3-15-2016

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*J Physiol* 594.6 (2016) pp 1553–1561



The Journal of Physiology

TECHNIQUES FOR PHYSIOLOGY

# Assessment of *in vivo* fetal growth and placental vascular function in a novel intrauterine growth restriction model of progressive uterine artery occlusion in guinea pigs

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**Key points**

- Intrauterine growth restriction (IUGR) is associated with short- and long-term detrimental cardiometabolic effects.
- Mice and rats are commonly used to assess IUGR, but differences in placental and fetal developmental physiology relative to those in humans highlight the need for alternative small animal IUGR models.
- We developed a guinea pig IUGR model by gradual occlusion of uterine arteries by ameroid constrictor implantation. In this model, reduced uterine blood flow was associated with IUGR, allowing *in vivo* assessment of fetal growth trajectory and umbilico-placental vascular function in conscious animals.
- The intervention induces placental vascular dysfunction and remodelling, as well as altered fetal abdominal growth resulting in an asymmetric IUGR and preserved brain growth.

**Abstract** Intra-uterine growth restriction (IUGR) is associated with short and long-term metabolic and cardiovascular alterations. Mice and rats have been extensively used to study the effects of IUGR, but there are notable differences in fetal and placental physiology relative to those of humans that argue for alternative animal models. This study proposes that gradual occlusion of uterine arteries from mid-gestation in pregnant guinea pigs produces a novel model to better assess human IUGR. Fetal biometry and *in vivo* placental vascular function were followed by sonography and Doppler of control pregnant guinea pigs and sows submitted to surgical placement of ameroid constrictors in both uterine arteries (IUGR) at mid-gestation (35 days). The ameroid constrictors induced a reduction in the fetal abdominal circumference growth rate (0.205 cm day$^{-1}$) compared to control (0.241 cm day$^{-1}$, $P < 0.001$) without affecting biparietal diameter growth. Umbilical artery pulsatility and resistance indexes at 10 and 20 days after surgery were significantly higher in IUGR animals than controls ($P < 0.01$). These effects were associated with a decrease in the relative luminal area of placental chorionic arteries (21.3 $\pm$ 2.2% *vs.* 33.2 $\pm$ 2.7%, $P < 0.01$) in IUGR sows at near term. Uterine artery intervention reduced fetal (~30%), placental (~20%) and liver (~50%) weights ($P < 0.05$), with an increased brain to

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DOI: 10.1113/JP271467

liver ratio ($P < 0.001$) relative to the control group. These data demonstrate that the ameroid constrictor implantations in uterine arteries in pregnant guinea pigs lead to placental vascular dysfunction and altered fetal growth that induces asymmetric IUGR.

(Received 20 August 2015; accepted after revision 21 December 2015; first published online 31 December 2015)
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## Introduction

Intrauterine growth restriction (IUGR) is a reduction in fetal growth potential (Figueras & Gardosi, 2011), clinically defined as a fetal weight less than the 10th percentile based on growth for gestational age (Sankaran & Kyle, 2009; Swanson & David, 2015). This condition is associated with increased perinatal morbidity and mortality (Halliday, 2009; Marsal, 2009) and with metabolic and cardiovascular alterations (Gluckman *et al.* 2008; Herrera *et al.* 2014). Diverse studies have focused on determining the cellular and molecular mechanisms involved in this condition, further characterizing the risk of developing chronic diseases later in life due to adverse intrauterine conditions (Gluckman *et al.* 2008; Negrato & Gomes, 2013). The mechanisms that determine IUGR are multifactorial, but impaired placental development and function are key factors (Valsamakis *et al.* 2006; Hendrix & Berghella, 2008).

The development of IUGR is often associated with reduced utero-placental blood flow and placental insufficiency. Reduced perfusion is characterized by decreased oxygen and nutrient delivery to the fetus (Herrera *et al.* 2014), usually related to alterations in placental structure and increased placental vascular resistance (Bamfo & Odibo, 2011). The latter alterations can be identified clinically by changes in Doppler ultrasound waveforms. Ultrasound examination of the uterine arteries evaluates vascular resistance and uterine perfusion, whereas fetal Doppler measurements provide information about cardiovascular and vascular function of the fetus itself (Bamfo & Odibo, 2011; Nardozza *et al.* 2012).

