ULTRASONOGRAPHIC, PHARMACOLOGIC AND PATHOLOGIC EXAMINATION OF THE FETOPLACENTAL CIRCULATION

Ph.D. Thesis

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I. Jakó M, Surányi A, Kaizer L, Domokos D, Gáspár R, Bártfai G.
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1.Abbreviations:

3DPD	3 dimensional power Doppler ultrasound
5-HT	t-hydroy-triptamine, serotonine
AC	abdominal circumference
AED	absent end diastolic flow
BPD	biparietal diameter
FI	flow index
FOD	frontooccipital diameter
FL	femur length
GABA	gamma-amino-butyric-acid
GDM	gestational diabetes mellitus
HC	head circumference
HE	hematoxyllin/ eosine
IOD	intraorbital distance
IUGR	intrauterine growth restriction
NADPH	nicotinamide-dinucleotide-phosphate
ns	not significant
OT	oxytocin
OTR	oxytocin receptor
PED	positive end diastolic velocity
RED	reverse end diastolic flow
S/D	peak systolic velocity/end diastolic velocity
SGA	small for gestational age
SUA	single umbilical artery
ThAPD	thoracal anterioposterior diameter
ThTD	thoracal transversal diameter
UCI	umbilical coiling index
UtBF	uterine artery blood flow
relUtBF	relative uterine artery blood flow
UtPI	uterine artery pulsatility index (peak systolic velocity-end diastolic velocity)/ mean velocity
VFI	vascularization flow index
VI	vascularization index
VOCAL	virtual organ computer aided analysis
VP	vasopressin
V1aR	type 1a vasopressin receptor
WHO	World Health Organisation

2.Summary

There are several known risk factors for placental insufficiency and intraurerine growth restriction (IUGR) but the exact pathophysiology is not clear yet. We examined the microvascular changes of placental and umbilical cord vessels in IUGR and healthy pregnancies where neither mathernal nor fetal conditions could justify the growth restriction and it derived from placental insufficiency. We also investigated the microvascular changes elicited by hyperglycaemia in gestational diabetes.

Conventional two-dimensional (2-D) ultrasound evaluation includes the morphology, anatomy, location, implantation, anomaly, color/power and pulsed Doppler ultrasound assessment of the placenta. The three-dimensional (3-D) reconstruction of the placenta gives information about 3-D placental vasculature and placental blood flow. The quantitative 3-D power Doppler (3-DPD) histogram analysis by Virtual Organ Computer-aided Analysis (VOCAL) program provides more details concerning qualitative assessments of the vascularisation and blood flow. A prospective case-conrol study was carried out in order to examine placental vascularisation using 3-DPD technique with VOCAL program in the second and third trimester of pregnancies complicated by gestational diabetes mellitus (GDM) and intrauterine growth restriction and we compared our data with those of the normal controls. After delivery we collected the placenta and umbilical cord, dissected vessel segments and performed tissue bath examinations via adding vasoactive agents in different doses and dosage patterns. All samples underwent pathological and histological examinations to find histopathologic alterations in the vessels that can justify the altered fetal growth. We compared our ultrasonographic data to pharmacological and pathological findings. A consecutive recruitment of pregnant women was carried out between January 2014. and May 2017. at the Department of Obstetrics and Gynecology, Szeged, Hungary.

a, In the first study set, pregnancies complicated by GDM and pregnancies complicated by IUGR were compared to control ones. The tested vasoactive agents were oxytocin and desmopressin in logarithmic, non-comulative dosage.

b, In the second study set, pregnancies were divided into two groups: non-pathological control group and IUGR group. The tested vasoactive agent was serotonin in logarithmic cumulative dosage with and without ketanserin incubation.

In case of IUGR and diabetic patients, significant deterioration of the 3-DPD indeces could be seen compared to the control group. Placental vascularisation in pregnancies complicated by IUGR is lower than in diabetic pregnancies and in controls. The difference is smaller, but significant regarding the flow index, meaning the amount of blood flowing through one vessel exceeds the control value compensating hypovascularization to a certain level. In GDM pregnancies despite of the general enlargement low placental flow is measured because of microvascular occlusions and calcification of the villi. Oxytocin elicited no significant changes in vascular tone in any of the vessels. Desmopressin, a partial agonist of the previous ligand, did not cause any significant change in vascular tone either. The contraction elicited by serotonin was stronger in IUGR umbilical arteries and the values of maximal contraction correlated with the values of the systolic/diastolic velocity ratio. The effect of ketanserin was more pronounced in IUGR umbilical cord arteries and veins. Regarding the placental vessels, both the contraction to serotonin and the effect of ketanserin was diminished in IUGR pregnancies related to healthy contols. In conclusion, 3-DPD assessment of placental vascularisation may provide new insights into normal and abnormal fetoplacental hemodynamics. Regarding the pharmacokinetic results, neither oxytocin, nor vasopressine have an active receptor on the placental and umbilical vessels. The reactivity to serotonin correlates with the umbilical artery velocymetry, thus suggesting that it has a role in regulating vascular tone. The difference between the IUGR and the control group in the effect of ketanserin can be explained by a higher relative density of 5-HT type 2 receptors in the study group. The pathologic examination revealed no vascular morphological alterations in the study groups. Samall, sporadic lesions could be observed in normal pregnancies, but the appearance of two or more alteration was characteristic to IUGR cases.

Our results suggest that serotonin has a role in the physiology of fetoplacental vasoregulation and its alterations can be observed in IUGR pregnancies. More studies are necessary to determine other vasoactive agents that actively regulate these vessels and show alterations in pathologic pregnancies. The possible therapeutic effect of ketaserin, should be investigated in IUGR pregnancies.

3. Introduction

3.1. Intrauterine Growth Restriction

Birthweight is one of the most sensitive – and also one of the most important – measures of the newborns' wellbeing. Birthweight is directly influenced by the health status of the mother. According to the World Health Organization (WHO), intrauterine growth restriction (IUGR) is diagnosed upon the estimated birthweight being below the 10th percentile of the recommended gender-specific birthweight for gestational age reference curves [1]. Although early preterm IUGR is associated with the highest rates of mortality and morbidity, late-pregnancy IUGR remains a leading cause of unexpected perinatal death and morbidity after 34 weeks of gestation [2–5]. Hence more than 50% of unexplained stillbirths are related to late growth restriction, the detection and follow-up of fetuses at risk are necessary for optimal management and planning of delivery [5–8]. Fetal biometry assessment and estimation of fetal weight by ultrasound plays a central role in the identification of fetuses at risk for IUGR-related adverse outcomes. Several methods are available to allow physicians to prenatally assess the likelihood of IUGR using biometric measurements. These include cross-sectional growth charts [9–11], estimated fetal weight (EFW) related charts [12–16], customized growth charts [17–18] and growth charts for longitudinal assessment. EFW is the most widely used reference value, using a cross-sectional age-specific percentile chart to determine any alteration in fetal growth. Either poor or excessive fetal growth determines the perinatal outcome. Estimated fetal weight is generally calculated through several steps: fetal biometric measurements are converted into a fetal weight estimate using one of several formulae, EFW is assessed using a given reference chart. If the observed estimate is found to be outside a predefined normal range, specific follow-up is necessary until delivery. This screening procedure requires a growth chart in which a given centile cut-off is chosen as a balance between high detection rate and affordable false-positive rate [18]. The most widely used cut-off is the 10th percentile of EFW, as suggested by the WHO and the American College of Obstetricians and Gynecologists too [1,18].

The newborn's gestational age is determined by the first day of the mother's last menstrual period and the embryonal size determined during the early ultrasound examination. IUGR newborns are distinguished from SGA (small for gestational age) newborns by being thin, with reduced subcutaneous fat tissue. Their mortality rate is 3-4 times higher, their morbidity

rate is also higher by 4-5% related to healthy infants. Mild or medium severe CNS damage, lack of glycogenic and fat deposits of the liver and the heart, make them susceptible to intrauterine hypoxia and uterine death as well as postnatal hypothermia and hypoglycaemia. Their postnatal adaptational ability is reduced [19]. The IUGR fetuses are less agile and less responsive to external stimuli, and their average heart rate is decreased [20]. With the severity of IUGR, the risk for premature birth, fetal distress, newborn hypoglycaemia (minimum plasma glucose <40 mg/dl), hypocalcaemia (minimum Ca <7 mg/dl) and polycythaemia (maximum capillary hbg ≥21 g/dl) increases. There is no conclusive evidence that this relationship would vary depending on the various etiologic factors [21]. IUGR complicates about 8% of pregnancies [22], and in some cases maternal factors play a role in the pathogenesis: age, body height, body weight, weight gain during pregnancy, vascular status, genetic abnormalities, uterine hypoplasia, uterine malformations, antiphospholipid syndrome, ethnicity, stress, medication, infection, chronic hypoxia, abnormal blood glucose, teratogenic noxa, previous pregnancy complicated by IUGR, drugs-, alcohol-, nicotine abuse. Growth restriction can be caused by fetal factors: congenital anomalies, chromosomal abnormalities [22-23], or by placental malformations: partial abruption, infarction, chorioangioma, small mass, small surface, calcification, cyst, abscess, and tumor. In addition, umbilical cord malformations may also be present: reduced umbilical circulation, umbilical cord knots, edema, compression, pathological adhesion to the placenta, inadequate length, cyst, teratoma, aneurysm, haematoma [24].

3.2. Gestational Diabetes

Apart from the IUGR study, another patient group, the gestational diabetes mellitus (GDM), was also considered important in the study of placental circulatory disorders. GDM affects approximately 14% of pregnancies and the mother's chance for type 2 diabetes ranges from 2.6 to 70.0% within 28 years postpartum [25-26]. The postprandial blood glucose value continues to increase during pregnancy while the insulin sensitivity decreases. To keep the blood glucose normal, the pancreas increases insulin production and increased insulin level increases insulin resistance [27]. The daily sugar requirements of the placenta and the fetus reach 150g in the third trimester. The glucose transport through the placenta is directly proportional to the maternal blood sugar level, and the elevation can increase the transport flow by up to five times through the GLUT-1 transporter [28]. Infant respiratory distress

syndrome (IRDS), cardiomyopathy, hypoglycaemia, hypocalcaemia, hypomagnesaemia and polycythaemia [29] are more common in fetuses exposed to high blood sugar level.

