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## Fetal echocardiography and pulsed-wave Doppler ultrasound in a rabbit model of intrauterine growth restriction.

--Manuscript Draft--

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**Key words:** intrauterine growth restriction, fetal echocardiography, Doppler ultrasound, fetal hemodynamics.

**Short Abstract:** (50 words maximum)

We describe examination of fetal cardiac function with contemporary functional fetal echocardiography and fetoplacental Doppler ultrasound using the VisualSonics VEVO 2100 microultrasound in a surgically induced model of intrauterine fetal growth restriction in a rabbit.

**Long Abstract:** (150 words minimum, 300 words maximum)

Fetal intrauterine growth restriction (IUGR) results in abnormal cardiac function that is apparent antenatally due to advances in fetoplacental Doppler ultrasound and fetal echocardiography. Increasingly, these imaging modalities are being employed clinically to examine cardiac function and assess wellbeing *in utero*, thereby guiding timing of birth decisions. Here, we used a rabbit model of IUGR that allows analysis of cardiac function in a clinically relevant way. Using isoflurane induced anesthesia, IUGR is surgically created at gestational age day 25 by performing a laparotomy, exposing the bicornuate uterus and then ligating 40-50% of uteroplacental vessels supplying each gestational sac in a single uterine horn. The other horn in the rabbit bicornuate uterus serves as internal control fetuses. Then, after recovery at gestational age day 30 (full term), the same rabbit undergoes examination of fetal cardiac function. Anesthesia is induced with ketamine and xylazine intramuscularly, then maintained by a continuous intravenous infusion of ketamine and xylazine to minimize iatrogenic effects on fetal cardiac function. A repeat laparotomy is performed to expose each gestational sac and a microultrasound examination (VisualSonics VEVO 2100) of fetal cardiac function is performed. Placental insufficiency is evident by a raised pulsatility index or an absent or reversed end diastolic flow of the umbilical artery Doppler waveform. The ductus venosus and middle cerebral artery Doppler is then examined. Fetal echocardiography is performed by recording B mode, M mode and flow velocity waveforms in lateral and apical views. Offline calculations determine standard M-mode cardiac variables, tricuspid and mitral annular plane systolic excursion, speckle tracking and strain analysis, modified myocardial performance index and vascular flow velocity waveforms of interest. This small animal model of IUGR therefore affords examination of *in utero* cardiac function that is consistent with current clinical practice and is therefore useful in a translational research setting.

## Introduction:

The burden of cardiovascular disease that results from fetal intrauterine growth restriction (IUGR) cannot be overstated. ~~After all, it~~ is the leading cause of stillbirth after congenital abnormalities.<sup>1</sup> IUGR refers to a fetus that fails to reach its growth potential and is commonly a result of placental insufficiency.<sup>2</sup> In survivors, cardiovascular ill health is evident across the life span with myocardial dysfunction apparent in infancy and childhood<sup>3,4</sup> and subsequent hypertension<sup>5</sup>, diabetes<sup>6</sup>, and obesity developing in adult life - ~~a slippery slope~~ all cumulative cardiac risk factors from birth towards premature death from ischemic heart disease.<sup>7</sup>

Developing animal models to characterize the maternal-fetal communication that establishes IUGR and the subsequent fetal response to reduced substrate availability is necessary if we are to both better understand the pathophysiology of IUGR-related cardiac impairment and to develop cardio-protective strategies to improve fetal and postnatal health. In this regard, many different models across different species have been described.<sup>8</sup> ~~Animal models IUGR is commonly induced by are generally based on~~ maternal under nutrition with a low protein diet in rodents, <sup>9</sup> surgical ablation or ligation of uterine blood flow in rodents and guinea pigs<sup>10</sup> or occlusion of the umbilical artery in sheep.<sup>11</sup> ~~occlusion~~<sup>10</sup> ~~However, or interruption of uterine blood flow.~~<sup>11</sup> ~~It is fair to say~~ apparent that no model fully recapitulates the human IUGR.<sup>12</sup>

In this current methodological study, we used a well validated approach of selective uteroplacental vascular interruption in a rabbit<sup>13-16</sup> that not only produces fetal cardiovascular responses observed with ultrasound clinically<sup>14</sup>, but also allows interrogation of fetal cardiac function with novel echocardiography using microultrasound technology of the VisualSonics VEVO 2100. While Doppler ultrasound of fetoplacental vessels remains the cornerstone of current antenatal surveillance of IUGR fetuses<sup>17</sup>, functional echocardiography is increasingly being utilized to provide new insights into disease pathophysiology and to assess fetal wellbeing.<sup>18</sup> Accordingly, here we take these advances from clinical research and describe an animal model that harbors not only this imaging sophistication but also provides the experimental platform to investigate mechanistic pathways and novel therapeutics.

## Protocol Text:

The following experimental protocol is approved by the Animal Ethics Committee, Katholieke Universiteit Leuven, Leuven, Belgium. We followed previously described surgical procedure<sup>13</sup> including some methodological changes, especially in anesthesia procedure.

### 1.) Inducing intrauterine growth restriction (IUGR).

1.1) Time dated pregnant rabbits (hybrid Dendermonde and New Zealand White) are housed in individual cages on a 12/12h light schedule with access to water and standard rabbit chow *ad libitum* from gestational age (GA) 18 days. At GA 25 days (full term is 31 days approximately), rabbits are transferred to the operating theatre for surgical creation of IUGR. Prior to surgery, randomly allocate a “case” (IUGR) and “control” uterine horn in the bicornuate rabbit uterus by using randomization software (GraphPad Software, USA)<sup>19</sup>. Sample size should be determined by power calculations by individual researchers for their question of interest.

### 1.2) Anesthesia Procedure.

Induction anesthesia is achieved by administering ketamine 35 mg/kg (Ceva Sante Animale, Brussels, Belgium) and xylazine 5 mg/kg (Ceva Sante Animale, Brussels, Belgium) intramuscularly in the hind leg. Anesthesia is then maintained with an inhalational mixture of 1-4% isoflurane (Isoba Vet; Abbott Laboratories Ltd., Queenborough, Kent, UK) in oxygen at 2 L/min. Place a pulse oximeter (Nellcor N-20, Covidien, Ireland) on the maternal second and third toe to continuously monitor heart rate and arterial oxygen saturation.

### 1.3) Surgical Procedure.

1.3.1) *Premedication.* Prior to commencing surgery, administer antibiotic prophylaxis Penicillin G, 300 000 IU (Kela Pharma, Hoogstraten, Belgium), uterine tocolytic medroxyprogesterone acetate 0.9 mg/kg (Depo-Provera, Pharmacia Upjohn, Puurs, Belgium) and analgesic buprenorphine 0.03 mg/kg (Temgesic; Schering-Plough) subcutaneously in a single 2.5 ml syringe.

1.3.2) *Rabbit preparation.* Place the rabbit on a warming pad for the duration of the surgery. Remove hair from the abdominal surgical field and apply antiseptic Povidone-iodine 7.5% (Betadine, Purdue, Connecticut, USA). A complete aseptic protocol is then employed.

1.3.3) *Abdominal laparotomy.* Infiltrate the incision site with local anesthetic (2 ml 1% lidocaine, B Braun Medical, Belgium) subcutaneously. The incision site should be approximately 5 cm in length in the midline, in the lower 2/3 of the abdomen. Once the skin is opened, use a mixture of sharp and blunt dissection to expose the rectus sheath. Care must

be taken to avoid the vascular maternal mammary glands located either side of the midline on entry (Figure 1A). Grasp the rectus sheath and peritoneum, tent to prevent inadvertent inclusion of abdominal contents, and carefully enter using sharp dissection (Figure 1B).