In order to understand the mechanisms underlying IUGR, several animal models have been developed. However, there are disadvantage with most existing models. Firstly, they rely on inducing IUGR in late gestation (Fung *et al.* 2012; Habli *et al.* 2013; Janot *et al.* 2014; Quibel *et al.* 2015). Secondly, these models are generally associated with high fetal mortality rates (Turner & Trudinger, 2009). Finally, they do not adequately represent IUGR in humans (Nathanielsz, 2006; Carter, 2007; Swanson & David, 2015). Animal models of human IUGR have been essential in elucidating the cellular and molecular mechanisms associated with this condition. However, there is much room for improvement as many of these models have significant endocrine and anatomical differences when compared with human pregnancy.

In the current study, we propose that the placement of ameroid constrictors around the uterine arteries of pregnant guinea pigs at mid-gestation is an IUGR model with important physiological similarities to humans (Carter, 2007; Swanson & David, 2015). Further, we hypothesize that this intervention induces IUGR through a progressive impairment in placental vascular function. To accomplish this, we modified the established IUGR model of uterine artery ligation/ablation (Turner & Trudinger, 2009) by placing an ameroid uterine artery constrictor at mid-gestation. This novel method consisted of progressive occlusion of uterine arteries by placing constrictors that gradually led to total occlusion. *In vivo* fetal biometry and umbilical Doppler ultrasound were performed during pregnancy, as well as fetal biometry at term to confirm altered fetal growth.

## Methods

All animal care, measures and experimental procedures were approved by the Ethics Committee of the Faculty of Medicine of the Pontificia Universidad Católica de Chile (1130801) and the Universidad de Chile (protocol CBA No. 0694 FMUCH) and conducted according to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). These measures and procedures were reported in accordance with the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines).

### Animals

Fourteen adult female Pirbright White guinea pigs (*Cavia porcellus*) were used for this study. All animals were housed in individual cages under standard conditions (30–35% humidity, 20–21°C and a 12:12 h light–dark cycle), with controlled food intake according to the body weight with a commercial diet (LabDiet 5025, Guinea Pigs, 20–30 g day$^{-1}$) and alfalfa hay, plus water *ad libitum*. Four- to five-month-old virgin sows in oestrus were paired with a fertile male for 2 days. After the mating period, the females were individually housed with daily monitoring of body weight, food intake and water consumption. Pregnancy was confirmed with ultrasonography at day

20–25, where the first day with the male was considered day 0 of pregnancy (term ~67 days).

### Experimental design and fetal ultrasound

After confirming pregnancy, the sows were randomly assigned to the control or IUGR group. Thereafter, pregnant guinea pigs were examined twice a week by ultrasound and Doppler as described (Turner & Trudinger, 2000) until 60 days of gestation. All *in vivo* evaluations were performed with conscious and manually restrained guinea pigs by experienced clinicians and veterinarians using an ultrasound sonograph (Sonovet R3, Samsung Medison). Every fetus was individually identified during pregnancy depending on the side and position in the uterus. In each examination, biparietal diameter (BPD) and abdominal circumference (AC) were registered. During each examination, umbilical artery function was assessed by colour Doppler ultrasound to determine the systolic/diastolic ratio, the resistance index and the pulsatility index (Turner & Trudinger, 2000, 2009). In addition, maternal and fetal heart rates were calculated from these readings.

### Surgical IUGR induction

At day 35 of gestation, all pregnant sows were subjected to aseptic surgery, either sham-operated (control) or progressive uterine artery occlusion (IUGR). Briefly, under general anaesthesia (ketamine 60 mg $kg^{-1}$, xylazine 4 mg $kg^{-1}$ and atropine 0.1 mg $kg^{-1}$ I.M.) an infra-umbilical midline laparotomy was performed, exposing the gravid uterus. For the IUGR group, ameroid constrictors (COR-2.00-SS, Research Instruments NW, Inc., Lebanon, OR, USA) (Harada *et al.* 1996; Mehl *et al.* 2005; Tang *et al.* 2005) were placed bilaterally around the base of each uterine artery (Fig. 1). The abdominal wall and skin were then sutured in layers with absorbable sutures (Vicryl 4/0, Ethicon, Cincinnati, OH, USA). Finally, surgical staples (Auto Suture, Condivien, Dominican Republic) were installed in the skin. As part of this procedure animals received analgesia (carprofen 4 mg $kg^{-1}$ S.C.) and prophylactic antibiotic (20 mg oxytetracycline $kg^{-1}$ S.C.) treatments. The skin staples were removed 7–8 days after surgery. The control group underwent the same surgical procedure, but without placement of the ameroid constrictors (sham-operated).

