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Case Reports

Intrauterine growth restriction model by hyperthermia: quantitative analysis using Doppler and contrast-enhanced ultrasound imaging

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Summary

Introduction: Vascular modifications mechanism in pregnancy with intrauterine growth restriction (IUGR) by placental defect is unknown. The objective of this study was to monitor and quantify uteroplacental perfusion during pregnancy in a RCIU rats model by Doppler (DUS) and contrast-enhanced ultrasound (CEUS). *Materials and Methods:* Thirty-six pregnant rats were randomized in three groups : control (1), hyperthermia 40°C (2), and 41°C (3). At gestational day (GD) 18 and 19, an hyperthemic stage (30 minutes) by increasing body temperature was performed for groups 2 (40°C) and 3 (41°C). DUS study was performed strictly after each hyperthermic stage. CEUS was performed on GD19 and GD20. Histological analysis of all placentas was performed after cesarian done after the last CEUS study. Weight birth of all fetuses was recorded. *Results:* Fetus' weight was significantly higher for group 1 compared to groups 2 and 3 ($p < 0.02$). The weight from group 3 fetuses was significant lower than this from fetuses from group 2 ($p < 0.01$). No significant difference was show for the DUS study. For CEUS study, a significant difference was noted between group 1 *vs.* 2 and 3 at G19 and G20. *Discussion*: IUGR model by hyperthermia showed a significant correlation between fetal weight birth and maternal body temperature. This IUGR is associated with a high fetal morbi-mortality with placental damage. Vascular modifications are more tissue destruction than structural modifications.

Key words: Uteroplacental perfusion; Doppler ultrasound; Contrast-enhanced ultrasound; Rat, Hyperthermia; Intrauterine growth restriction.

Introduction

Intrauterine growth restriction (IUGR) affects 10% of the current pregnancies [1]. Several causes inherent to maternal, fetal, and/or placental sides can initiate this disease: maternal arterial pressure disorders, chronic maternal diseases, fetal chromosomal aberrations, fetal infections, defects of placenta insertion or placental ischemic disorders [2]. Consequences are responsible for maternal and fetal morbidity and mortality [3].

The most frequent cause is a high arterial blood pressure during pregnancy. The mechanism involved is a decrease in the utero-placental blood flow which is the consequence of a worse trophoblastic invasion by spiral arteries [2]. The premature entrance of maternal blood into the villous trophoblast contributes to abnormal development of the villous tree too [2]. However the initial vascular changes of this placental failure still remains unknown.

Various models are proposed in the literature to explain the physio-pathological system of the human IUGR such as caloric limitation, anemia, hyperthermia or surgical models. The hyperthermia model is known to induce an IUGR in pregnant rats [4] without fetal abnormalities before the 14th day of gestation [5].

The use of Doppler ultrasound (DUS) to assess utero-placental blood flow is currently the reference method for the clinical management of high-risk pregnancy [6]. A recent study showed the interest of the contrast-enhanced ultrasound (CEUS) [7]. Microbubble-based CEUS improves blood perfusion imaging in various organs [8, 9]. CEUS offers a new opportunity to monitor the utero-placental circulation and quantify the intervillous space flow velocity in obstetrics. Various studies in pregnant women showed an important contribution of ultrasound contrast agents in the placental vascular examinations [10]. The increased signal intensity within utero-placental circulation improves largely the detection of ischemia zones or nasty bruise frequently observed in placental insufficiency. CEUS assessment of placental perfusion completes the data provided by DUS in rats.

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The aim of this study was to monitor and quantify uteroplacental perfusion during pregnancy in a RCIU rats model by the complementary use of two ultrasonographic methods. DUS by measuring resistance index (RI), pulsatility index (PI) and blood velocity of uterine, umbilical, and cerebral arteries and CEUS, by quantifying the intervillous blood flow.

Materials and Methods

Thirty-six Sprague-Dawley pregnant rats with time dated gestational (GD) were received at GD08 and randomized into three groups.