1.3.4) *Uterine exteriorization.* Surround the surgical field with gauze drapes soaked in warmed saline. Carefully grasp and exteriorize both sides of the bicornuate uterus. Care must be taken not to place either the vaginal or ovarian end of each uterine horn under tension, as this may result in unwelcome bleeding, pain and uterine contractility. The assistant continuously irrigates the exposed uterus with warmed saline to prevent uterine contractions. Palpate and record the number of fetuses present in each horn. By convention, the fetus at the ovarian end is designated the first fetus. One uterine horn is the case horn and the other is the control horn. Identify the previously randomly allocated case horn for IUGR induction and then replace the control horn back into the abdomen (internal control fetuses). ~~one uterine horn the case horn and the other the control horn. R~~

replace the control horn back into the abdomen (internal control fetuses).

1.3.5) *Surgically induced IUGR.* Identify the uteroplacental vessels to each gestational sac in the designated case horn (Figure 1C). Surgically ligate 40-50% of these vessels using a 5-0 polyglactin suture (Ethicon Vicryl, Johnson and Johnson Medical, USA) (Figure 1D-E). In practice, there are often three vascular bundles supplying each gestational sac. In this case, the largest vascular bundle is ligated. Once complete, the uterus is again irrigated with warmed saline and carefully returned to the abdomen.

1.3.6) *Abdominal closure.* Close the abdomen in two layers using a continuous running 2-0 polyglactin suture (Ethicon Vicryl, Johnson and Johnson Medical, USA). Close the skin with subcuticular 3-0 polyglactin suture (Ethicon Vicryl, Johnson and Johnson Medical, USA). Spray aluminiumaluminum aerosol onto the wound to prevent the rabbit from later interfering with the sutures.

1.3.7) *Recovery.* Cease anesthesia and closely observe recovery of respiration. The rabbit is then housed as described above with access to food and water *ad libitum* and observed daily.

## **2.) Performing fetal echocardiography and pulsed-wave Doppler ultrasound**

2.1.) The same rabbit is then transferred at GA 30 days for echocardiographic and ultrasound evaluation. The rabbit is placed on a warming pad for the duration of the examination.

### **2.2.) Anesthesia Procedure.**

2.2.1) Induction anesthesia is achieved by administering ketamine 35 mg/kg (Ceva Sante Animale, Brussels, Belgium) and xylazine 5 mg/kg (Ceva Sante Animale, Brussels, Belgium)

intramuscularly. Once sedate, obtain intravenous access using a 24 Gauge cannula (BD Insyte-W, Becton Dickinson Infusion Therapy Systems, Utah, USA) inserted into a peripheral auricular vein.

2.2.2) Maintain anesthesia with a continuous infusion of ketamine and xylazine: 2 ml ketamine (100 mg/ml) and 1.5 ml xylazine (20 mg/ml) to 46.5 ml of normal saline, then infused via a syringe pump at 40 ml/hr. Secure a three-way-tap and attach a 5ml syringe of rescue anesthesia: 2 ml ketamine (100 mg/ml) and 1.5 ml xylazine (20 mg/ml). Boluses of 0.5 ml rescue anesthesia are rarely required. Administer analgesic buprenorphine 0.03 mg/kg (Temgesic; Schering-Plough) subcutaneously prior to surgery. Oxygen 2L/min is applied by mask. Place a pulse oximeter (Nellcor N-20, Covidien, Ireland) on the maternal second and third toe to continuously monitor heart rate and arterial oxygen saturation.

2.3.) *Abdominal laparotomy and uterine exteriorization.* Refer to 1.3.3 and 1.3.4.

2.4.) *Fetal positioning for echocardiographic and ultrasound examination.*

2.4.1). Ensure-Place the rabbit ~~is~~ in a lateral position facing towards ~~you~~the researcher. This facilitates easier retrieval of a single gestational sac for examination and prevents excessive traction on uteroplacental vessels. As much as possible, try to exteriorize only one gestational sac at a time to minimize exposure. Ensure the exposed gestational sac is placed on gauze irrigated with warmed saline. To ensure standardized results between different study groups, generally only the fetus at the ovarian and vaginal ends of each uterine horn are used in the ultrasound examination.

2.4.2). Fetal cardiac function is very susceptible to the effect of uterine exteriorization, change in temperature and pressure from the ultrasound transducer over the placental vessels. To minimize these environmental and iatrogenic confounding effects on cardiac function, the ultrasound examination must begin immediately after exteriorization of the gestational sac and should be systematic and efficient. In our experience, changes in fetal cardiac function seldom occur in the first 5 minutes of exposure. If fetal bradycardia is encountered, remove the ultrasound probe, reposition the fetus to prevent kinking of fetoplacental vessels, irrigate with warmed saline or briefly return the gestational sac back into the maternal abdomen. If fetal bradycardia is persistent, the examination for this fetus should be abandoned in favor of examining the next fetus.

2.5.) *Fetal echocardiography.*

2.5.1). The Visualsonics VEVO 2100 (Toronto, Canada) high-resolution microultrasound platform with cardiac and strain analysis software installed is required. A VisualSonics MS-250 ~~13–24 MHz~~ transducer (VisualSonics, Toronto, Canada) is used for data acquisition. This has a centre frequency of 21 MHz with a bandwidth 13–24 MHz. The geometric focus is 15 mm, the maximum image width is 23 mm and the maximum image depth is 30 mm. The

footprint is 28 x 5.75 mm. Data is acquired in accordance with the American Society of Echocardiography guidelines and standards for performance of the fetal echocardiogram.<sup>2049</sup>

2.5.2). *Data acquisition 1: lateral view.* At the level of the lateral four-chamber-view, record a B-mode cine loop of at least five cardiac cycles to determine strain, strain rate, velocity and displacement. This is analyzed offline using the VevoStrain (Visualsonics, Toronto, Canada) algorithm as previously described in the journal in detail.<sup>219</sup> M-mode echocardiography indices can then be obtained in this standard view. Offline measurements of both cardiac structure and function can then be analyzed; internal ventricular and septal diameters in systole and diastole, volumes, stroke volume, fractional shortening, ejection fraction and cardiac output ~~are then obtained.~~<sup>219</sup>

2.5.3). *Data acquisition 2: apical view.* At the level of the apical four-chamber-view, B-mode cine loop can be repeated for VevoStrain analyses as described above. M-mode echocardiography is used to assess tricuspid (TAPSE) and mitral annular plane systolic excursion (MAPSE) and longitudinal strain and strain rate.<sup>224,232</sup> This apical view also permits assessment of valvular flow velocity waveforms and the modified myocardial performance index as previously described.<sup>243</sup>

## 2.6). *Fetoplacental pulse-waved Doppler ultrasound.*

Flow velocity waveforms (FVW) of the umbilical artery are obtained by locating the umbilical vessels using color Doppler and then placing the pulsed Doppler sample gate over the umbilical artery on a free loop of umbilical cord.<sup>254</sup> The middle cerebral artery (MCA) FVW is located by placing the pulsed Doppler sample gate just beyond the origin of MCA once the Circle of Willis is located by color Doppler.<sup>265</sup> The ductus venosus (DV) FVW is obtained by placing the pulsed Doppler sample gate at the proximal portion of the DV seen with color Doppler where it originates from the intrahepatic umbilical vein either in a sagittal or transverse view of the fetus.<sup>265</sup> The pulsatility index (PI) is calculated offline using the VisualSonics cardiovascular analysis software.

## Representative Results:

An asymmetrical growth restricted fetus and placenta from uteroplacental vascular ligation is compared to a normal control fetus and placenta in Figure 1F. Asymmetrical growth is confirmed by reduced neonatal birth weight and increased head circumference:abdominal circumference ratio to controls. Representative results from fetoplacental Doppler studies are shown in Figure 2. A normal low resistance pattern of positive end-diastolic flow in a control fetus is shown. With progressive increases in placental resistance seen in IUGR fetuses, absence and then reversed end diastolic flow is apparent. Figure 3 demonstrates a normal high resistance middle cerebral artery Doppler signal in a healthy fetus and a positive *a wave* ductus venosus in the same fetus. In IUGR fetuses, an increased pulsatility index of the ductus venosus and reversal of the *a wave* can be seen.<sup>14</sup> Representative results from M-mode fetal



echocardiography ~~in~~ are then shown in Figure 4. This lateral view allows calculation of internal ventricular diameters and volumes. ~~–Tand–~~ the apical view allows calculation of TAPSE and MAPSE. Figure 5 demonstrates speckle tracking of velocity vectors and the resultant regional strain curves to calculate strain, strain rate, displacement and velocity.