There are many, noncorresponding data regarding the prevalence of GDM in Hungary. In a populationbased screening program, 75 g OGTTs were offered to all pregnant women between 24–28 weeks of gestation and evaluated according to WHO criteria. In that study 8.7% of pregnant women were diagnosed with GDM, and the risk increased linearly with maternal age. Women with the highest BMI (≥ 29.2 kg/m2) had decreased risk compared to women with a BMI of 26.1–29.1 kg/m2, and percentage of body fat >90th percentile, caesarean section, and cord C-peptide >90th percentile. The first line of management of women with gestational diabetes is medical nutrition therapy and a given minimum of exercise. Patients who fail to maintain normal glycemic values via diet and exercise therapy receive insulin [30]. As umbilical cord vessels represent a suitable model for the study of vascular alterations brought about by GDM, the aim of the present work was to compare the ultrasonographic vascular flow measurements to pathological microvascular changes, and also to test the vasoreactivity of the vessels. The selected agents were oxytocin, which is present naturally at the time of pregnancy, and vasopressin, an oxytocin receptor agonist. Oxytocin and vasopressin are both peptide hormones, and oxytocin is widely used for the augmentation of contractions in labor in clinical practice. The effects of these peptides are mediated via transmembrane receptors. Both the oxytocin receptor (OTR) and the V1a vasopressin receptor (V1aR) are expressed in human myometrium. The expression of OTR is significantly higher in gravid uterus while V1aR expression is not significantly elevated compared to non-gravid uterus [31]. Oxytocin gene and receptor expression have also been shown in human chorion, deciduas and amnion.

3.3. Placental circulation

The functional and structural integrity of the microcirculation of the placenta is essential for the satisfactory functioning of intra-uterine transport of nutrients, gas and metabolites [32]. These transport processes are crucial for proper growth and maturation of the fetus [33]. The size, weight and shape of the placenta can vary within wide extremes [34], and the nutritional transport rate is proportional to the placental size [32-34]. The relationship between placental morphometry and unfavorable perinatal outcome is known [35]. The smaller size [36], the decreased surface [37] and the reduced volume [38] correlate with the prevalence of IUGR.

The placenta is gradually growing during pregnancy [39-40], but its mass and volume in IUGR pregnancies is below normal value [41-42]. The placental ratio (placental weight / newborn weight) was first determined by William A. Little, with normal value between 0.10 and 0.18 [43]. In the literature we can find data that a smaller placenta can functionally compensate for its size and the placenta of IUGR infants can have almost normal mass and volume by the time of delivery [44-48]. With ultrasound, the volume of the placenta can be measured and the growth restriction of the placenta can precede fetal growth restriction by weeks [49]. Pathological lesions affecting the placental vessels may further complicate the circulation (calcification, reduced capillarisation, decreased cytotrophoblastic proliferation, chronic vascular stenosis, infarction, fibrin deposition) that can prevent fetal development, although a single pathologic alteration does not usually cause severe IUGR and in milder forms may be present in healthy pregnancies too [50-51].

3.4. Ultrasonographic examination of the placenta

3.4.1. 2-dimensional ultrasonography

The functionality of the placenta is characterized by the Doppler flowmetry of the uterine arteries, umbilical arteries and arteria cerebri media, and fetal biometry is used to monitor intrauterine fetal growth. The flow velocity in the umbilical arteries shows low resistance in the third trimester of healthy pregnancies [52]. The increase in the resistance of the umbilical artery is a sign of circulatory insufficiency, the rate of increase in vascular resistance correlates with the damage of tertiary villi int he placenta. In the case of IUGR fetuses, the velocity of uterine artery and umbilical artery flow is abnormal; the decreased, but positive (PED), absent (AED) or reversed (RED) end-diastolic flow are tipical signs of poor fetal growth [53].

For IUGR fetuses, amount of blood flow through uterine artery at a time (UtBF) and its calculated value for fetal weight (relUtBF) are significantly lower. These circulatory differences also exist in mid-term pregnancies when no other signs of intrauterine growth restriction are observed. Uterine pulsatility index (UtPI) is always reduced with abnormal UtBF and relUtBF. However, abnormal UtPI does not necessarily mean abnormal intrauterine circulation and some of the fetuses are born with normal weight and are healthy. This implies placental compensatory factors with the fact that they have not shown a close correlation

between blood flow through uterine arteries and umbilical arteries during a time unit. IUGR still remains a diagnostic challenge, since ultrasonographic biometry has only a 50% detection rate [53-54]. Most studies in literature have compared the data of ultrasonographic flow measurements with the microscopic ultrastructural differences of the placenta [53-55] or the mass of the newborn with the macroscopic form and size changes of the placenta [56].

3.4.2. 3-dimensional ultrasonography

For 2-D, 3-D and color Doppler examinations Voluson 730 ultrasound equipment (GE Medical System, Kretztechnik, Austria) and RAB 2-5 MHz convex transducer was used. The vascularisation of placentas was assessed in the second and third trimester of pregnancies complicated by GDM, IUGR and normal pregnancies using 3-DPD technique. For „placental vascular biopsy" we applied the *„Mercé-type sonobiopsy"* at insertion of the umbilical cord, the most vascularised part of the placenta. The stored 3-D volume images were analyzed via VOCAL program pertaining to the computer software 4-D View (GE Medical Systems, Austria, version 10.4). The VOCAL program calculates automatically the indices from gray-scale and color values of the acquired spherical sample. The vascularisation index (VI, the volume occupied by vessels in a particular tissue segment), the flow index (FI, the amount of blood flowing through the examined volume in a given time) and the vascularization flow index (VFI, derived from the combination of the previous two indeces) can be asessed to examine the functional capacity of the placenta. The value of these indeces does not change during normal pregnancy, the development of the vascular network is proportional to the placental growth. In case of IUGR pregnancies with AED in the umbilical cord artery, all three parameters, while in IUGR pregnancies complicated with PED only the VI and VFI were significantly lower [57]. This is due to the lack of elasticity of the arteries, the insufficient capillary network with variable wall thickness [58-59].

3.5. Pharmacology

3.5.1. Oxytocin and Vasopressin

Since the placenta and umbilical cord does not have an autonomic innervation, the tone is mediated by humoral factors. Mast cells along the blood vessels are potent sources of these factors [60]. Such regulative vasoactive substance may be oxytocin (OT), which is physiologically present during pregnancy and passes through the placenta [61] and arginine-

vasopressin (AVP). Both are peptide hormones, they elicit constriction via transmembrane receptors [31]. The expression of the OT gene and receptor (OTR) can be detected in the epithelial cells of chorionic, deciduous and amniotic cells after birth. Structural matching between the two peptides (approximately 80%) and the homology of the OT and AVP receptors may result in cross reaction [62]. The expression of the vasopressin receptor 1a (V1aR) mRNA is known in sheep's placenta, but not proven in human, and the absence of vasopressin mRNA is also known [63]. Pharmacological studies disclose controversial data regarding vasodilator function of these receptors and ligands in the vascular wall. Only the presence of the OTR gene could have been demonstrated in the umbilical vein [64]. Other studies, however, suggest the type 2 vasopressin receptor in the background of vasodilation [64-65]. According to our best knowledge and information, the presence of these receptors in the umbilical cord arteries has not been studied and the description of the fetal origin of the ligand exists only in hypothetic form.

3.5.2. Serotonin and Ketanserin

In in vitro perfused umbilical arteries from uncomplicated term pregnancies, serotonin induces a dominating pressure increase usually preceded by a transient vasodilatation [66]. It has been established that serotonin stimulates both, contraction and relaxation of blood vessels [67-69]. Due to the efforts made to identify the serotonin receptors involved in vasoregulation, thirteen different mammalian G-protein coupled 5-HT receptor types have been identified by molecular cloning, which have been grouped into seven families [70]. Among the five 5-HT receptor subtypes, 5-HT1A to 5-HT1F, inhibit adenylate cyclase activity. The three 5-HT2 receptor subtypes, 5-HT2A to 5-HT2C, stimulate the hydrolysis of phosphatidylinositol. 5-HT4, 5-HT6, and 5-HT7 receptors enhance adenylate cyclase activity. No functional coupling has yet been described for the 5-HT receptor subtypes [71]. 5-HT 3 receptors, which are receptorchannel complexes, may also represent a heterogenous group [72]. Using organ bath experiments, it has previously been demonstrated that 5-HT2C-like and 5-HTl-like receptors induce vessel relaxation in an endothelium-dependent fashion [73-76]. Other 5-HTl-like receptors were reported to trigger relaxation of blood vessels independent of the presence of an intact endothelium [77]. Smooth muscle contraction is induced independently of the endothelium by activation of 5-HT2A receptors [67,69]. It is clear that serotonin regulates vasoconstriction and vasorelaxation in a complex way which

involves the interaction of several serotonin receptor subtypes with conflicting functional effects. In the absence of highly specific receptor ligands, it has become much more difficult to characterize pharmacological responses as being mediated by a certain 5-HT receptor subtype. The molecular analysis of 5-HT receptor mRNA expression in vascular tissues revealed that only five of the 13 known G-protein coupled 5-HT receptor mRNAs are expressed in blood vessels (5-HT1Dp, 5-HT2A, 5-HT2B, 5-HT4 and 5-HT7) [78]. 5-HT2A receptor mRNA was shown to be expressed in rat aortic smooth muscle cells [79] and 5-HT 7 mRNA in human coronary artery [80]. The expression of 5-HT2B and 5-HT4 receptor mRNAs in blood vessels had not been proven before (*Table 1)*.