All animal used were carried out in accordance with the *Directive for the protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes*, from the Council of the European Communities (86/609/EEC). Pregnant rats were kept in individual plastic cages in a conventional animal facility. They were fed *ad libitum* with usual diet and supplied with unlimited water.

Three groups were randomly determined: control group (1, n=12), hyperthermia group 40°C (2, n=12), and hyperthermia group 41°C (3, n=12). On GD18, each animal was anesthetized by inhalation of 3% Isoflurane and rectal temperature was monitored during all experimentations.

For groups 2 and 3 on GD18 and GD19, a 30-minute hyperthermic stage was achieved by increasing body temperature of pregnant rats to 40°C (2) and 41°C (3). The temperature was increased using a homeothermic blanket with rectal temperature monitoring. After 30 minutes, the heating system was switched off and rectal temperature decreased to the baseline value during three minutes.

The total duration of anesthesia (45 minutes) was identical in the three studied groups at each date. The control group temperature was kept constant at 37°C.

Both groups underwent DUS studies on GD18 and GD19, just after each hyperthermic stage. The uterine RI and the umbilical and cerebral fetal PI were measured with an ultrasound machine and a linear array transducer (15L8) at 14 MHz. These parameters were measured to the right and to the left uterine horns of the animal. The same operator performed all measurements. A one-mm pulsed Doppler gate was used and the insonating angle was < 30°. Doppler waveforms were obtained with the standardized method previously described [7].

The first CEUS was performed on GD19 after the DUS study and on GD20 for all groups. Ultrasound scans were obtained and a 21-MHz (MS250) probe at a mechanical index of 0.43. A 23-gauge catheter was placed in a tail vein to inject the contrast agent. A bolus of 0.3 ml of contrast agent was then injected. After image acquisition, tissue perfusion was analyzed quantitatively from the linear raw data. Motion artifacts were corrected and four regions of interest (ROIs) were manually defined: the mesometrial triangle, the placenta, the umbilical cord, and the fetus. Time intensity curves for each ROI were computed with VevoCQ software. Two parameters, including the rise time (RT) time to have the maximal signal) and the RPA (related perfused area for 50% of the maximal signal) were calculated for each ROI from the time-intensity curves [11].

On GD20, the fetuses were delivered by cesarean section and weighed after DUS and CEUS studies. Placentas and uterine horns were fixed in formol-acetic acid solution. Tissue samples were then embedded in paraffin, cut in approximately 5-mm sections and prepared with a conventional hematoxylin/eosin staining protocol. The tissue sections were examined by light microscopy. The quality of the histological sections, tissue accountability, slides labeling, and tissue placement were good and considered adequate for the present study by the pathologist. In blinded review, the pathologist was seeking for necrotic and hemorrhage areas.

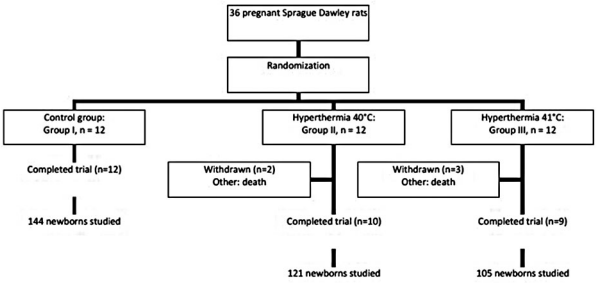

Figure 1. — Flowchart of experiments.

Randomization was performed by a computer-generated random-allocation sequence. Results are presented as the standard error of mean (SEM). Continuous variables were compared with a non-parametric Wilcoxon test. *P* values < 0.05 were considered to be statistically significant. Statistical analysis was carried out with R statistical software (version 2.15.0).

Results

Two and three rats were excluded in the groups 2 and 3, respectively (died during the hyperthermic stage). Twelve rats were therefore examined for the group 1, ten for the group 2, and nine for the group 3 (Figure 1). The total population of fetuses in the present study was 370, distributed as following: 144 in the group 1 (38.9%), 121 in the group 2 (32.7%), and 105 in the group 3 (28.4%) (Figure 1).