## Discussion:

We have used a previously validated approach of surgically reducing uteroplacental blood flow in a rabbit to produce IUGR<sup>13-16</sup> and later examining fetal cardiac function<sup>14</sup> to describe microultrasound technology and cardiac function analysis available on the VisualSonics VEVO 2100. The ability to reproduce fetoplacental Doppler changes of human IUGR fetuses in a small animal model and to then allow examination of cardiac function with recently described echocardiography is likely to progress fetal cardiac research in a clinically relevant way.

Small animal models commonly rely on maternal caloric restriction or low protein consumption<sup>9</sup>, however these are limited by an inability to demonstrate reduced placental blood flow, the primary mechanism of IUGR in developed countries.<sup>276</sup> Further, surgical bilateral uterine artery ligation in rats, while commonly reported, does not reproducibly result in growth restriction.<sup>287</sup> In this current methodology in rabbit fetuses, we show placental insufficiency to be evident by an absent or reversed end-diastolic flow of the umbilical artery Doppler (UA AREDF), consistent with sonographic findings in human IUGR. It has been shown experimentally that increased resistance in this Doppler signal reflects downstream impedance to blood flow in the placental vascular bed and is indicative of placental insufficiency.<sup>298</sup> The presence of UA AREDF is apparent when 50 – 70% of the villous vasculature is dysfunctional.<sup>3029,319</sup> Clinically, UA AREDF is a powerful predictor of hypoxia and poor perinatal outcome, and there is level 1-evidence to support its use in the management of high-risk pregnancy.<sup>17</sup>

In this model, if UA AREDF is observed the researcher can be confident that the primary surgery was successful in producing severe placental insufficiency and that further echocardiographic assessment is likely to be fruitful. Fetal echocardiography has recently branched out from a predominantly diagnostic domain of congenital abnormalities to now providing detailed functional assessments of cardiac function.<sup>18</sup> Both Doppler and M-mode can be used to assess fetal ventricular function and derive measures of cardiac output.<sup>324</sup> More recently, novel indices of cardiac performance in the fetus have been described such as speckle tracking and strain measurement<sup>332</sup>, the myocardial performance index<sup>243,343</sup>, tissue Doppler<sup>354</sup> and three-dimensional (3D) techniques.<sup>324</sup> An important feature of this current study is that these recent advances can also be performed on this small-scale ~~animal-rabbit~~ model using the Visualsonics VEVO 2100 microultrasound and cardiac function analysis software. Furthermore, as previously described<sup>14</sup> this model also allows assessment of the fetal hemodynamic response in other vascular territories, in particular the middle cerebral artery, ductus venosus and aortic isthmus, which are used widely in clinical practice when examining the growth-restricted fetus.<sup>365</sup> Similarly, the effects of administering

glucocorticoids in preparation for preterm birth may also be examined.<sup>37,38</sup> This rabbit model offers further advantages in terms of internal controls in the opposite horn of the uterus, a similar villous and hemochorial placentation to human pregnancy<sup>13</sup>, low cost, availability and relatively easy handling.

There are, nevertheless, several limitations of this model that must be addressed. The major limitation is iatrogenic fetal bradycardia during echocardiography. Maternally administered inhalational isoflurane can result in fetal bradycardia<sup>39</sup> and should be kept to a minimum or as in our case not used at all during echocardiography. Instead, we substituted this for an intravenous infusion of ketamine and xylazine, which has recently been shown in rabbits not to alter mean arterial pressure<sup>40,39</sup>, thereby presumably maintaining placental (and thus fetal) perfusion. Despite this approach, exposure of the gestational sac to the external environment, handling and pressure from the ultrasound transducer can all cause fetal bradycardia temporarily. We describe in the method ways to minimize this effect, however for accurate results we believe echocardiographic and fetoplacental Doppler ultrasounds are best limited to around 5 minutes of total exposure. With increasingly complex echocardiographic techniques and learning curves now described in the clinical literature<sup>41</sup>, meticulous planning is required beforehand to ensure standardized results. Several of the echocardiographic measurements described in this study, for example valvular annular plane systolic excursion and speckle tracking with strain analysis, are not established in current obstetric clinical practice, despite their use in adult cardiology.<sup>42,43</sup> Nevertheless, given the recent research interest in these novel measurements in fetal medicine, we chose to include them in our methodology to inform researchers that they are feasible to obtain when using this rabbit model. The analysis of these specific parameters in IUGR fetuses is beyond the scope of this methodological study. Another limitation Further, the rabbit has a natural tendency in the rabbit for IUGR based on fetal position<sup>44</sup>, therefore the well-perfused ovarian and vaginal ends of each horn are recommended for fetal case allocation. Furthermore, in this methodology, a gestational age of 25 days is used to surgically induce IUGR. This is based on previously described successful experiments, however the expected mortality rate is 50%.<sup>13,14</sup> Lastly, the fetal size precludes chronic instrumentation that is useful in sheep models for the placement of hemodynamic flow probes for later non-euthanized measurements and repeated blood sampling.<sup>19</sup>

In conclusion, selective ligation of the uteroplacental vessels to produce IUGR in the rabbit with subsequent microultrasound examination of cardiac function represents an animal model that is consistent with contemporary clinical practice and therefore useful to bidirectional translational researchers.

#### Disclosures:

The author Professor Jan D'hooge has a research agreement with VisualSonics. The remaining authors have no disclosures.

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**Table of specific reagents and equipment:**

Name of Reagent/Material	Company	Catalog Number	Comments
Ketamine	Ceva Sante Animale		<a href="http://www.ceva.com/en">http://www.ceva.com/en</a>
Xylazine	Ceva Sante Animale		<a href="http://www.ceva.com/en">http://www.ceva.com/en</a>
Depot Provera	Pharmacia Upjohn		
Penicillin G	Kela Pharma		<a href="http://www.kela.be">http://www.kela.be</a>
Lidocaine	B Braun Medical		<a href="http://www.bbraun.com/">http://www.bbraun.com/</a>
Temgesic	Schering-Plough		<a href="http://www.merck-animal-health-usa.com/">http://www.merck-animal-health-usa.com/</a>
Isolurane	Isoba Vet; Abbott Laboratories Ltd		<a href="http://www.abbottanimalhealth.com/index.html">http://www.abbottanimalhealth.com/index.html</a>
Ethicon	Johnson and Johnson		<a href="http://www.ethiconproducts.co.uk/products/sutures">http://www.ethiconproducts.co.uk/products/sutures</a>
Ethicon	Johnson and Johnson		<a href="http://www.ethiconproducts.co.uk/products/sutures">http://www.ethiconproducts.co.uk/products/sutures</a>
Ethicon	Johnson and Johnson		<a href="http://www.ethiconproducts.co.uk/products/sutures">http://www.ethiconproducts.co.uk/products/sutures</a>
VEVO 2100	VisualSonics	SN100-0032	<a href="http://www.visualsonics.com/">http://www.visualsonics.com/</a>
Aquasonic Gel	Parker Laboratories	01 02	<a href="http://www.parkerlabs.com/ultrasound_products.html">http://www.parkerlabs.com/ultrasound_products.html</a>
<a href="#">Nellcor N-20PA Pulse oximeter</a>	<a href="#">Covidien</a>		<a href="http://www.nellcor.com/prod/PRODUCT.ASPX?S1=POX&amp;S2=MON&amp;id=282&amp;V=O">http://www.nellcor.com/prod/PRODUCT.ASPX?S1=POX&amp;S2=MON&amp;id=282&amp;V=O</a>

## Figure Legends

Figure 1: Surgical technique of creating IUGR in a rabbit model. A: Midline laparotomy exposing rectus sheath, arrow = mammary glands; B: Safe entry into the abdominal cavity; C: arrow = uteroplacental vessels supplying gestational sac; D: suture method; E: arrow = completed ligation of uteroplacental vessel; F: representative control and IUGR fetus and placenta.