Receptor Subtype	**Location**	**Physiological Action**	**Agonist**	**Antagonist**
5-HT1A	Raphe nuclei, Hippocampus, Cholinergic heteroreceptor in myenteric plexus	*Inhibit adenylate cyclase and activate receptor operated* K^+ *channel. Inhibit voltage gated* Ca^{2+} *channel* Neuronal inhibition, Facilitate Ach and nor adrenaline release, Cholinergic nerve terminal in myenteric plexus, Hyperphagia	Buspirone Ipsapirone Flesinoxan Quetiapine	Spiperone Sibutramine
5-HT1B	Subiculum substania nigra, Vascular smooth muscle	*Inhibit adenylate cyclase* Control release of Ach and nor adrenaline, Contraction of vascular smooth muscle	Sumatriptan Ergotamine	Methiothepin Cynopindolol
5-HT1D	Cranial blood vessel, Vascular smooth muscle	*Inhibit adenylate cyclase* Vasoconstriction of intracranial blood vessel smooth muscle	Sumatriptan Zolmitriptan Nortriptan Ergotamine	Methiothepin Ergotamine
5-HT1E	Cortex striatum, m-RNA in vascular tissue	*Inhibit adenylate cyclase* Unknown	5-HT	Methiothepin
5-HT1F	Spinal cord hippocampus, Uterus, mesentery, vascular smooth muscle	*Inhibit adenylate cyclase* Trigeminal (V) neuro inhibition in guinea pig and rat	No selective agonist or antagonist are available	
5-HT2AD	Cerebral cortex, GI, vascular and bronchial	*Phospholipase C activation*	α-methyl 5-HT	Ketanserin Cyproheptadi

Receptor Subtype	Location	Physiological Action	Agonist	Antagonist
	smooth muscle, platelets	Neuro excitation, Broncho constriction, Platelet aggregation, Smooth muscle contraction	5-CT Sumatriptan	n Pizotifin Methylsergide Risperidone Olanzapine Clozapine
5-HT2B	Cerebellum, hypothalamus, Vascular endothelium, stomach	*Phospholipase C activation* Endothelium dependant vaso relaxation via NO production and stomach fundus contraction	5-CT Sumatriptan BW723C86	RS127445 SB204741
5-HT2C	Choroid plexus, hippocampus, hypothala-mus	*Phospholipase C activation* Modulation of transferin production and modulation of CSF volume	α-methyl 5-HT 5-CT Quipazine	Methylsergide Olanzapine Mesulergine
5-HT3	Area postrema, Abdominal visceral, Afferent neuron	*Ligand gated ion channel* vomiting by vagal neuro excitation, Stimulate nociceptive (pain mediating) nerve ending led to pain	2-me5-HT	Ondensetron Tropisetron Granisetron
5-HT4	Hippocampus, GIT	*Activation of adenylate cyclase* Neuronal excitation, Increase GI motility	Mosapride Cisapride Zacopride	GR113808 SB204070
5-HT5A	Olfactory bulb, Hebenula	Unknown	No selective agonist or antagonist are available	
5-HT5B	Olfactory bulb, Hebenula	Unknown	No selective agonist or antagonist are available	
5-HT6	Caudate putamen, Hippocampus, Superior cervical ganglia	*Activation of adenylate cyclase* Modulation of CNS Ach release	No selective agonist available	SB271046 Methiothepin
5-HT7	Hypothalamus, Gastrointestinal and vascular smooth muscle	*Activation of adenylate cyclase* Smooth muscle relaxation	5-HT Sumatriptan 8-OH DPAT	SB258719 Methiothepin

Table 1. Serotonin receptor subtypes and physiology [81, shortened]. (8-OH DPAT: 8-Hydroxy-2-(di-n-propylamino) Tetraline; 5-CT: 5 Carboxamidotryptamine; CSF: Cerebrospinal Fluid; HT: Hydroxytryptamine)

The human umbilical artery (HUA) is a unique mammalian artery that lacks autonomic innervation [80-82]. Thus, the vascular tone of HUA that modulates fetoplacental circulation, is regulated by local mediators such as prostaglandins and 5-HT or some ions such as potassium and calcium [79-80]. 5-HT produced contractions through the release of calcium from intracellular stores have been reported in the absence of extracellular calcium. It is consistent with our observation that 5-HT induced cumulative concentration-dependent contractions decreased significantly in the absence of calcium [80].

Serotonin is a potent regulatory factor in foetoplacental circulation and it can also be found physiologically in measurable quantity in the umbilical cord blood. Its contractile effect was confirmed in both normal and preeclampic pregnancies. The effect on vasoconstriction was not altered if the pregnancy was treated with prednisolone, azathioprine, acetylsalicylic acid, propranolol, furosemide, nifedipine, labetolol, indomethacin or cyclosporine-A [83]. Its dose-effect curve is associated with mild vasodilation, which disappears at a dose of 10^{-7}M and is dominated by contraction afterwards [84-85].

Ketanserin is a selective 5-HT2a antagonist but shows measurable affinity for the 5-HT2c receptor, α1 and α2-adrenergic receptors, and 5-HT1d, 5-HT2b, 5-HT6, and 5-HT7 receptors. It is used in pre-eclampsia for the treatment of maternal hypertension, as it has only peripheral vascular effect, it has no chrono-, dromo- or bathmotrop effect. The optimal therapeutic dose has not yet been determined [80, 86]. It has a half-life of 12-48h, and it is transported through the placenta fairly enough to develop its effect on the fetal circulation [87].

4. Aims and hypotheses:

Our objectives were to investigate the fetoplacental circulation and the vasoregulation of the umbilical and placental vessels in healthy pregnancies and pregnancies complicated by intrauterine growth restriction and gestational diabetes. Our hypothesis is that in case of placental insufficiency and compromised fetal growth a misregulation of vascular resistance and vascularization leads to the unfavourable neonatal outcomes. In order to proove our hypothesis we assessed the following data and investigated their relationship:

1) We asessed ultrasonographic biometry, flowmetry and 3-dimensional ultrasonographic indeces of the afore mentioned pregnancy groups. Ultrasonographic data was compared to blood vessel response to vasoactive agents.
2) We investigated whether the vasoreactivity to the investigated agents is altered in the fetoplacental circulation in pregnancies complicated by IUGR or GDM.
3) We investigated the pathological and histological alterations in the placenta and umbilical cord, whether these findings can affect or be a result of compromised vasoregulation and may lead to restricted fetal growth.

5. Materials and Methods

5.1.Patient recruitment and ultrasound examination

We recruited pregnant patients at the Department of Obstetrics and Gynecology, University of Szeged, between January 2014. and May 2017. Patients have read and signed the Informed Consent (Ethics Registry Number: 49870-3773 / 2014 / EKU 586) after which estimated fetal weight was determined by Hadlock 'B' formula [16]:

$$Log10\ EFW = 1{,}335\text{-}0{,}0034\ ACxFL + 0.0316BPD + 0.0457AC + 0.1623FL$$

IUGR and control group were set up based on the EFW, in the IUGR group the estimated fetal weight in the 2nd and 3rd trimester of pregnancy was below the 10th percentile. The weight percentile value established on the basis of a gender-specific percentile curve recommended by the International Society of Ultrasound in Obstetrics and Gynecology [88]. Pregnant women who had been suspected for IUGR based on EFW but a newborn with birthweight over the 10th percentile was delivered, were excluded from the study. Additionally, premature deliveries, newborns with genetic malformation, chromosomal or developmental disorders, pregnancies complicated by hypertension (> 140/90 mmHg), diabetes (fasting blood glucose up to 24 weeks,> 6.9 mmol / l) were excluded from the control and IUGR group.

The GDM group consisted of pregnant patients diagnosed at the 24-28th week 75 g oral glucose tolerance test based on the WHO diagnostic criteria [89]. All diabetic pregnant blood glucose levels were controlled by diet, those who needed insulin therapy were excluded from this study. None of the recruited patients had immunologic, cardiovascular, gastrointestinal or pulmonological disease. Patients with twin pregnancy, history of habitual abortion or assisted reproduction, fetal developmental malformations were excluded.

All ultrasonographic measurements were performed by the same examiner to eliminate interobserver errors. The intraobserver errors were evaluated by repeated measurements of the 3-DPD indices at the initiation of the study. All pregnancies were examined in a semirecumbent position with "Obstetrics / 2-3 trimester" in 2D mode. The fetal biometry consisted of biparietal diameter (BPD), frontooccipital diameter (FOD), head circumference (HC), abdominal circumference (AC) and femur length (FL). Conventional color Doppler assay was used to determine the flow values of the umbilical artery. 3D power Doppler

Ultrasound volume image was made at the placental insertion of the umbilical cord. We performed the Mercé type sonobiopsy to calculate the flow index (FI), the vascularization index (VI), and the vascularization flow index (VFI) by VOCAL program.

The data on delivery, birth mode, duration of labour and gestational weeks were recorded. We asessed the Apgar values, body mass and body length of the newborns, from which Rhohner's ponderal index can be calculated with a normal value between 2.2 and 2.9. Patients assigned in the IUGR group who delivered normal weight newborns were excluded from the study.

5.2.Pharmacologic studies:

The umbilical cord and placenta were immediately placed into 1000ml Krebs-Henseleit buffer solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 2.5 mM $CaCl_2 \cdot 2H_2O$, 25 mM $NaHCO_3$; 11.7mM dextrose), at pH 7.4 and 4°C. The cord was removed from the placenta and perfused with the solution. The cold nutritive solution slowed the metabolism of endothelial cells and prevented postpartum blood clotting. The buffer was freshly prepared every week and stored at 4°C. For the tissue bath experiments fresh buffer was prepared and heated up to 37°C. The tissues were taken to the Department of Pharmacodinamics and Biopharmacy, Faculty of Pharmacy, University of Szeged and the examination took place within 24 hours after delivery. The umbilical vessels were dissected from a 6 cm section at the placental end of the cord. The placental vessels were identified at the cord insertion and we introduced a rat central venous cannula during preparation to follow up the vessels to the periphery. All vessels were cut into 3-5 mm long segments and mounted on stainless steel hooks *(Figure 1)*.