The maximal temperature was 41.0 ± 0.07°C in group 3 and 40.0 ± 0.1°C in the group 2 at 30 minutes; significantly higher than in the control group ($p < 0{,}001$). No difference in weight gain for pregnant rats was found according to body temperature between the groups.

One resorption and one hemorrhagic fetus were registered in all fetuses in the group 1 (1.38% of intrauterine deaths), one hemorrhagic fetus and six resorptions (5.79% of intrauterine deaths) in group 2, and nine hemorrhagic fetuses, ten resorptions, and 11 intrauterine deaths (28.57% of fetal loss) in the group 3. A significant difference between the group 3 *vs.* 1 and 2 was shown ($p < 0.01$).

For pregnant rats, offspring number per female was 12.0 ± 2.8 in group 1, 12.1 ± 2.6 in group 2, and 11.7 ± 2.1 in group 3. No significant difference among the three groups was shown.

Fetus weight was significantly higher in group 1 compared to groups 2 and 3 ($p = 0.012$ *vs,* group 2 and $p < 0.001$ *vs.* group 3). The weight of group 3 fetuses was significantly lower than this measured for group 2 fetuses ($p < 0.01$) (Figure 2).

The left and the right uterine arteries of all animals were

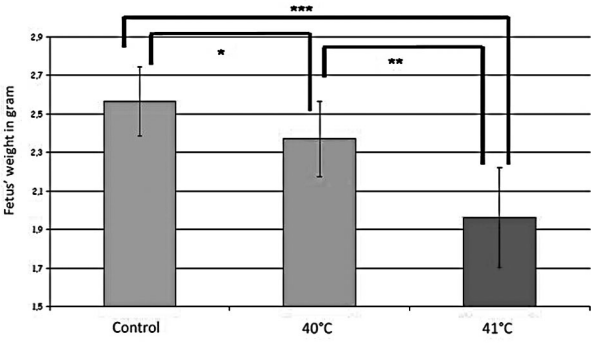

Figure 2. — Average of the fetus' weights in grams according to the groups.

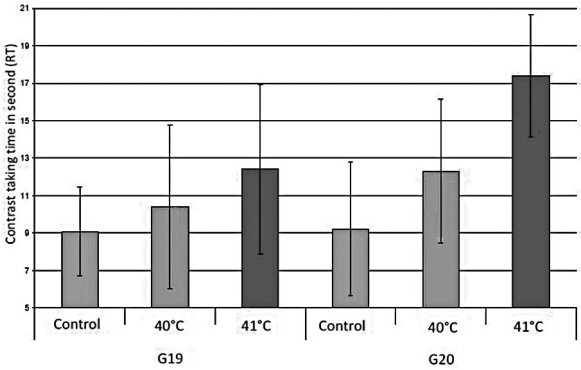

Figure 3. — Contrast taking time (RT) on the 19th and 20th days of pregnancy.

identified by DUS imaging during 15 ±1 minutes in all cases. Because no significant difference was observed between the two sides in any measurements, the authors grouped these data together for quantitative analysis and further statistical analysis.

G19 uterine RI was 0.62 ± 0.18 for the group 1, 0.52 ± 0.06 for the group 2, and 0.53 ± 0.08 for the group 3. No significant difference in uterine RI was observed among groups 1 and 2 ($p = 0.051$) and group 3 ($p = 0.07$), despite a downward trend RI in the groups 2 and 3. No difference was noted between groups 2 and 3 ($p = 0.832$).

Umbilical IP was, respectively, 2.5 ± 0.28 in group 1, 2.32 ± 0.38 in group 2, and 2.19 ± 0.25 in group 3. There was no significant difference between groups 1 and 2 in spite of a marked tendency ($p = 0.054$). On the other hand, a significant difference was reported between groups 1 and 3 ($p = 0.004$). No significant difference was found between groups 2 and 3 ($p = 0.287$).

The cerebral IP was not different among the groups: 1.87 ± 0.33 in group 1, 2.06 ± 0.43 in group 2, and 1.88 ± 0.39 in group 3 ($p = 0.211$ and $p = 0.899$ *vs.* group 1, respectively). No difference was observed between the groups 2 and III ($p = 0.287$).