Figure 2: Doppler ultrasound of the umbilical artery. A: positive end-diastolic flow (EDF); B: absent end-diastolic flow (AEDF); C: Reversed end-diastolic flow (REDF).

Figure 3: A: Doppler ultrasound of the ductus venosus, s = s wave (ventricular systolic contraction), d = d wave (early ventricular diastole), a = a wave (atrial contraction); B: Doppler ultrasound of the middle cerebral artery.

Figure 4: M mode echocardiography. A: lateral four-chamber view, LVID = left ventricular internal diameter, IVSD = intraventricular septal diameter, RVID = right ventricular septal diameter, ESD = end systolic diameter, EDD = end diastolic diameter; B: apical view demonstrating tricuspid annular plane systolic excursion (TAPSE); C: apical view demonstrating mitral annular plane systolic excursion (MAPSE).

Figure 5: Fetal cardiac strain analysis. A: Region of interest defined by endo- and epi-cardium of the left ventricle; B: Strain rate curves of six myocardial segments, SR = peak systolic strain rate; C: Strain curves of six myocardial segments, Str = peak systolic strain. Cardiac motion depicted by M mode demonstrating end diastole (ED) and end systole (ES).



**Title:** Fetal echocardiography and pulsed-wave Doppler ultrasound in a rabbit model of intrauterine growth restriction.

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**Key words:** intrauterine growth restriction, fetal echocardiography, Doppler ultrasound, fetal hemodynamics.

**Short Abstract:** (50 words maximum)

We describe examination of fetal cardiac function with contemporary functional fetal echocardiography and fetoplacental Doppler ultrasound using the VisualSonics VEVO 2100 microultrasound in a surgically induced model of intrauterine fetal growth restriction in a rabbit.

**Long Abstract:** (150 words minimum, 300 words maximum)

Fetal intrauterine growth restriction (IUGR) results in abnormal cardiac function that is apparent antenatally due to advances in fetoplacental Doppler ultrasound and fetal echocardiography. Increasingly, these imaging modalities are being employed clinically to examine cardiac function and assess wellbeing *in utero*, thereby guiding timing of birth decisions. Here, we used a rabbit model of IUGR that allows analysis of cardiac function in a clinically relevant way. Using isoflurane induced anesthesia, IUGR is surgically created at gestational age day 25 by performing a laparotomy, exposing the bicornuate uterus and then ligating 40-50% of uteroplacental vessels supplying each gestational sac in a single uterine horn. The other horn in the rabbit bicornuate uterus serves as internal control fetuses. Then, after recovery at gestational age day 30 (full term), the same rabbit undergoes examination of fetal cardiac function. Anesthesia is induced with ketamine and xylazine intramuscularly, then maintained by a continuous intravenous infusion of ketamine and xylazine to minimize iatrogenic effects on fetal cardiac function. A repeat laparotomy is performed to expose each gestational sac and a microultrasound examination (VisualSonics VEVO 2100) of fetal cardiac function is performed. Placental insufficiency is evident by a raised pulsatility index or an absent or reversed end diastolic flow of the umbilical artery Doppler waveform. The ductus venosus and middle cerebral artery Doppler is then examined. Fetal echocardiography is performed by recording B mode, M mode and flow velocity waveforms in lateral and apical views. Offline calculations determine standard M-mode cardiac variables, tricuspid and mitral annular plane systolic excursion, speckle tracking and strain analysis, modified myocardial performance index and vascular flow velocity waveforms of interest. This small animal model of IUGR therefore affords examination of *in utero* cardiac function that is consistent with current clinical practice and is therefore useful in a translational research setting.

## Introduction:

The burden of cardiovascular disease that results from fetal intrauterine growth restriction (IUGR) cannot be overstated. It is the leading cause of stillbirth after congenital abnormalities.<sup>1</sup> IUGR refers to a fetus that fails to reach its growth potential and is commonly a result of placental insufficiency.<sup>2</sup> In survivors, cardiovascular ill health is evident across the life span with myocardial dysfunction apparent in infancy and childhood<sup>3,4</sup> and subsequent hypertension<sup>5</sup>, diabetes<sup>6</sup>, and obesity developing in adult life - all cumulative cardiac risk factors from birth towards premature death from ischemic heart disease.<sup>7</sup>

Developing animal models to characterize the maternal-fetal communication that establishes IUGR and the subsequent fetal response to reduced substrate availability is necessary if we are to both better understand the pathophysiology of IUGR-related cardiac impairment and to develop cardio-protective strategies to improve fetal and postnatal health. In this regard, many different models across different species have been described.<sup>8</sup> IUGR is commonly induced by maternal under nutrition with a low protein diet in rodents,<sup>9</sup> surgical ablation or ligation of uterine blood flow in rodents and guinea pigs<sup>10</sup> or occlusion of the umbilical artery in sheep.<sup>11</sup> However, it is apparent that no model fully recapitulates the human IUGR.<sup>12</sup>

In this current methodological study, we used a well validated approach of selective uteroplacental vascular interruption in a rabbit<sup>13-16</sup> that not only produces fetal cardiovascular responses observed with ultrasound clinically<sup>14</sup>, but also allows interrogation of fetal cardiac function with novel echocardiography using microultrasound technology of the VisualSonics VEVO 2100. While Doppler ultrasound of fetoplacental vessels remains the cornerstone of current antenatal surveillance of IUGR fetuses<sup>17</sup>, functional echocardiography is increasingly being utilized to provide new insights into disease pathophysiology and to assess fetal wellbeing.<sup>18</sup> Accordingly, here we take these advances from clinical research and describe an animal model that harbors not only this imaging sophistication but also provides the experimental platform to investigate mechanistic pathways and novel therapeutics.

## **Protocol Text:**

The following experimental protocol is approved by the Animal Ethics Committee, Katholieke Universiteit Leuven, Leuven, Belgium. We followed previously described surgical procedure<sup>13</sup> including some methodological changes, especially in anesthesia procedure.

### **1.) Inducing intrauterine growth restriction (IUGR).**

1.1) Time dated pregnant rabbits (hybrid Dendermonde and New Zealand White) are housed in individual cages on a 12/12h light schedule with access to water and standard rabbit chow *ad libitum* from gestational age (GA) 18 days. At GA 25 days (full term is 31 days approximately), rabbits are transferred to the operating theatre for surgical creation of IUGR. Prior to surgery, randomly allocate a “case” (IUGR) and “control” uterine horn in the bicornuate rabbit uterus by using randomization software (GraphPad Software, USA)<sup>19</sup>. Sample size should be determined by power calculations by individual researchers for their question of interest.

#### *1.2) Anesthesia Procedure.*

Induction anesthesia is achieved by administering ketamine 35 mg/kg (Ceva Sante Animale, Brussels, Belgium) and xylazine 5 mg/kg (Ceva Sante Animale, Brussels, Belgium) intramuscularly in the hind leg. Anesthesia is then maintained with an inhalational mixture of 1-4% isoflurane (Isoba Vet; Abbott Laboratories Ltd., Queenborough, Kent, UK) in oxygen at 2 L/min. Place a pulse oximeter (Nellcor N-20, Covidien, Ireland) on the maternal second and third toe to continuously monitor heart rate and arterial oxygen saturation.

#### *1.3) Surgical Procedure.*

1.3.1) *Premedication.* Prior to commencing surgery, administer antibiotic prophylaxis Penicillin G, 300 000 IU (Kela Pharma, Hoogstraten, Belgium), uterine tocolytic medroxyprogesterone acetate 0.9 mg/kg (Depo-Provera, Pharmacia Upjohn, Puurs, Belgium) and analgesic buprenorphine 0.03 mg/kg (Temgesic; Schering-Plough) subcutaneously in a single 2.5 ml syringe.

1.3.2) *Rabbit preparation.* Place the rabbit on a warming pad for the duration of the surgery. Remove hair from the abdominal surgical field and apply antiseptic Povidone-iodine 7.5% (Betadine, Purdue, Connecticut, USA). A complete aseptic protocol is then employed.