### Killing at near-term

At ~90% of pregnancy, approximately 60–63 days of gestation, the guinea pigs and their fetuses were killed with a maternal anaesthetic overdose (sodium thiopentone 200 mg $kg^{-1}$ I.P., Opet, Laboratorio, Chile). Once cardio-respiratory arrest was confirmed, the fetuses and their placentae were dissected and weighed.

### Chorionic artery histology

Placentae were immersed in 4% formaldehyde for 24 h and then washed in phosphate-buffered saline (PBS) 1× and embedded in paraffin. Chorionic plate sections were cut in 5 $\mu$m serial slides and treated with haematoxylin–eosin and van Gieson staining procedures. The vascular wall thickness of the chorionic plate arteries was calculated as described previously (Herrera *et al.* 2008).

### Data and statistical analyses

Biometry growth curves were analysed with a Pearson test to assess correlations. Thereafter data were analysed with linear regression and functions were compared by ANCOVA. Data for scalar units were expressed as median and interquartile range, whilst ratios, percentages and Doppler indexes were expressed as means ± SEM for normal distributions. The Shapiro–Wilk test was used to determine normality in the data distribution, followed by a parametric (Student's *t* test) or non-parametric (Mann–Whitney) test, as appropriate. Doppler data was averaged weekly and compared by a two-way ANOVA with a Bonferroni *post hoc* test. Differences were considered significant when $P \leq 0.05$ (Prism 5.0; GraphPad Software, La Jolla, CA, USA).

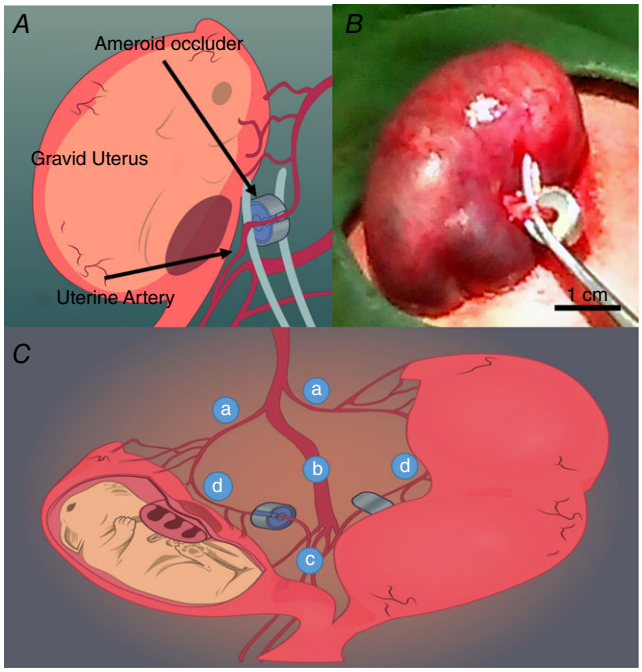


**Figure 1. Ameroid occludder placement**
Schematic representation (*A* and *C*) and photograph (*B*) of the placement site of the ameroid constrictors in the uterine artery of a pregnant guinea pig at 35 days of gestation. *C* shows the maternal artery supply to the uterus in gunea pigs; a, ovarian arteries; b, aorta; c, uterine arteries; d arcade arteries.

## Results

### Animal handling and surgical procedures

The surgical intervention took an average of 31.5 ± 3.4 min for the sham-operated group and 31.2 ± 7.2 min for IUGR group. Of the 14 pregnant sows (9 control, 5 IUGR), 37 fetuses were studied (24 from the control group, 13 from the IUGR group). There were three spontaneous abortions, one in the sham-operated group and two belonging to different mothers from the IUGR group. All abortions occurred within a couple of days after surgery and did not result in pregnancy cessation.

### Fetal biometry and umbilical Doppler during gestation

Before surgery, fetal biometries and Doppler measurements were similar in the two groups. For a better analysis of the IUGR group, we considered data starting on day 35 of gestation (right after surgery). The biparietal diameter (BPD) growth curve was represented by a linear correlation ($r^2_{\text{Control}} = 0.9725$; $r^2_{\text{IUGR}} = 0.9197$) without significant differences in the slope (Fig. 2*A*). Similarly, the AC growth curve presented a linear trajectory with a comparable correlation for the two groups ($r^2_{\text{Control}} = 0.9665$; $r^2_{\text{IUGR}} = 0.9502$); however the regression slope of the IUGR group was markedly lower ($m_{\text{IUGR}} = 0.205$)