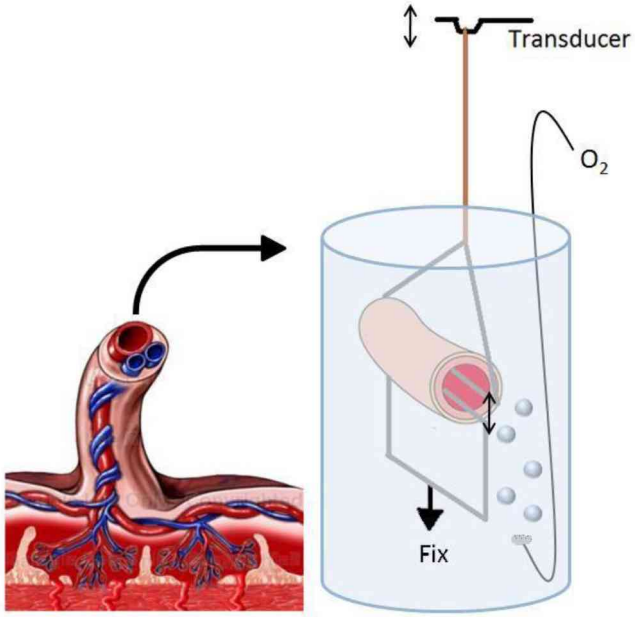


Figure 1. The schematic drawing of the tissue bath chamber.

The isolated tissue bath computer complex consists of five main parts; eight chambers and buffer tanks, with a thermostat, amplifier and transducer, a gas cylinder, pressure regulator with carbongen gas (95% O_2 + 5% CO_2) and a computer unit. During incubation the umbilical cord vessels (dissected from Wharton's jelly) were placed in chambers number 1-2 (arteries) and 3-4 (veins), placental vessels in number 5-6 (arteries) and 7-8 (veins) filled with freshly prepared Krebs-Henseleit solution at 37°C and marched to 2g initial tension, bubbled with carbogen gas. During the incubation, tissues were washed through with fresh solution in every fifteen minutes and reached equilibrium within 60 minutes, so that the spontaneous vessel tones would stabilize, and logarithmic single dose levels (10^{-10}M-10^{-7}M) of oxytocin (Sigma-Aldrich O3251) or desmopressin (vasopressin analogue, Sigma-Aldrich D0650000) was added to the system. The buffer solution was exchanged between each doses. The time elapsed between the doses was calculated on the basis of the half-life of the active substance, 15 minutes in case of oxytocin.

In the second stage of our work under similar conditions, serotonin (Sigma-Aldrich H9523) was added to the vessels in the same pattern as oxytocin. We administered serotonin in logarithmic concentration (10^{-9}M-10^{-5}M) in every 6 minutes without washouts. To isolate receptor subtypes, a new study was performed by incubating the blood vessels with 10^{-8}M ketanserin (Sigma-Aldrich S006) for 6 minutes and testing for the cumulative dose-response

curve of the aforementioned serotonin. In the 10^{-9}M-10^{-5}M range of ketanserin, we found that 10^{-8}M concentrations alone did not affect the tone.

5.3. Pathological examination:

The volume of placenta was determined by water displacement method. The tissues were assigned a numeric code after delivery and restored for futher examinations.

The pathological study was performed after 3 to 7 days of formalin fixation, measuring the placental weight and volume according to the Royal College of Pathologists Guideline [90]. After measuring the diameters, the placenta was cut into 1 cm thick stripes along the longest diameter and its thickness was determined at the umbilical cord insertion. We prepared histological samples from the placental end of the umbilical cord, cut 4mm slices perpendicular to the longitudinal axis. The slices were dehydrated with ethanol and embedded in paraffin. 4 micrometer of paraffin slices were cut and deparaffinized, rehydrated, and after hematoxylin-eosin (HE) staining evaluated under an Axio Vision SE64 Rel. 4.9.1. microscope. We digitally recorded the longest cross-sectional diameter of the cord, the cross-sectional area of blood vessels and the thickness of the vessel wall. Due to the coiling of the umbilical cord, the vascular image was never a perfect mathematical cross section, therefore the arterial wall was measured at the following locations: the most and the least distorted location and a third measurement was performed at 3/6/9 o'clock depending on the previous two data. The measurements were done blindly, the samples wre identified only by their numeric code. Histological samples of the placenta (3mm x 10mm x 20mm) were taken from the umbilical cord insertion, the peripherial area, the maternal and the fetal side, and from the pathological alteration, if there were any.

5.4.Statistical analysis:

When comparing maternal and fetal parameters with normal distribution, Student's t-test was used. The total thickness of the umbilical cord, the vessel wall thickness and the various lumen shapes and the dose-response curve of the active compounds were analyzed by ANOVA. The area under the curve (AUC) was determined by the ISOSYS Program software (SOFT-02-ISO S.P.E.L. ADVANCE ISOSYS, MDE Kft. 1062 Budapest) at each dose and each vessel. The AUC data were then transformed to csv files and in Microsoft Excel program the relationships of AUC values were determined by the

*(-fx=(f3/f3)*100)-100*

formula.

Data showing non-normal distribution were analyzed with Kruskall-Wallis probe with Bonferroni correction. Differences were considered significant when $p < 0.05$. Linear regression was studied between placental morphometry and newborn parameters. The Pearson's correlation was judged if two variables co-varied; r can vary from −1 (perfect negative correlation) through 0 (no correlation) to +1 (perfect positive correlation). If r was over 0.5 it was considered to be strong; moderate if it was between 0.3 and 0.5; and weakened if it was below 0.3. For statistical analysis, Prism 6 (2016, Graph Pad Software Inc. La Jolla, California, USA) software was used.

6. Results

6.1. Results of IUGR/GDM/control study with Oxytocin and Desmopressin

The age of the pregnant patients (mean ± SD) in the control group was 32.17 ± 1.8 years, IUGR was 29.75 ± 1.1 years and 34.2 ± 2.2 years in the GDM group without significant difference. The number of previous pregnancies in the control group was 1.00 ± 0.4, in the IUGR it was 1.75 ± 0.5 and 2.80 ± 0.4 in the gestational diabetic group ($p = 0.0076$), of which the number of births in the same order 0.83 ± 0.3; 0.75 ± 0.3; and 1.80 ± 0.8. There were two IUGR pregnancies in the history of the diabetic group, and no such preceding events in the other two groups. There were no significant differences between medications and drugs taken during pregnancy, and proportion of those who did not take any medication. Among the mentioned medications were Augmentin, Elevit, Femibion, Magne B6, Maltofer, Neo-ferro-folgamma, and Noacid in one month prior to delivery. In the delivery room the caesarean

section frequency was 17% in the control group, 75% in the IUGR group and 40% in the GDM group. *Table 2* summarizes the data of the newborns. Although the physical parameters of the IUGR newborns differed more from the controls (CTRL), the Apgar scores in the GDM group were worse. The normal value of Rhohrner's ponderal index is between 2.2 and 2.9, below 2.2 is dysmaturity and above 2.9 is macrosomy.

	CTRL (n=:		IUGR (n=10)		GDM (n=5)		p value
	mean	±SD	mean	±SD	mean	±SD	
Birthweight (g)	3221	266.1	**2350***	194.0	**3627#**	363.5	*0.0023 #0.0411
GA (weeks)	39.20	1.23	38.00	1.10	38.22	2.54	ns
Rhohner's ponderal index	2.69	0.25	2.57	0.12	2.85	0.35	ns
1' Apgar	9.46	0.52	**8.25***	1.63	**8.20#**	1.10	*0.0360 #0.0213
5' Apgar	10.00	0.00	9.50	0.29	9.00	1.23	ns
10' Apgar	10.00	0.00	10.00	0.00	10.00	0.00	ns
Umbilical cord pH	7.37	0.02	7.33	0.04	7.37	0.03	ns
Body length (cm)	50.00	0.86	**45.00***	1.16	48.80	0.37	*0.0112
HC (cm)	34.50	0.43	**31.75***	0.48	34.00	0.58	*0.0030
AC (cm)	32.17	0.75	**28.50***	0.64	33.00	1.05	*0.0088

*Table 2. Data of the newborns. * p≤0.05 (CTRL and IUGR), # * p≤0.05 (CTRL and GDM)*

In all pathological histological studies, 3 vessels were found throughout the length of the cords, the data of the 3-7 days formalin-fixed samples were compared in *Table 2*. Typically, the cords are twisted counterclockwise. The umbilical coiling index (UCI) was determined by the total number of 360° turns on the 5 cm section. The coiling pattern was determined on the basis of a study published by LM Ernst in 2013 [91]. In the case of umbilical cords from IUGR pregnancies, the undulating pattern was uniformly found, while in the other groups the rope pattern was dominant (*Figure 3*).

	CTRL (n=22)		IUGR (n=10)		GDM (n=5)		p value
	mean	±SD	mean	±SD	mean	±SD	
Cord length (cm)	58.00	9.15	62.50	12.50	59.60	7.13	ns
UCI	1,43	0,77	**0,33***	0,30	2,00	1,00	*0,0418
coiling direction (left, %)	66.00		100.00		80.00		ns
Cord cross-sectional area, via ultrasonography (cm^2)	1.24	0.35	1.05	0.01	**0.94#**	0.10	#0.0027
Vascular cross-sectional area (mm^2)	4.26	1.01	**2.37***	0.07	3.69	0.50	*0.0058
Wharton's jelly/ vascular cross-sectional area ratio	10.97	1.86	8.17*	0.52	5.78#	1.92	*0.0046 #0.0056
Placental weight (g)	463.80	56.77	458	4.69	535.40	78.53	ns
Birth weight/ placental weight	7.38	0.76	**4.71***	0.56	6.29	0.56	*0.0112

*Table 3. Pathological examination of the placenta and umbilical cord. * p≤0.05 (CTRL and IUGR), # * p≤0.05 (CTRL and GDM)*

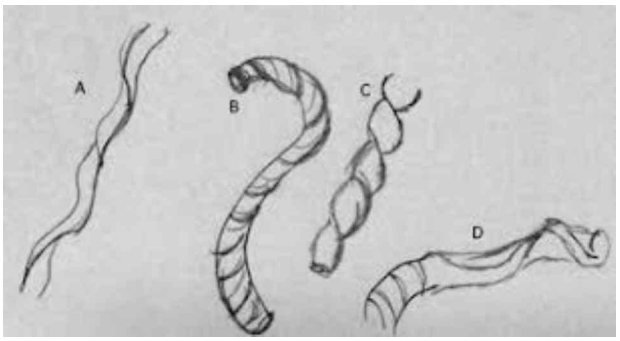


Figure 2. Schematic drawing of rhe umbilical cord coiling patterns. A: undulating, B: rope, C: segmented, D: linked.