1.3.3) *Abdominal laparotomy.* Infiltrate the incision site with local anesthetic (2 ml 1% lidocaine, B Braun Medical, Belgium) subcutaneously. The incision site should be approximately 5 cm in length in the midline, in the lower 2/3 of the abdomen. Once the skin is opened, use a mixture of sharp and blunt dissection to expose the rectus sheath. Care must be taken to avoid the vascular maternal mammary glands located either side of the midline on

entry (Figure 1A). Grasp the rectus sheath and peritoneum, tent to prevent inadvertent inclusion of abdominal contents, and carefully enter using sharp dissection (Figure 1B).

1.3.4) *Uterine exteriorization.* Surround the surgical field with gauze drapes soaked in warmed saline. Carefully grasp and exteriorize both sides of the bicornuate uterus. Care must be taken not to place either the vaginal or ovarian end of each uterine horn under tension, as this may result in unwelcome bleeding, pain and uterine contractility. The assistant continuously irrigates the exposed uterus with warmed saline to prevent uterine contractions. Palpate and record the number of fetuses present in each horn. By convention, the fetus at the ovarian end is designated the first fetus. One uterine horn is the case horn and the other is the control horn. Identify the previously randomly allocated case horn for IUGR induction and then replace the control horn back into the abdomen (*internal control fetuses*).

1.3.5) *Surgically induced IUGR.* Identify the uteroplacental vessels to each gestational sac in the designated case horn (Figure 1C). Surgically ligate 40-50% of these vessels using a 5-0 polyglactin suture (Ethicon Vicryl, Johnson and Johnson Medical, USA) (Figure 1D-E). In practice, there are often three vascular bundles supplying each gestational sac. In this case, the largest vascular bundle is ligated. Once complete, the uterus is again irrigated with warmed saline and carefully returned to the abdomen.

1.3.6) *Abdominal closure.* Close the abdomen in two layers using a continuous running 2-0 polyglactin suture (Ethicon Vicryl, Johnson and Johnson Medical, USA). Close the skin with subcuticular 3-0 polyglactin suture (Ethicon Vicryl, Johnson and Johnson Medical, USA). Spray aluminum aerosol onto the wound to prevent the rabbit from later interfering with the sutures.

1.3.7) *Recovery.* Cease anesthesia and closely observe recovery of respiration. The rabbit is then housed as described above with access to food and water *ad libitum* and observed daily.

## **2.) Performing fetal echocardiography and pulsed-wave Doppler ultrasound**

2.1.) The same rabbit is then transferred at GA 30 days for echocardiographic and ultrasound evaluation. The rabbit is placed on a warming pad for the duration of the examination.

### **2.2.) Anesthesia Procedure.**

2.2.1) Induction anesthesia is achieved by administering ketamine 35 mg/kg (Ceva Sante Animale, Brussels, Belgium) and xylazine 5 mg/kg (Ceva Sante Animale, Brussels, Belgium) intramuscularly. Once sedate, obtain intravenous access using a 24 Gauge cannula (BD Insyte-W, Becton Dickinson Infusion Therapy Systems, Utah, USA) inserted into a peripheral auricular vein.

2.2.2) Maintain anesthesia with a continuous infusion of ketamine and xylazine: 2 ml ketamine (100 mg/ml) and 1.5 ml xylazine (20 mg/ml) to 46.5 ml of normal saline, then infused via a syringe pump at 40 ml/hr. Secure a three-way-tap and attach a 5ml syringe of rescue anesthesia: 2 ml ketamine (100 mg/ml) and 1.5 ml xylazine (20 mg/ml). Boluses of 0.5 ml rescue anesthesia are rarely required. Administer analgesic buprenorphine 0.03 mg/kg (Temgesic; Schering-Plough) subcutaneously prior to surgery. Oxygen 2L/min is applied by mask. Place a pulse oximeter (Nellcor N-20, Covidien, Ireland) on the maternal second and third toe to continuously monitor heart rate and arterial oxygen saturation.

2.3.) *Abdominal laparotomy and uterine exteriorization.* Refer to 1.3.3 and 1.3.4.

2.4.) *Fetal positioning for echocardiographic and ultrasound examination.*

2.4.1). Place the rabbit in a lateral position facing towards the researcher. This facilitates easier retrieval of a single gestational sac for examination and prevents excessive traction on uteroplacental vessels. As much as possible, try to exteriorize only one gestational sac at a time to minimize exposure. Ensure the exposed gestational sac is placed on gauze irrigated with warmed saline. To ensure standardized results between different study groups, generally only the fetus at the ovarian and vaginal ends of each uterine horn are used in the ultrasound examination.

2.4.2). Fetal cardiac function is very susceptible to the effect of uterine exteriorization, change in temperature and pressure from the ultrasound transducer over the placental vessels. To minimize these environmental and iatrogenic confounding effects on cardiac function, the ultrasound examination must begin immediately after exteriorization of the gestational sac and should be systematic and efficient. In our experience, changes in fetal cardiac function seldom occur in the first 5 minutes of exposure. If fetal bradycardia is encountered, remove the ultrasound probe, reposition the fetus to prevent kinking of fetoplacental vessels, irrigate with warmed saline or briefly return the gestational sac back into the maternal abdomen. If fetal bradycardia is persistent, the examination for this fetus should be abandoned in favor of examining the next fetus.

2.5.) *Fetal echocardiography.*

2.5.1). The Visualsonics VEVO 2100 (Toronto, Canada) high-resolution microultrasound platform with cardiac and strain analysis software installed is required. A VisualSonics MS-250 transducer (VisualSonics, Toronto, Canada) is used for data acquisition. This has a centre frequency of 21 MHz with a bandwidth 13–24 MHz. The geometric focus is 15 mm, the maximum image width is 23 mm and the maximum image depth is 30 mm. The footprint is 28 x 5.75 mm. Data is acquired in accordance with the American Society of Echocardiography guidelines and standards for performance of the fetal echocardiogram.<sup>20</sup>

2.5.2). *Data acquisition 1: lateral view.* At the level of the lateral four-chamber-view, record a B-mode cine loop of at least five cardiac cycles to determine strain, strain rate, velocity and displacement. This is analyzed offline using the VevoStrain (Visualsonics, Toronto, Canada) algorithm as previously described in the journal in detail.<sup>21</sup> M-mode echocardiography indices can then be obtained in this standard view. Offline measurements of both cardiac structure and function can then be analyzed: internal ventricular and septal diameters in systole and diastole, volumes, stroke volume, fractional shortening, ejection fraction and cardiac output.<sup>21</sup>

2.5.3). *Data acquisition 2: apical view.* At the level of the apical four-chamber-view, B-mode cine loop can be repeated for VevoStrain analyses as described above. M-mode echocardiography is used to assess tricuspid (TAPSE) and mitral annular plane systolic excursion (MAPSE) and longitudinal strain and strain rate.<sup>22,23</sup> This apical view also permits assessment of valvular flow velocity waveforms and the modified myocardial performance index as previously described.<sup>24</sup>

## 2.6). *Fetoplacental pulse-waved Doppler ultrasound.*

Flow velocity waveforms (FVW) of the umbilical artery are obtained by locating the umbilical vessels using color Doppler and then placing the pulsed Doppler sample gate over the umbilical artery on a free loop of umbilical cord.<sup>25</sup> The middle cerebral artery (MCA) FVW is located by placing the pulsed Doppler sample gate just beyond the origin of MCA once the Circle of Willis is located by color Doppler.<sup>26</sup> The ductus venosus (DV) FVW is obtained by placing the pulsed Doppler sample gate at the proximal portion of the DV seen with color Doppler where it originates from the intrahepatic umbilical vein either in a sagittal or transverse view of the fetus.<sup>26</sup> The pulsatility index (PI) is calculated offline using the VisualSonics cardiovascular analysis software.