At GD20, uterine IR was 0.54 ± 0.07 in group 1, 0.56 ± 0.06 in group 2, and 0.58 ± 0.07 in group 3. There was no significant difference among groups 1 and 2 ($p = 0.443$), and 2 ($p = 0.208$) or between the groups 2 and 3 ($p = 0.609$).

Umbilical IP was 2.62±0.37 in group 1, 2.75 ± 0.49 in-group 2, and 2.89 ± 0.34 in group 3. No significant difference was reported between groups 1 and 2 ($p = 0.370$), between groups 1 and 3 ($p = 0.084$), as well as between groups 2 and 3 ($p = 0.386$).

The cerebral IP was 1.52 ± 0.23 in group 1, 1.58 ± 0.29 in group 2, and 1.46 ± 0.23 in group 3. No significant difference was found between all groups for this index (group 1 *vs.* 2, $p = 0.494$; group 1 *vs.* 3, $p = 0.463$; group 2 *vs.* 3, $p = 0.178$).

CEUS imaging was used to monitor and quantify the perfusion of the first feto-placental unit on each horn. No contrast enhancement was observed in the umbilical vein of the fetus. Quantification of placental perfusion was based on objective parameters from linear raw data, before signal compression, including RT and RPA.

At GD19, RT was 9.09 ± 2.36 seconds in group 1, 10.04 ± 4.36 seconds in group 2, and 12.4 ± 4.55 seconds in group 3. The comparative analysis showed a significant difference between groups 1 and 3 ($p = 0.027$). However, no significant difference was revealed between groups 1 and 2 ($p = 0.546$), as well as between groups 2 and 3 ($p = 0.335$) (Figure 3). At GD20, RT was 9.21 ± 3.57 seconds in group 1, 12.3 ± 3.83 seconds in group 2, and 17.4 ± 3.28 seconds in group 3. No significant difference was shown between groups 1 and 2 ($p = 0.102$). A significant difference was noted between groups 1 and 3 ($p < 0.001$), as well as between groups 2 and 3 ($p = 0.010$) (Figure 3).

The measurement of area (in percentage), which had mostly 50% of maximal signal intensity, showed that at GD19, there was no difference between the groups for the assessment of viable placenta. At GD20, the RPA was significantly lower in group 1 compared to group 3 ($p < 0.001$), as well as for groups 1 and 2 ($p = 0.015$). No significant difference was noted between groups 2 and 3 (Table 1).

Placental volume averaged 924 ± 121 mm^3 in group 1, 949 ± 162 mm^3 in group 2, and 1008 ± 205 mm^3 in group 3. No significant difference was shown among the groups ($p = 0.689$ in group 1 *vs.* group 2, $p = 0.164$ in group 1 *vs.* group 3, and $p = 0.336$ in group 2 *vs.* group 3).

The histological analysis showed after objective observation in group 1, 84.8% of normal placenta, 2.2% of necrosis placenta, 8.7% with fibrin zone, and 6.5% with edema. In group 2, there was 82.5% of normal placenta, 5% of edema, 5% of necrosis form, and 12.5% of hemorrhagic form. In group 3, the microscopic analysis showed only 11.1% of normal placenta. There was also 38.9% of necrosis form and 36.1% of hemorrhagic form. Indeed, in

Table 1. — *Related perfused area (RPA) superior 50% of the maximal intensity,*

		RPA 50%	
		G19	G20
Control	Average	35.5	30.2
	SD	10.1	7.59
Hyperthermia 40°C	Average	37.0	40.6
	SD	15.2	14.3
Hyperthermia 41°C	Average	43.4	48.9
	SD	17.5	13.8

Table 2 — *Placental forms in % after histological analysis.*

	Group 1	Group 2	Group 3
Hemorrhagic	0	12.5	136.1
Ischemic	0	0	0
Necrotic	2.2	5.0	38.9
Fibrin	8.7	0	5.6
Edema	6.5	0	30.6
Congestive	0	5.0	11.1
Normal	84.8	82.5	11.1

group 3, congestive form represented 11.1%, edema 30.6%, and fibrin form 5.6%; for this reason 75% of abnormal placenta was found in this group (Table 2).

