoked changes in the adrenoreceptor status of different tissues were highly inconsistent.

It is well known, that DM increases the AR density in the prostate and brain [66, 67] and also reduces the number of α-ARs in the heart muscle [68-71]. The aorta has been reported to produce increased reactivity to NA without changing the affinity [72, 73]. The explanation of the entire above mentioned phenomenon must be the change in receptor expression. To investigate if the observed changes in α- and β-AR activity could also be correlated with decreased receptor expression, RT-PCR experiments were conducted. In the uterine samples of STZ-treated rats the α_{1B}-AR density was found elevated, whereas the α_{1D}-AR density substantially decreased. No differences were detected concerning the α_{1A}-ARs. Our data suggest that the α_{1B}-AR population must be responsible for the increased contractility observed during isolated organ experiments.

The relaxing effect of terbutaline was less pronounced in DM, however the RT-PCR experiments showed no difference in the β_2-AR density. Experimental diabetes has been shown to decrease the isoprenalin-stimulated adenylyl cyclase activity of a prostatic membrane preparation from STZ-treated rats [74]. It seems possible that DM decreases the G-protein activation mediated by β_2-ARs therefore resulting in an uncoupling of the receptor and its signal transduction mechanism, rather than actually decreasing the β_2-AR density. The relaxing effect of terbutaline on the uterus decreases in late pregnancy, possibly with the same mechanism [75]. Our results are in agreement with the previous report of a decreased relaxing effect of salbutamol on gastrointestinal smooth muscle in alloxan-induced diabetic rats [76].

Pharmacological analysis of electric field stimuli induced mechanical responses in isolated smooth muscle strips is a useful in vitro technique for clarifying autonomic innervation and contractility. Peripheral neuropathy is such a well-characterized consequence of DM, that it can be used as a marker to describe the progression of the disease. According to our experiments, DM resulted in a substantial decrease in the uterine contractility induced by electric field stimuli, both in non pregnant and in pregnant animals. In GDM direct neural stimuli generated by electric field, could not result in any notable contraction activity, presenting a state very similar to that of a completely denervated uterus at term. This means that even if the duration of the diabetic period was only as short as 5 days, it still seemed to be enough to nearly completely eliminate any contraction responses for direct neural stimulation. This suggests that the myometrium might be more sensitive to the effects of DM, since DM induced peripheral neuropathy in other organs needs more time to develop.

Since pregnancy induced adrenergic denervation plays a crucial role in the isolation of the fetoplacental unit, we also conducted experiments to determine the involvement of the OT system during this process. Normally the uterine tissue only becomes responsive for OT in the last trimester of pregnancy, but the present set of experiments indicate that diabetes modulates this effect, and the uterus becomes responsive to this neuropeptide sooner than seen in the control group. At the onset of labor, preterm and at term the vasopressin V_{1a} and oxytocin receptors are elevated to a moderate degree in the myometrium [77]. Increased uterine sensitivity to OT was registered in guinea pigs, using nerve stimulation even with parameters that were not able to cause any actual contractions [78]. Our RT-PCR experiments revealed a substantially higher expression of OTRs in the myometrium of diabetic animals, and this can explain the increased uterotonic effect, witnessed in GDM. The elevated contractility

registered for OT in GDM and also the RT-PCR results for OTRs suggest that, the uterine sensitivity for OT increases in GDM. Subsequently we can conclude that diabetes may have a "pregnancy accelerating" effect, meaning that the pregnant uterus in GDM shows the characteristics of a more further developed pregnancy, earlier than normal. This "accelerating" effect of diabetes stands true for the adrenergic and oxytocin system, but the other regulating factors of the uterus still need to be investigated. The presented results can suggest a phenomenon more general, which can contribute to the characteristic consequences of GDM, such as pre-term delivery.

Acknowledgements

First of all I would like to express my gratitude to my supervisor, Dr. István Zupkó, whose expertise, understanding, and patience, added considerably to my research experience. I appreciate his vast knowledge and skill in many areas, and his assistance in writing reports or doing research experiments.

I thank Dr. György Falkay, the head of the PhD program Pharmacodynamics, Biopharmacy and Clinical Therapy for giving me the possibility to work with his group and providing the professional background for my studies and research.

A very special thanks goes out to my colleagues, Gábor Csík, Borbála Réthy, Dénes Csonka and Renáta Minorics for the cooperation during research, for their help in every day work and friendship.

I would also like to thank my family for the support they provided me through my entire life.

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