## **Representative Results:**

An asymmetrical growth restricted fetus and placenta from uteroplacental vascular ligation is compared to a normal control fetus and placenta in Figure 1F. Asymmetrical growth is confirmed by reduced neonatal birth weight and increased head circumference:abdominal circumference ratio to controls. Representative results from fetoplacental Doppler studies are shown in Figure 2. A normal low resistance pattern of positive end-diastolic flow in a control fetus is shown. With progressive increases in placental resistance seen in IUGR fetuses, absence and then reversed end diastolic flow is apparent. Figure 3 demonstrates a normal high resistance middle cerebral artery Doppler signal in a healthy fetus and a positive *a wave* ductus venosus in the same fetus. In IUGR fetuses, an increased pulsatility index of the ductus venosus and reversal of the *a wave* can be seen.<sup>14</sup> Representative results from M-mode fetal echocardiography are then shown in Figure 4. This lateral view allows calculation of internal ventricular diameters and volumes. The apical view allows calculation of TAPSE and MAPSE. Figure 5 demonstrates speckle tracking of velocity vectors and the resultant regional strain curves to calculate strain, strain rate, displacement and velocity.

## Discussion:

We have used a previously validated approach of surgically reducing uteroplacental blood flow in a rabbit to produce IUGR<sup>13-16</sup> and later examining fetal cardiac function<sup>14</sup> to describe microultrasound technology and cardiac function analysis available on the VisualSonics VEVO 2100. The ability to reproduce fetoplacental Doppler changes of human IUGR fetuses in a small animal model and to then allow examination of cardiac function with recently described echocardiography is likely to progress fetal cardiac research in a clinically relevant way.

Small animal models commonly rely on maternal caloric restriction or low protein consumption<sup>9</sup>, however these are limited by an inability to demonstrate reduced placental blood flow, the primary mechanism of IUGR in developed countries.<sup>27</sup> Further, surgical bilateral uterine artery ligation in rats, while commonly reported, does not reproducibly result in growth restriction.<sup>28</sup> In this current methodology in rabbit fetuses, we show placental insufficiency to be evident by an absent or reversed end-diastolic flow of the umbilical artery Doppler (UA AREDF), consistent with sonographic findings in human IUGR. It has been shown experimentally that increased resistance in this Doppler signal reflects down stream impedance to blood flow in the placental vascular bed and is indicative of placental insufficiency.<sup>29</sup> The presence of UA AREDF is apparent when 50 – 70% of the villous vasculature is dysfunctional.<sup>30,31</sup> Clinically, UA AREDF is a powerful predictor of hypoxia and poor perinatal outcome, and there is level 1-evidence to support its use in the management of high-risk pregnancy.<sup>17</sup>

In this model, if UA AREDF is observed the researcher can be confident that the primary surgery was successful in producing severe placental insufficiency and that further echocardiographic assessment is likely to be fruitful. Fetal echocardiography has recently branched out from a predominantly diagnostic domain of congenital abnormalities to now providing detailed functional assessments of cardiac function.<sup>18</sup> Both Doppler and M-mode can be used to assess fetal ventricular function and derive measures of cardiac output.<sup>32</sup> More recently, novel indices of cardiac performance in the fetus have been described such as speckle tracking and strain measurement<sup>33</sup>, the myocardial performance index<sup>24,34</sup>, tissue Doppler<sup>35</sup> and three-dimensional (3D) techniques.<sup>32</sup> An important feature of this current study is that these recent advances can also be performed on this small-scale rabbit model using the Visualsonics VEVO 2100 microultrasound and cardiac function analysis software. Furthermore, as previously described<sup>14</sup> this model also allows assessment of the fetal hemodynamic response in other vascular territories, in particular the middle cerebral artery, ductus venosus and aortic isthmus, which are used widely in clinical practice when examining the growth-restricted fetus.<sup>36</sup> Similarly, the effects of administering glucocorticoids in preparation for preterm birth may also be examined.<sup>37,38</sup> This rabbit model offers further advantages in terms of internal controls in the opposite horn of the uterus, a similar villous and hemochorial placentation to human pregnancy<sup>13</sup>, low cost, availability and relatively easy handling.



There are, nevertheless, several limitations of this model that must be addressed. The major limitation is iatrogenic fetal bradycardia during echocardiography. Maternally administered inhalational isoflurane can result in fetal bradycardia<sup>39</sup> and should be kept to a minimum or as in our case not used at all during echocardiography. Instead, we substituted this for an intravenous infusion of ketamine and xylazine, which has recently been shown in rabbits not to alter mean arterial pressure<sup>40</sup>, thereby presumably maintaining placental (and thus fetal) perfusion. Despite this approach, exposure of the gestational sac to the external environment, handling and pressure from the ultrasound transducer can all cause fetal bradycardia temporarily. We describe in the method ways to minimize this effect, however for accurate results we believe echocardiographic and fetoplacental Doppler ultrasounds are best limited to around 5 minutes of total exposure. With increasingly complex echocardiographic techniques and learning curves now described in the clinical literature<sup>41</sup>, meticulous planning is required beforehand to ensure standardized results. Several of the echocardiographic measurements described in this study, for example valvular annular plane systolic excursion and speckle tracking with strain analysis, are not established in current obstetric clinical practice, despite their use in adult cardiology.<sup>42,43</sup> Nevertheless, given the recent research interest in these novel measurements in fetal medicine, we chose to include them in our methodology to inform researchers that they are feasible to obtain when using this rabbit model. The analysis of these specific parameters in IUGR fetuses is beyond the scope of this methodological study. Another limitation relates to the natural tendency in the rabbit for IUGR based on fetal position<sup>44</sup>, therefore the well-perfused ovarian and vaginal ends of each horn are recommended for fetal case allocation. Furthermore, in this methodology, a gestational age of 25 days is used to surgically induce IUGR. This is based on previously described successful experiments, however the expected mortality rate is 50%.<sup>13,14</sup> Lastly, the fetal size precludes chronic instrumentation that is useful in sheep models for the placement of hemodynamic flow probes for later non-euthanized measurements and repeated blood sampling.<sup>11</sup>

In conclusion, selective ligation of the uteroplacental vessels to produce IUGR in the rabbit with subsequent microultrasound examination of cardiac function represents an animal model that is consistent with contemporary clinical practice and therefore useful to bidirectional translational researchers.

### **Disclosures:**

The author Professor Jan D'hooge has a research agreement with VisualSonics. The remaining authors have no disclosures.

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**Table of specific reagents and equipment:**

Name of Reagent/Material	Company	Catalog Number	Comments
Ketamine	Ceva Sante Animale		<a href="http://www.ceva.com/en">http://www.ceva.com/en</a>
Xylazine	Ceva Sante Animale		<a href="http://www.ceva.com/en">http://www.ceva.com/en</a>
Depot Provera	Pharmacia Upjohn		
Penicillin G	Kela Pharma		<a href="http://www.kela.be">http://www.kela.be</a>
Lidocaine	B Braun Medical		<a href="http://www.bbraun.com/">http://www.bbraun.com/</a>
Temgesic	Schering-Plough		<a href="http://www.merck-animal-health-usa.com/">http://www.merck-animal-health-usa.com/</a>
Isolurane	Isoba Vet; Abbott Laboratories Ltd		<a href="http://www.abbottanimalhealth.com/index.html">http://www.abbottanimalhealth.com/index.html</a>
Ethicon	Johnson and Johnson		<a href="http://www.ethiconproducts.co.uk/products/sutures">http://www.ethiconproducts.co.uk/products/sutures</a>
Ethicon	Johnson and Johnson		<a href="http://www.ethiconproducts.co.uk/products/sutures">http://www.ethiconproducts.co.uk/products/sutures</a>
Ethicon	Johnson and Johnson		<a href="http://www.ethiconproducts.co.uk/products/sutures">http://www.ethiconproducts.co.uk/products/sutures</a>
VEVO 2100	VisualSonics	SN100-0032	<a href="http://www.visualsonics.com/">http://www.visualsonics.com/</a>
Aquasonic Gel	Parker Laboratories	01 02	<a href="http://www.parkerlabs.com/ultrasound_products.html">http://www.parkerlabs.com/ultrasound_products.html</a>
Nellcor N-20PA Pulse oximeter	Covidien		<a href="http://www.nellcor.com/prod/PRODUCT.ASPX?S1=POX&amp;S2=MON&amp;i">http://www.nellcor.com/prod/PRODUCT.ASPX?S1=POX&amp;S2=MON&amp;i</a>

## Figure Legends

Figure 1: Surgical technique of creating IUGR in a rabbit model. A: Midline laparotomy exposing rectus sheath, arrow = mammary glands; B: Safe entry into the abdominal cavity; C: arrow = uteroplacental vessels supplying gestational sac; D: suture method; E: arrow = completed ligation of uteroplacental vessel; F: representative control and IUGR fetus and placenta.