The correlation study between the placenta and the state of the fetus during the cesarian (alive or dead aspects) showed that 97.8% of placental analyses were performed in a live fetus for the control group, 95% in the hyperthermia 40°C group, and 91.7% in the hyperthermia 41°C group. The authors showed 2.2% of resorbed fetuses for the control group and 2.5% for the hyperthermia 40°C group. For the hyperthermia 41°C group, they had 2.8% of resorbed fetuses and 5.6% of hemorrhagic fetuses.

Discussion

The purpose of this study was to evaluate and quantify utero-placental perfusion during pregnancy in a RCIU rat model by hyperthermia. The design of this study used two hyperthermia groups (40°C and 41°C) and investigated a "depending dose" effect to assess placental vascular and structural abnormalities by DUS and CEUS methods to characterize the possible role of those abnormalities in the development of IUGR.

The hyperthermia episode was constant and allowed to obtain homogeneous and comparable groups. IUGR, being significant for the hyperthermia groups with regards to the control group in this study, allowed to confirm that hyperthermia induces it. Furthermore, the significant difference between group 40°C and group 41°C is in favor of a positive correlation between the degree of hyperthermia and the fetal weight consequences. This was not previously reported in the literature. A level of 40°C is a physiological temperature that could be observed in infectious origin fever. A level of 41°C is an extreme physiological fever which was not previously studied in relation to this extreme value. The exploration of this last level allows to evaluate its impact on IUGR. In rat species, little research indicated extreme hyperthermia as 41°C or 42°C, but the experiment was performed on GD10 [12]. Previous papers which compared hyperthermia episode to the delivery did not evaluate such extreme temperature as 41°C [13].

Episode of hyperthermia 41°C induces a significant IUGR associated with a pregnant female mortality of 25 %. Furthermore in this group, the fetal mortality is important and significantly increased compared to the other groups. The difference in the fetal mortality shows a teratogenic direct effect of hyperthermia more than vascular modifications. In case of spontaneous delivery at GD21-22, the number of live newborns would probably reduce in relation to placental structural defects more than vascular structural modifications. Hyperthermia of 41°C induces a maximal growth restriction with an increase of morbidity and mortality after natural birth. Placental underperfusion in rat model is known to induce IUGR by a reduced plasma volume expansion [14].

Placental structural defects hypothesis could be explained by the tendency to a decrease in uterine RI between the control group and the other groups at GD19, confirmed at GD20. It excludes delayed maternal vascular effects after the hyperthermia episode in spite of the implementation of IUGR [15]. At GD19, the significant difference in umbilical IP between control and hyperthermia 41°C group, which was not confirmed at GD20, was also not in favor of delayed vascular consequences of hyperthermia despite the development of IUGR. Cerebral PI values confirmed this hypothesis. According to these global results, there are no delayed maternal and fetal vascular consequences despite the establishment of a fetal restriction.

The difference for RT between the control group and the hyperthermia 41°C group at GD19 could be in relation to a vasodilatation process after the recent hyperthermia episode.

The increase of RPA at GD20 for 40°C and 41°C groups with a significant difference between control and 41°C groups suggests that placental damages exist secondary to the vasoconstriction rather than vascular changes themselves. The existence of large ischemic areas using the histological analysis with installation of necrosis correlating with the contrast ultrasonographic study consolidates this hypothesis.

Conclusion

This study demonstrates that IUGR model by hyperthermia highlights a significant correlation between fetal weight birth and maternal body temperature. IUGR is as-

sociated to a high fetal morbi-mortality with placental damage. Vascular modifications are similar to tissue destruction more than structural modifications. The hemodynamic modification investigated by DUS and CEUS, and the histological modifications were not similar to human physiopathology. More studies are necessary to understand the vascular IUGR mechanism.

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