In the umbilical cord blood of IUGR fetuses, we could see perivascular haematoma and once haemangioma cavernosum, several monocytes, and mild funisitis and an edemic cord in GDM. There was no significant difference between control and IUGR samples. There was no real knot, stenosis, compression, meconium staining, maceration, thrombosis, or other pathology, discoloration or unpleasant odors. The cross-sectional surface area was compared to the ultrasonographic measurements and it can be seen that although the tissue has shrunk

during formaline tissue fixation, the proportions of the individual groups did not change significantly.

Table 4 shows the data obtained by VOCAL analysis of the 3-D ultrasound scan. The arteria umbilicalis flowmetry (S/D ratio) showed wider variability than the 3DPD indeces.

	CTRL (n=7)		IUGR (n=10)		GDM (n=5)		p value
	mean	±SD	mean	±SD	mean	±SD	
FI	49.16	1.76	**38.48***	1.74	**35.50#**	5.89	*0.0115 #0.0318
VI	10.08	0.49	**4.53***	0.61	**4.90#**	1.85	*0.0004 #0.0136
VFI	4.92	0.16	**2.70***	0.74	**1.83#**	1.01	*0.0194 #0.0069
a. umbilicalis S/D	2.26	0.04	3.01	0.50	2.51	0.08	ns

*Table 4. The 3DPD indeces of the placenta. * $p \leq 0.05$ (CTRL and IUGR), # * $p \leq 0.05$ (CTRL and GDM)*

The umbilical cord vessels (dissected from Wharton's jelly) were placed in Krebs-Henseleit buffer solution and after incubation the spontaneous vessel tone was taken as the reference value. The change in vascular tone elicited by logarithmic non-cumulative oxytocin dose was not significant in the arteries or veins in any of the groups, and the measurement results reflect the dynamic variability of baseline equilibrium. Since no significant difference could be observed among the three groups, the values are represented in one diagram (*Figure 3*). Since oxytocin elicits vascular effect via type 1 vasopressin receptor, a more powerful agonist, desmopressin, was administered during the experiment, but it was again found that there was no significant alteration in vascular tone. Based on our results, there is probably no type 1 vasopressin receptor in the veins of the umbilical cord or is present only in inactive form. The viability of the vessels were tested with contractions elicited by 10^{-5}M Serotonin.

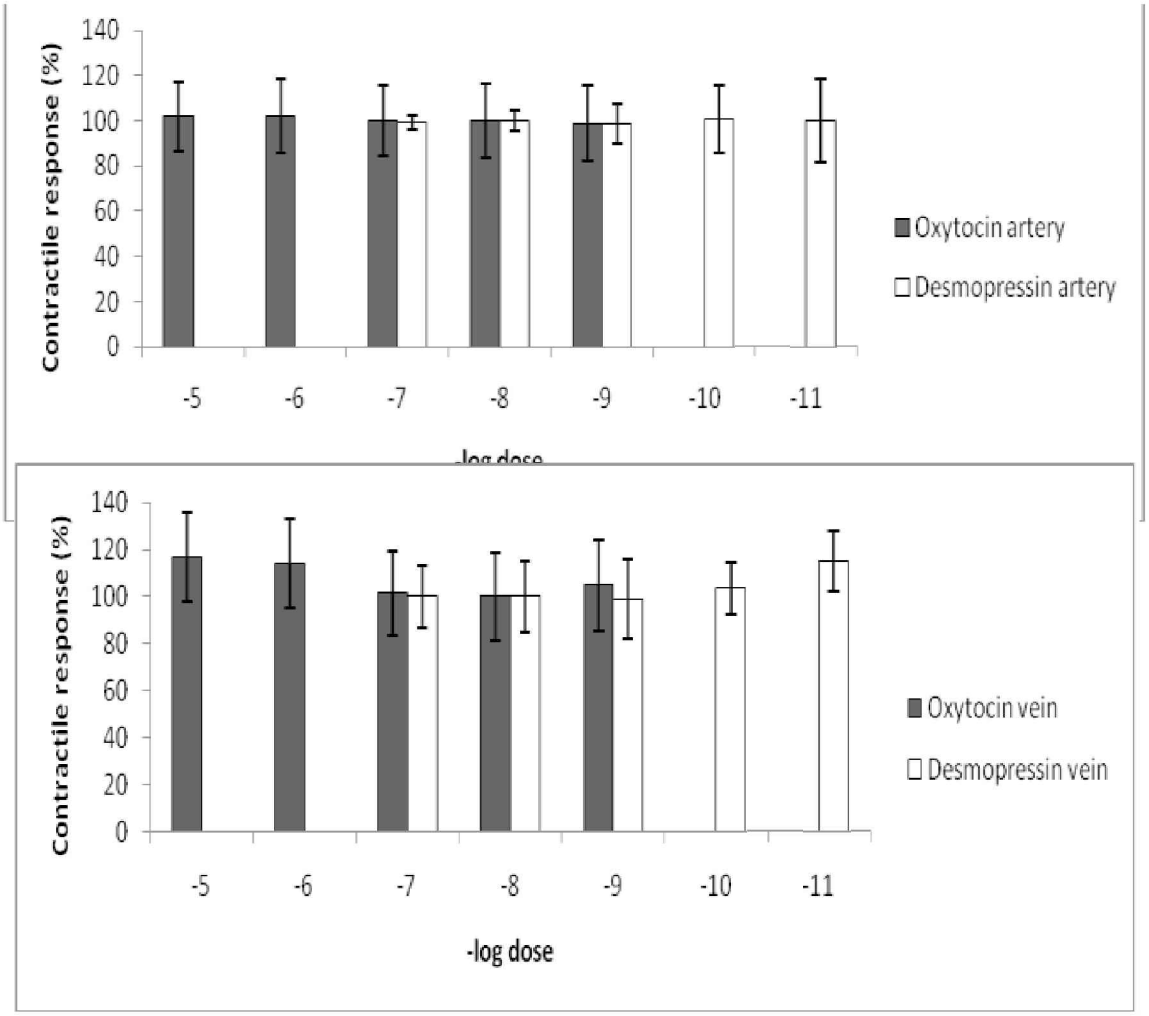


Figure 3. The vascular tone in case of oxytocin and desmopressin administration. Despite the wide range of dosage, no significant change in vascular tone could be observed. (n=37)

6.2. Results of IUGR/control study with Serotonin and Ketanserin

Table 5 shows the clinical data of the two groups. There were no siginificant difference in blood parameters that could alter the blood viscosity meaning that the difference in oxygen and nutrient supply is rather due to vascular permeability regulation. The systolic/diastolic flow velocity in the umbilical arteries of IUGR fetuses were elevated, the birth weight and Apgar scores were lower related to the control (CTRL) group.

	IUGR (n=18)		CTRL (n=46)		p value
	mean	±SD	mean	±SD	
MCV (fL)	84.48	0.96	88.25	1.26	ns
RBC (T/L)	4.07	0.03	4.03	0.10	ns
Hgb (g/L)	123.80	4.48	121.50	2.31	ns
Htk (L/L)	0.35	0.01	0.36	0.01	ns
PLT (Giga/L)	235.50	24.73	197.80	11.81	ns
MPV (fL)	10.65	0.66	12.01	0.32	ns
Prothrombin time (sec)	12.80	0.10	12.93	0.13	ns
INR	0.97	0.01	0.98	0.01	ns
APTT (sec)	33.93	0.26	32.03	0.47	ns
arteria umbilicalis S/D	**3.39***	0.38	2.22	0.11	0.0006
maternal age (year)	29.75	1.11	25.72	3.64	ns
maternal BMI (kg/m^2)	28.48	2.35	22.46	1.83	ns
parity	0.85	0.16	1.48	0.34	ns
Birthweight (g)	**2110.00***	194.0	3367.73	435.04	0.0023
Gestational age (weeks)	37.29	1.10	38.58	1.58	ns
1' Apgar score	**7.71***	12.14	8.38	1.85	0.036
5' Apgar score	8.86	1.46	9.46	1.13	ns
10' Apgar score	19.57	1.13	9.92	0.28	ns
male (%)	50.00	-	45.94	-	ns
female (%)	50.00	-	54.05	-	ns

*Table 5. Clinical data of the two groups. * p≤0.05.*

Both FI and VI are significantly lower in IUGR pregnancies. It is clear from the two data that there is a more prominent difference in the number of vessels in a unit volume, than the blood flowing through a corresponding volume of the placenta in a certain time. Reducing the vascular resistance increases the blood flow through a given volume of the placenta. By reducing resistance, the placenta can compensate hypovascularization to a certain level, a significant reduction in the blood flow still can be observed but not as pronounced as in

vascularization. The umbilical artery S/D ratio did correlate well with the maximum response to serotonin (*Figure 4*).