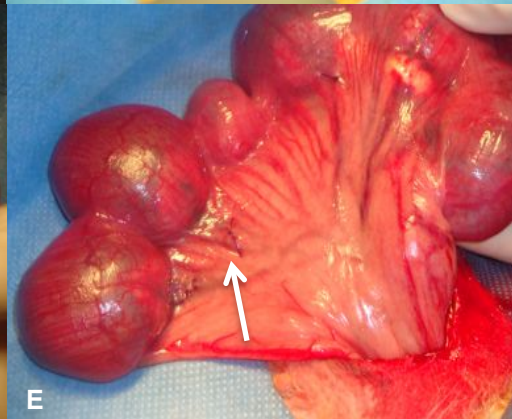
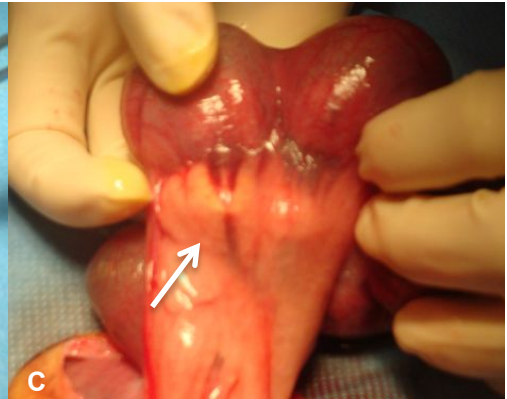
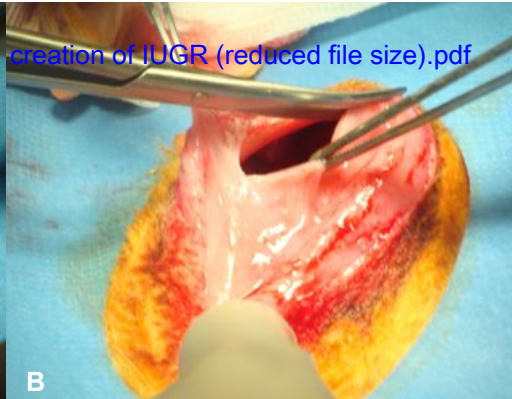
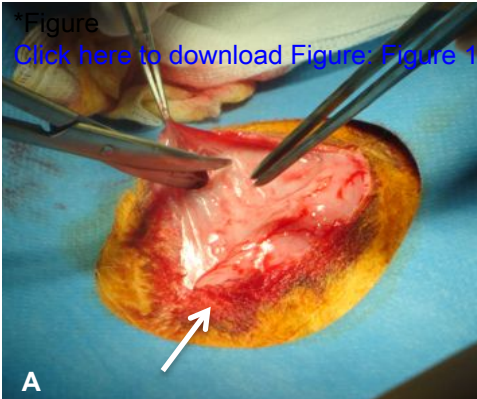
Figure 2: Doppler ultrasound of the umbilical artery. A: positive end-diastolic flow (EDF); B: absent end-diastolic flow (AEDF); C: Reversed end-diastolic flow (REDF).

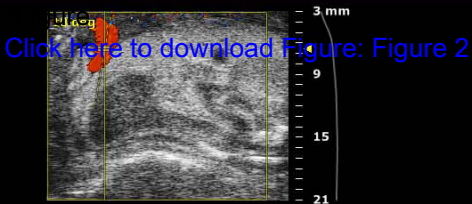
Figure 3: A: Doppler ultrasound of the ductus venosus, s = s wave (ventricular systolic contraction), d = d wave (early ventricular diastole), a = a wave (atrial contraction); B: Doppler ultrasound of the middle cerebral artery.

Figure 4: M mode echocardiography. A: lateral four-chamber view, LVID = left ventricular internal diameter, IVSD = intraventricular septal diameter, RVID = right ventricular septal diameter, ESD = end systolic diameter, EDD = end diastolic diameter; B: apical view demonstrating tricuspid annular plane systolic excursion (TAPSE); C: apical view demonstrating mitral annular plane systolic excursion (MAPSE).

Figure 5: Fetal cardiac strain analysis. A: Region of interest defined by endo- and epi-cardium of the left ventricle; B: Strain rate curves of six myocardial segments, SR = peak systolic strain rate; C: Strain curves of six myocardial segments, Str = peak systolic strain. Cardiac motion depicted by M mode demonstrating end diastole (ED) and end systole (ES).

\*Figure  
[Click here to download Figure: Figure 1 creation of IUGR \(reduced file size\).pdf](#)





A

EDF

B

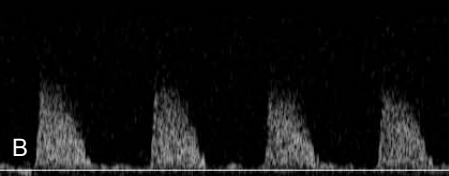
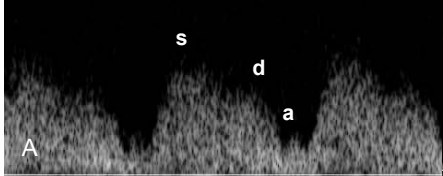
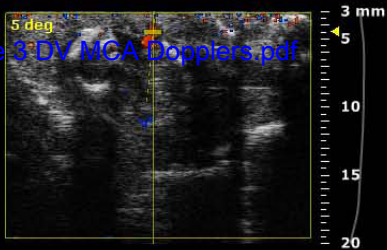
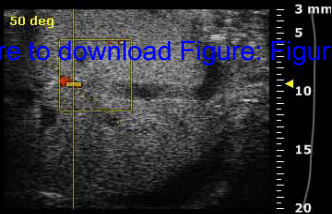
AEDF

C

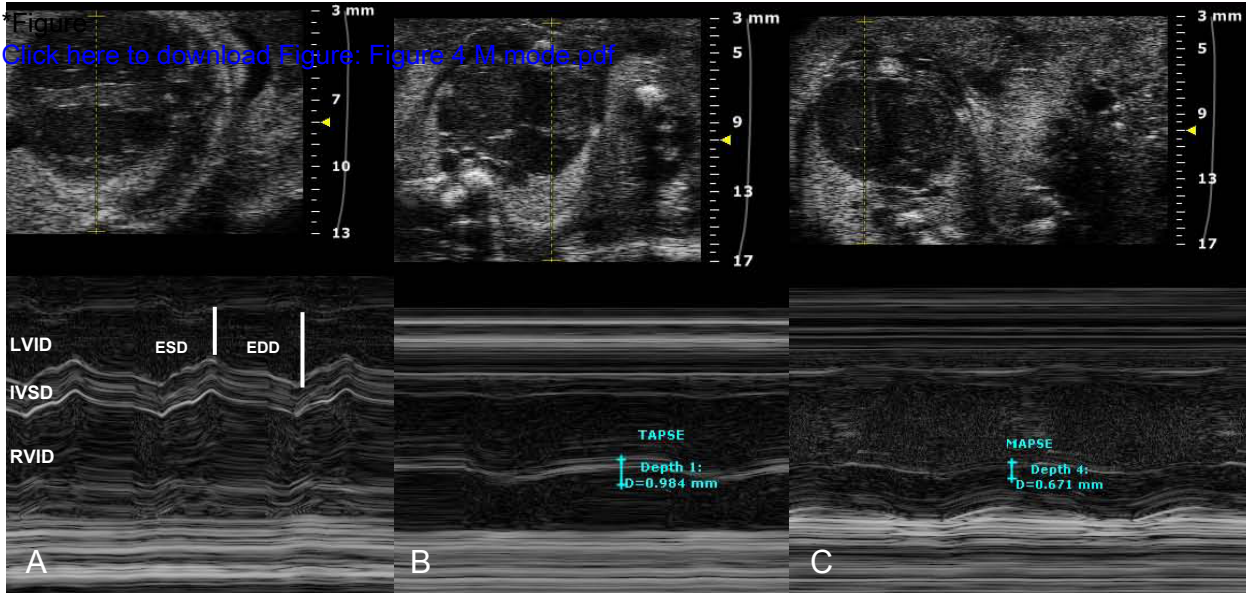
REDF



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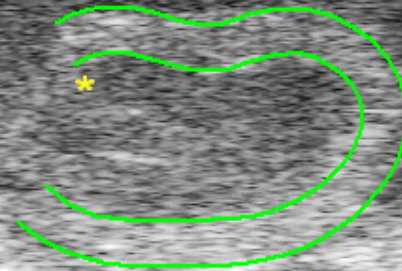
\*Figure  
[Click here to download Figure: Figure 4 M mode.pdf](#)



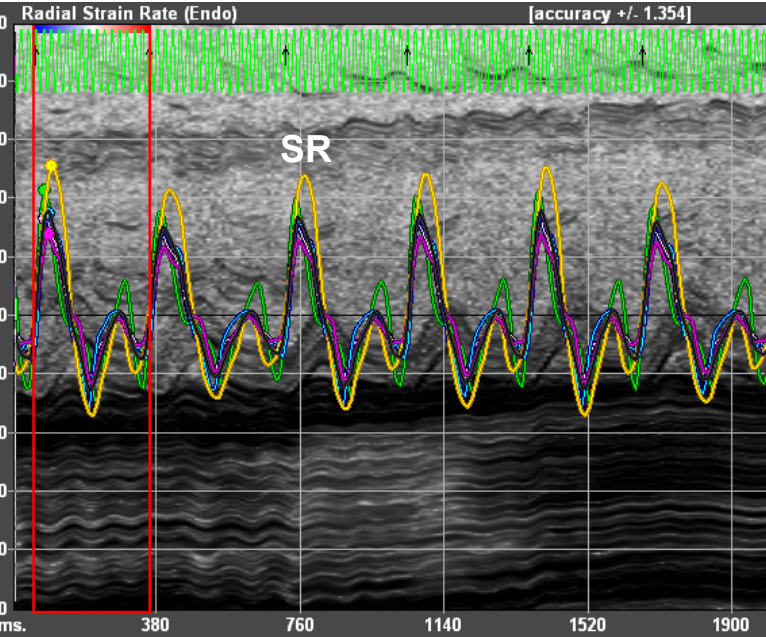
Figure

[Click here to download Figure: Figure 5 strain.pdf](#)

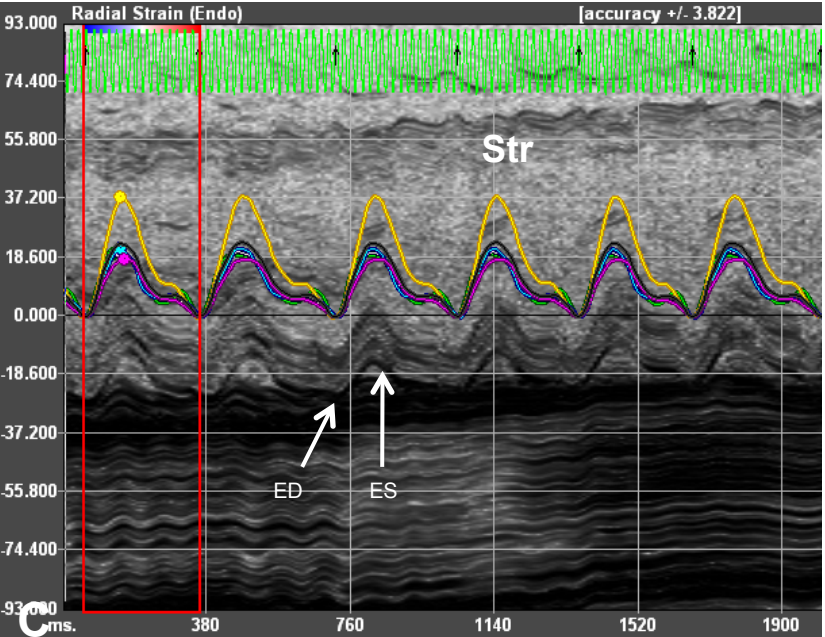
**A**



**B** ms.



**C** ms.



Name of Reagent/Material	Company	Catalog Number
Ketamine	Ceva Sante Animale	
Xylazine	Ceva Sante Animale	
Depot Provera	Pharmacia Upjohn	
Penicillin G	Kela Pharma	
Lidocaine	B Braun Medical	
Temgesic	Schering-Plough	
Isolurane	Isoba Vet; Abbott Laboratories Ltd	
Ethicon	Johnson and Johnson	
Ethicon	Johnson and Johnson	
Ethicon	Johnson and Johnson	
VEVO 2100	VisualSonics	SN100-0032
Aquasonic Gel	Parker Laboratories	01 02
Nellcor N-20PA Pulse oximeter	Covidien	

## Comments

<http://www.ceva.com/en>

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<http://www.visualsonics.com/>

[http://www.parkerlabs.com/ultrasound\\_products.html](http://www.parkerlabs.com/ultrasound_products.html)

<http://www.nellcor.com/prod/PRODUCT.ASPX?S1=POX&S2=MON&id=282&V>

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*JoVE (Journal of Visualized Experiments)*

17 Sellers St., Cambridge, MA 02139

13 November, 2012

Dear Alexa,

*Re: Fetal echocardiography and pulsed-wave Doppler ultrasound in a rabbit model of intrauterine growth restriction.*

On behalf of my co-authors I am pleased to submit a revised version of the above named manuscript for consideration for publication by *JOVE*. I thank the reviewers and the editorial board for their comments. Please find below the specifics of the revised manuscript:

Editorial comments: "you" has been changed as advised.

Reviewer 1: The introduction has been changed as per the reviewer's suggestions and more detailed specifics of the ultrasound transducer have been incorporated. With regard to representative results, we have provided description of the Doppler waveforms and provided representative images. This has been expanded and referencing to our previously published work has been included. These are the most clinically relevant indices. For some of the more novel echocardiographic indices, we have not included specific data as we believe this to be beyond the scope of this methodological study. Instead, we have included in the discussion a more detailed explanation about the current standing of these measurements in a research versus clinical setting. As explained in the manuscript, we felt it important to include the methodology of these indices in our manuscript given the research interest in novel functional echocardiography, and to show it is feasible in this rabbit model, however the analysis remains too preliminary to publish differences in IUGR fetuses. This relates to ongoing research outside the realms of this methodological manuscript.

Reviewer 2: Specifics for randomization procedure have been included.

Reviewer 3: The introduction and discussion have been amended to further highlight the rationale for using rabbit fetuses, compared to other models in particular studying mice and humans. As explained, there is poor reproducibility in mice studies and lack of placental insufficiency in malnourished rodent models making the rabbit model a useful and clinically relevant model to study. Cardiac structure is analysed and has been made more obvious in the methods by describing ventricular and septal diameters. With regards to anaesthesia, there is a lengthy discussion about the effects of anaesthesia on fetal cardiac parameters. We have also now included how we monitor maternal vital signs during surgery and echocardiography to prevent this influencing fetal cardiac function. Gestational age and timing is now included in the discussion. The rationale for lateral positioning of the mother has been included to make this clearer. The spelling mistake we could identify was aluminium, which has been changed to the American spelling aluminum.

We indeed thank you for receiving our revised manuscript and we look forward to receiving your editorial comments.

Yours sincerely,



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