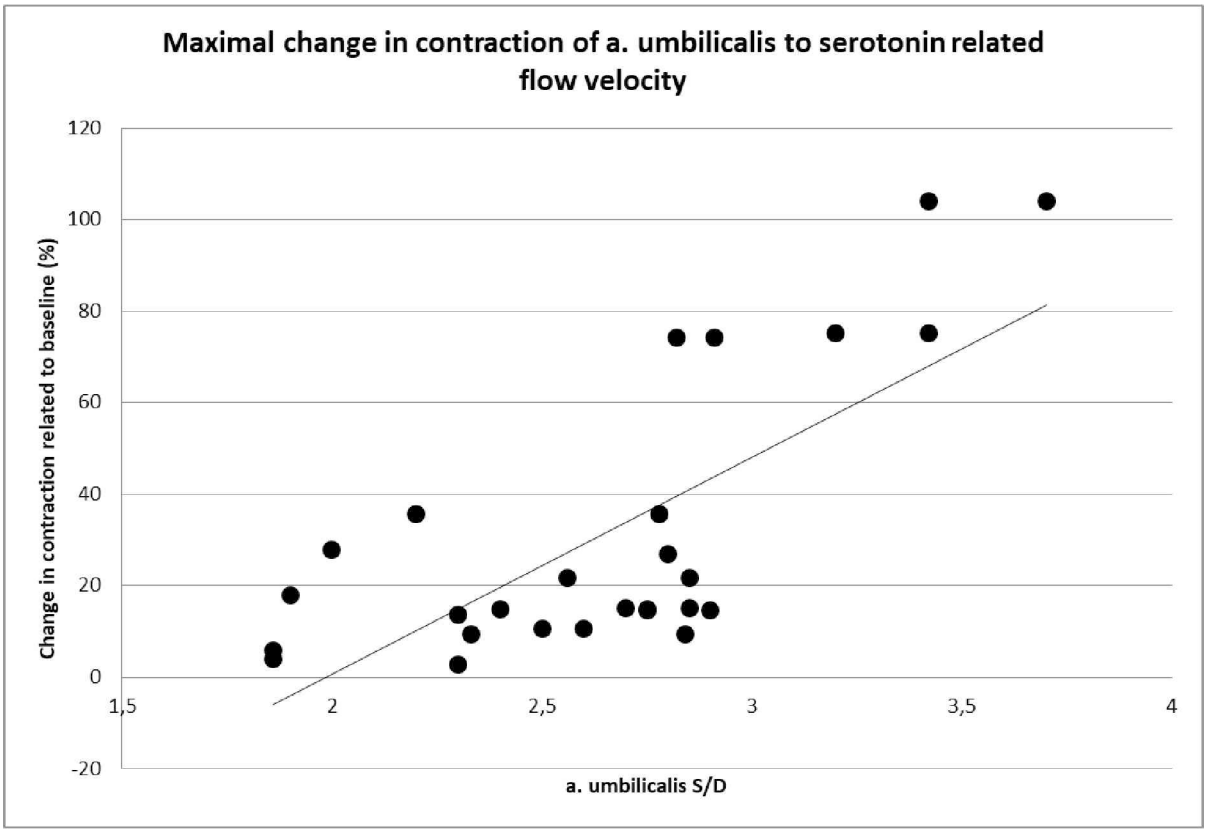
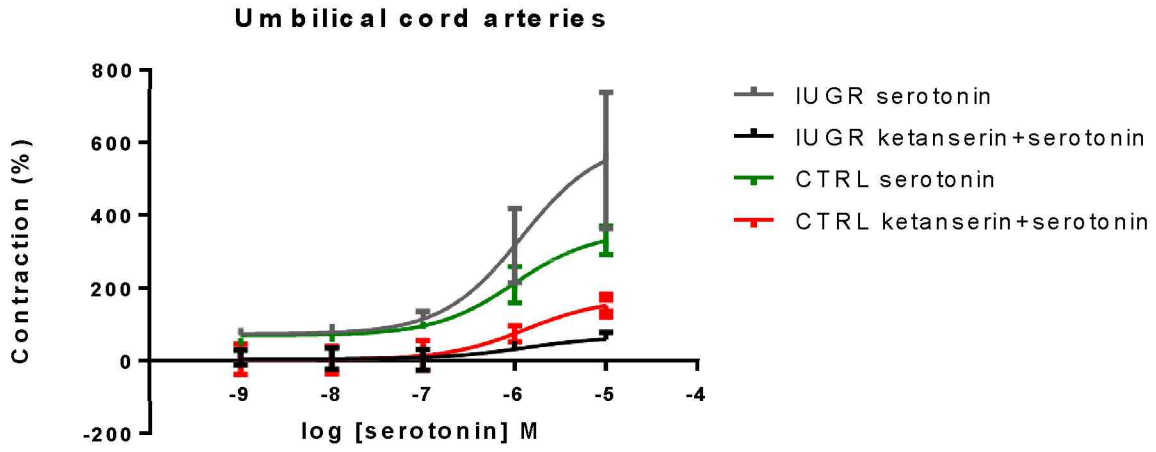


Figure 4. The maximal change in contraction of umbilical arteries elicited by serotonin correlates with the S/D flow velocity measured via Doppler ultrasound in utero. r= 0,5328.

Serotonin is stored in the umbilical cord in perivascular mastocytes. When released into the vessels, it has a vasoconstricting effect on the 5-HT1-2 and 7 receptors, which was evaluated on a total of 240 vessel segments. It elicited significant contraction in control and IUGR umbilical arteries at the concentrations of 10^{-6}M (*Figure 5A*). In case of umbilical veins significant contraction occured at 10^{-7}M concentration while in IUGR at 10^{-6}M (*Figure 5B*). The placental arteries contracted at 10^{-6}M concentrations in controls and only at 10^{-5}M in IUGR (*Figure 5C*). In the placental veins the effect reached significance at 10^{-7}M in controls, but no significant reaction could be obsereved in IUGR (*Figure 5D*). However, ketanserin inhibition results in a significant reduction in contraction and this effect is more relevant in IUGR fetuses (n = 30) than in healthy pregnancies (n = 48). While in the umbilical arteries the

reaction to serotonin without ketanserin incubation does not show significant difference, after ketanserin incubation, the reduction in IUGR vessel reactivity is more prominent and in controls the difference reaches significancy only at 10^{-7}M concentration. In case of umbilical veins the control group shows more contractility over 10^{-7}M concentration, but after ketanserin incubation the reactivity of the IUGR veins is nearly eliminated yet the control vessels show as much reactivity as the IUGR without the antagonist. Both the placental arteries and veins are more contractile to serotonin in the control group but the IUGR vessels differ at only 10^{-7}M and 10^{-6}M concentrations respectively. After ketanserin incubation the responsiveness to the agonsit decrease in both groups, in controls over 10^{-6}M an in IUGR over 10^{-7}M. In case of IUGR placental veins neither the contraction to serotonin nor the effect of ketanserin reaches significance. This difference between the case and the control group may be due to the higher relative density of the 5-HT2 receptor in the umbilical veins of IUGR fetuses, resulting a smaller contraction on the fewer 5-HT1 receptor and reduced effectiveness of ketanserin on the elicited contraction.

A



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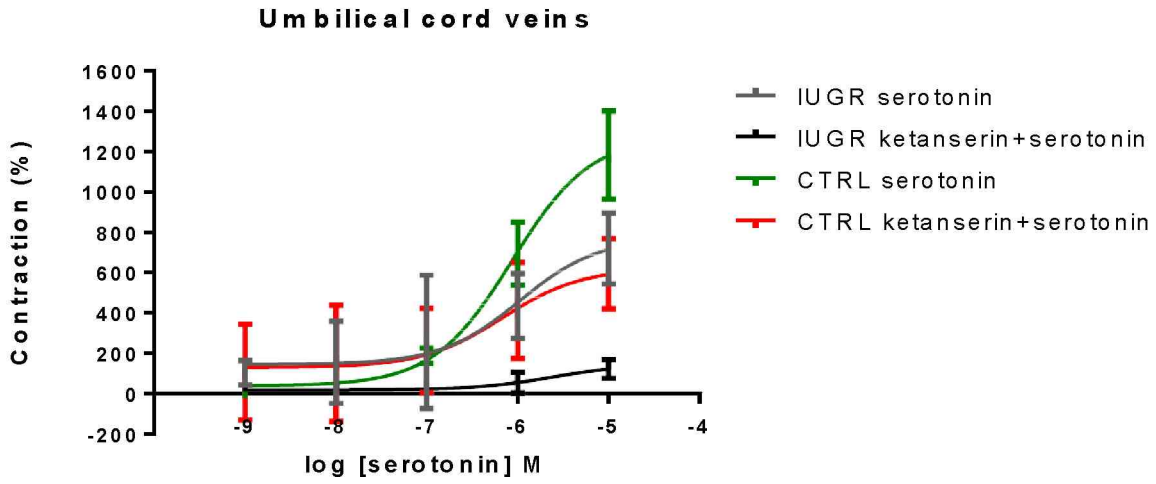
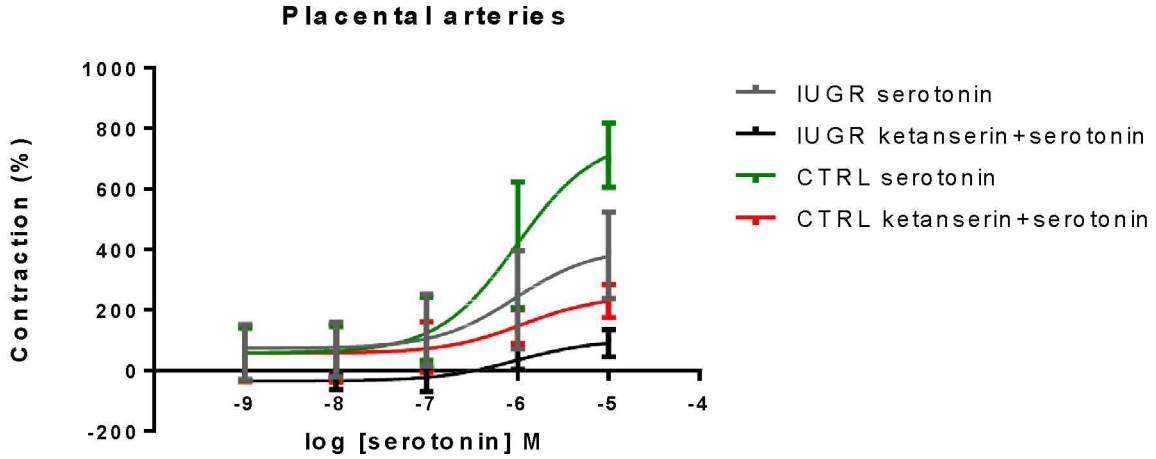


C



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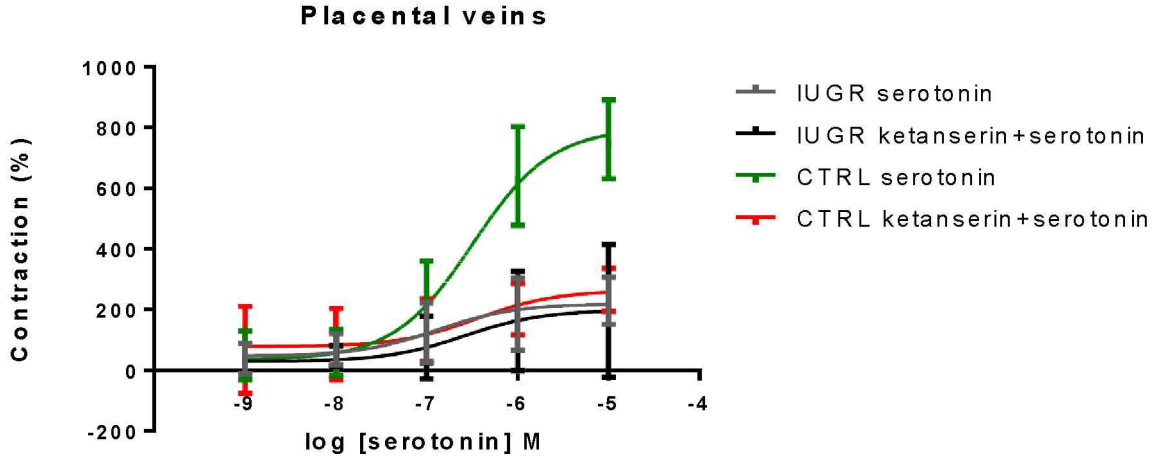

Figure 5. Contraction elicited by serotonin with and without ketanserin incubation.

The morphometric examination of the placenta, showed that the volume measured after birth is more strictly correlated with the weight of the newborn than the mass or volume of the placenta after formalin fixation *(Figure 6)*.

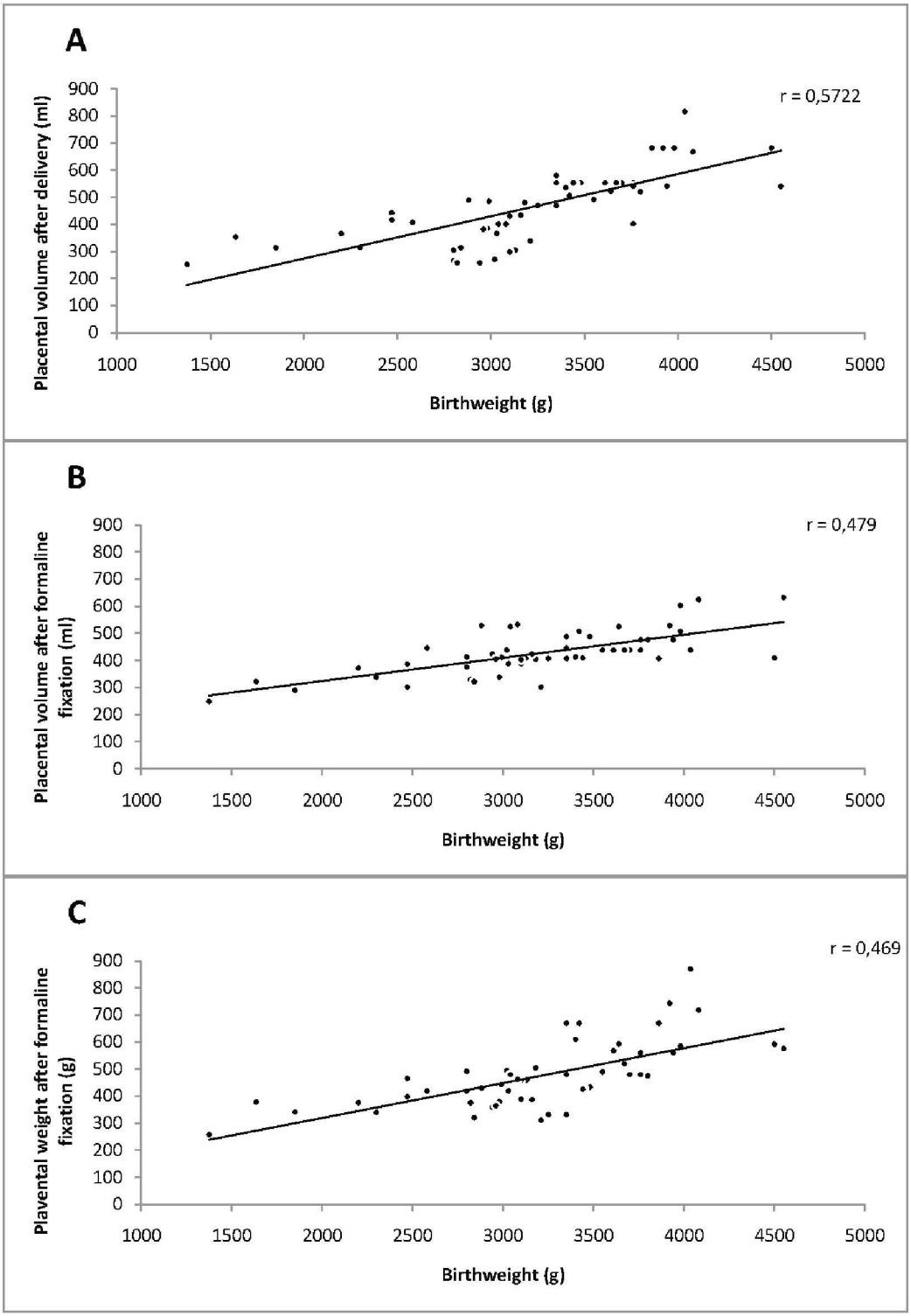

Figure 4. The morphometric examination of the placenta related to birth weight.

	IUGR (n=18)		CTRL (n=46)	
	n	%	n	%
focal calcification	2	11,11	5	10,87
hypovascularized villi	4	22,22	2	4,35
hypoplasia villi	4	22,22	7	15,22
intervillous fibrin deposition	3	16,67	5	10,87
syncytial nod	3	16,67	1	2,17
amnion nodosum	0	0,00	1	2,17
villitis	0	0,00	1	2,17
hematoma	0	0,00	2	4,35
maternal side nonconversion	2	11,11	3	6,52

Table 6. Histopathological alterations in the placenta.

The results of the histological studies are shown in *Table 6* and *Table 7*. On the maternal side of the placenta we observed the so called nonconversion of the arteries, which is similar to the anomaly seen in the uterine artery. In this case, the smooth muscle around the vessel is retained and abnormally increases the resistance. This phenomenon can be seen in uterine artery via ultrasound as a drop in flow velocity between the systolic and diastolic phase (notch). However, these cases did not coincide with the alteration seen in the uterine artery. Arterial cross-sectional area shows significant difference between the two groups and has a slight correlation with birth weight ($r = 0.336$).

	IUGR (n=18)		CTRL (n=46)		
	Mean	±SD	Mean	±SD	p
vena umbilicalis wall thickness (µm)	663.8	41.6	605.4	20.6	ns
vena umbilicalis lumen cross-section (μm^2)	1563000.0	1054000.0	1803000.0	514365.0	ns
arteria umbilicalis wall thickness (µm)	636.4	41.0	678.2	15.0	ns
arteria umbilicalis lumen cross-section (μm^2)	**146825.0***	18369.0	526659.0	77690.0	0.0296
obliterated lumen ratio	0.71	0.29	0.89	0.13	ns
UCI	1.05	0.27	1.52	0.44	ns
Cord cross-sectional area (mm^2)	93.20	8.34	116.10	6.24	ns
number of histological changes of the placenta (described in Table 6)	**2.13****	0.30	0.70	0.11	<0.0001
placental volume (ml)	**315.30****	23.44	492.50	17.95	<0.0001
placental volume after formaline fixation (ml)	**368.80****	23.15	461.50	15.01	0.0018
placental weight after formaline fixation (g)	**401.80***	18.33	468.90	15.49	0.0226
longest placental diameter (cm)	15.50	1.15	15.86	0.20	ns
shortest placental dimaeter (cm)	**13.11**	0.46	14.39	0.29	0.0196
placental thickness (cm)	2.58	0.14	2.71	0.08	ns
placental co-efficient (placental weight/fetal weight)	**0.19****	0.01	0.14	0.01	<0.0001

*Table 7. Histopathological data of the umbilical cord and the placenta. * $p \leq 0.05$, ** $p \leq 0.01$.*

7. Discussion

Perinatal complications, such as necrotizing enterocolitis, low Apgar score, hypoxic brain damage, need for respiratory support, chronic lung disease, retinopathy, and prolonged perinatal intensive therapy are more likely in case of IUGR newborns. 2D ultrasonographic examination of the umbilical arteries showed that the flow velocity curve may be normal in growth restricted fetuses as compared to their gestational age, but its abnormal appearance can be considered as a diagnostic signal for intrauterine growth restriction. Compared to normal pregnancies, the systolic / diastolic flow index remains permanently high in IUGR fetuses and correlates with later complications. The flow rates were similar in our measurements. There is a correlation between abnormal uterine pulsatility index (UtPI) and the early develpoment of IUGR, but some of the fetuses are also born with abnormal UtPI and normal birthweight, and IUGR is also present with normal uterine artery flow [53].

The umbilical artery blood flow of GDM pregnancies showed a normal S/D ratio. Of the 3DPD indices, the VI has been low, so the placenta is hypovascularized related to normal and the blood vessels are damaged by high blood sugar levels. Increased glucose transport leads to the activation of pentose phosphate and NADPH oxidase resulting in excessive free radical formation. The free radical's proinflammatory effect leads to atherosclerosis [92]. Initial ischemic signs include syncytial knot, hypovascularized villus, interstitial calcification, and extravascular fibrin deposition. Their combined, long-term consequence is the increase of their arterial resistance [93]. The humoral vascular tone regulation of the placenta and umbilical cord plays a key role in this process. In case of GDM pregnancies, the umbilical coiling index exceded the control, while the IUGR umbilical cord showed significantly lower number of turns. The thin, hypocoiled cord with less Wharton's jelly is unprotected against mechanical impacts, that makes the fetus and the newborn more vulnerable.

Romani et al. examined the effect of nicotine and its degradation product on umbilical endothelial cells in similar experimental settings and found that storage at 4°C for 24 hours did not alter vasoreactivity [94]. Since direct contraction was observed neither for oxytocin nor for desmopressin, it can be assumed that there is no type 1 vasopressin receptor on the umbilical cord. The vascular rings were contracted with serotonin to confirm their viability. In a placental perfusion model, vasopressin (30pg / ml to 60,000pg / ml) administered on the maternal side was measured at the fetal side with a maximum of 3110 pg / ml, which was

$3.11x10^{-8}$M. The highest concentration we have studied is 10^{-7}M. If we could not contract the umbilical cord in vitro due to decreased receptor number, it can not be contracted with in vivo vasopressin dose because the amount of active substance does not reach the umbilical vessels [95]. Holcberg et al. studied the effect of oxytocin in the veins of meconium stained placentas. While there was no change in basal tone in the normal group, vessel contraction of meconium-impregnated placenta was observed [89].

Maternal oxytocin can pass through the placenta and reach the fetal brain, and induce the hyperpolarization of GABA-ergic neurons in the fetal hippocampus and neocortex during delivery. Reduction of GABA-mediated excitation induced by oxytocin has been demonstrated and completely eliminated by Atosiban [96]. Since hypoxic brain damage is the leading cause of fetal death, the important conclusion is that oxytocin has an inhibitory cortical and hippocampal neuronal effect by which it reduces fetal brain oxygen and nutrient requirements, and therefore it is less sensitive to hypoxia. It is therefore assumed that oxytocin does not bind to the receptors in the placenta without meconuium impregnation, and since there is no receptor in the umbilical vessels, it can exert its hyperpolarizing effect in the fetal brain [96-97].

Serotonin can be found in detectable amount in umbilical cord blood after birth and may also play a role in occlusion of the umbilical vessels [60]. In vascular smooth muscle cells, serotonin exerts a contractile effect on its own receptor by increasing cytoplasmic calcium levels through receptor and voltage-dependent calcium channels or intracellular calcium release. Serotonin-induced vasoconstriction may be similar to or exceeds that of potassium chloride and is reduced in calcium-free environment [60,66]. In the case of umbilical arteries, the initial small vasodilation is followed by a dominant contraction. Endothelial receptors and release of nitric oxide are responsible for the short dilatation, after removal of the endothel this transient reaction disappears [66]. We studied the reactivity to serotonin in umbilical and placental vessels in normal and IUGR term deliveries. Regarding the umbilical cord arteries, the contraction of the IUGR vessel rings was stronger but with ketanserin inhibition its reactivity decreased significantly ($p < 0.05$) and remained below the controls. In the case of umbilical veins, the control group's veins reacted with higher intensity but the difference persisted with ketanserin inhibition. The presence of serotonin in the umbilical cord increases the resistance in the arteries and in veins. In IUGR pregnancies the vascular tone in umbilical

arteries is more elevated by serotonin than controls, resulting increased resistence and abnormal blood flow velocity. The physiologic effect of serotonin can be seen in the correlation between its effect and S/D ratio and their relationship with perinatal outcome. Ketanserin in the umbilical circulation would decrease vascular resistance thus providing beneficial circulatory environment for the fetus. This effect is more prominent in IUGR fetuses. In the placenta IUGR vessels were less contractile and this difference persisted with the presence of ketanserin. The difference in the vascular tone regulatory capacity can be seen in the 3DPD indices too; the difference in VI is more prominent than in the FI. Although there are fewer vessels in a certain volume of the placenta in IUGR, these vessels are less contractile to serotonin, a humoral regulator, providing smaller vascular resistance and more blood flowing to the fetus. These results suggest that serotonin has a major role in regulating fetoplacental blood flow and might play a role in the circulatory environment in intrauterine growth restriction. Fetoplacental circulation might be improved by ketanserin. Regarding that the umbilical and placental circulation is balanced by several other humoral factors that can distort our data, additional studies are required to map the receptor spectrum of the foetoplacental unit to improve fetal circulation.

Based on the pathological data it can be concluded that the volume of placenta shows a stronger correlation with the weight of the newborn than the weight of the placenta. Although morphometric results show differences between IUGR and control cases, they do not reflect the functional capacity of the placenta. A large placenta with continuous infarcts and calcifications is found among the upper region of the weight percentile chart, but the fetus can be IUGR due to insufficient transport function. By measuring the volume of the placenta and the 3DPD indeces, however, we can obtain information that correlates with birth weight. For the purpose of improving IUGR diagnostics and pregnancy care, a percentile curve or diagnostic flowchart can be constructed from the aforementioned data. Our results also show that histological and ultrastructural differences may be present in milder forms in normal pregnancies, but two or more of these differences already pose a significant reduction in the compensative capacity of the placenta and can be diagnostic sign for IUGR. Due to the coiling of the umbilical cord, the image seen on the histological section is rarely a mathematical cross-section. We often see a distorted oval or bean-shaped lumen in the samples. We tried to eliminate this by standardizing the measurement points. Based on the

results obtained, only the cross-section area of the arteries showed deviation, which corresponds to the data reported in the literature [50-51]. This difference also plays a role in the differences in flow in the arteria umbilicalis, but the causative relationship is not clear yet.

Based on the results of this study, we can make the following statements:

The volume of the placenta is more closely related to the perinatal outcome than the placental weight. The volume of the placenta can be measured ultrasonographically in utero and therefore may be an informative part of IUGR diagnostics. However the functional volume of the placenta is more important than the absolute volume, to determine this, the 3DPD ultrasonographic indeces allow the measurement of VI, FI and VFI values that correlate with clinical data of the newborn. With the establishment of percentile or cut off values it is possible to improve the monitoring of IUGR pregnancies.

In regulating the circulation of umbilical cord and placenta, the effect of oxytocin and desmopressin was investigated. Neither oxytocin nor desmopressin elicited a significant increase in vascular tone in healthy, IUGR or GDM pregnancies. Serotonin-induced contraction in umbilical cord arteries correlates with clinical data. The response of the placenta to serotonin reflects the value of the 3-dimensional ultrasonographic flow index.

The physiological role of serotonin in vascular tone regulation can be assumed but needs further research. The different contractility of the vessels in IUGR and their altered response in ketanserin inhibition can be explained by difference in receptor-density and/ or difference in receptor subtypes. The altered density of 5-HT1 receptor itself can play a role in the pathogenesis of IUGR and the relatively higher density of 5-HT2 subtype can be another predisposing factor. As ketanserin passes from maternal blood to the fetoplacental circulation, its therapeutic use in IUGR should be investigated.

Further studies are needed to map the receptor spectrum of placenta and umbilical cord vessels to understand the regulation of vascular tone. Based on our initial results, inadequate fetal development can be studied from a new etiologic perspective and a new therapeutic approach can be possible.

8. Conclusion

A significant reduction in placental 3-DPD indeces could be measured in pathological pregnancies (eg.: IUGR and GDM). The reduction of the vascularization index was more prominent, than the flow index suggesting that the regulation of the vascular resistance can compensate the placental hypovascularization. Hence there is no innervation in the placenta and umbilical cord, the humoral regulation is responsible for the compensation.

The first examined vasoregulator factors were oxytocin and its more potent agonist, desmopressin. None of them elicited any alteration in the vascular tone. It is not likely possibble that these agents play a major role in regulating fetal blood supply. In our second study set we examined the vascular effect of serotonin. 5-HT not only elicited a strong vasoconstriction, its effect showed signifcant difference in IUGR pregnancies. When we preincubated the vessels with ketanserin, a selective 5-HT2 receptor antagonist, the difference between the two groups became more prominent. Our results suggest that the difference in the serotonin receptor density and the ratio of the 5-HT1 and 5-HT2 type receptors might play a role in the pathogenesis of placental insufficiency and intrauterine growth restriction. Moreover, since ketanserin can pass the placental barrier these receptors might serve as target points in clinical therapy. Further studies are needed to confirm the significance of serotonin in vasoregulation of the fetoplacental circulation.

Our results of histopathologic examination shows that no pathological alteration could be identified as a clear cause of placental insufficiency. However two or more minor pathologies were characteristic for IUGR placentas. These findings did not lead us closer to a more specific prenatal diagnostic method but does support our hypothesis about the importance of vasoregulation.

9. The new results of the thesis

1. Among the placental 3-DPD indices the vascularisation index shows a more prominent decrease than the flow index suggesting that the vasoregulation of the fetoplacental circulation can compensate the hypovascularization of the placenta.

2. Oxytocin and desmopressin are not likely potent vasoregulators of the fetoplacental circulation. Their effect was only observed on meconium-stained tissues.

3. Serotonin elicits a dose-dependent vasoconstriction on both arteries and veins in both the placenta and the umbilical cord.

4. The reactivity to serotonin is altered in IUGR pregnancies. This difference is magnified if the tissues are incubated with ketanserin, a selective 5-HT2 receptor antagonsit.

5. Serotonin seems to be one of the major humoral factors that determine the amount of blood flowing through the fetoplacental circulation.

6. The difference in the receptor density of the IUGR and control placentas suggest that serotonin /serotonin receptors might play a role in the pathogenesis of IUGR.

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