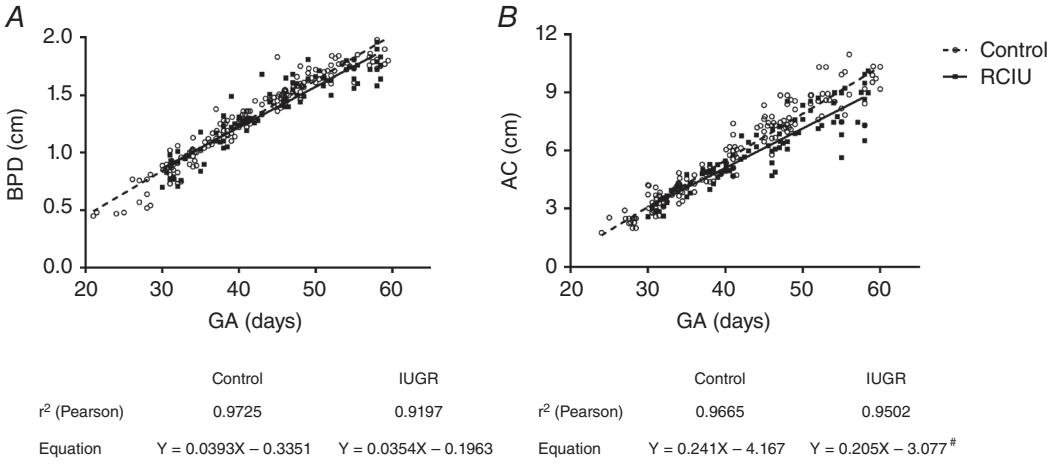


**Figure 2. Fetal biometry during gestation determined by ultrasound**
Biparietal diameter (BPD) (*A*) and abdominal circumference (*B*) growth rate during gestation in control (open circles, dash lines) and IUGR (filled circles, continous lines) fetal guinea pigs from. $^{\#}P < 0.001$ *vs*. control, ANCOVA test.

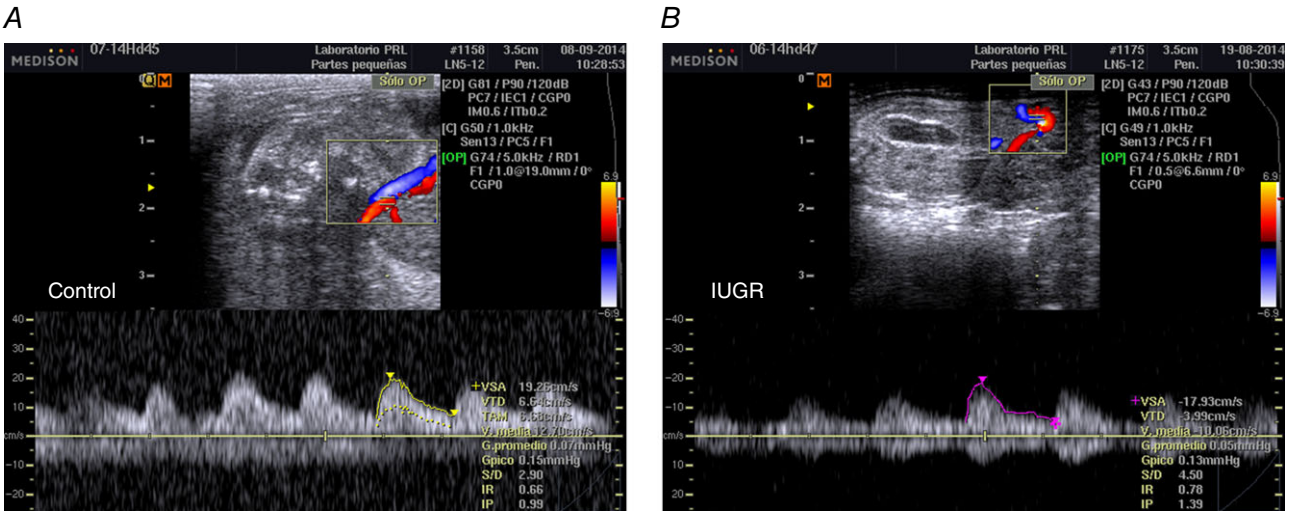


**Figure 3. Representative umbilical Doppler velocimetry in control and IUGR fetus**
Representative screen pictures of the umbilical Doppler analysis in control (*A*) and IUGR (*B*) fetuses at 45 and 47 days of gestation, respectively.

than that of the control group ($m_{Control} = 0.241$, $P < 0.001$; Fig. 2*B*).

The umbilical Doppler showed a progressive decrease in pulsatility and resistance indexes during gestation in the control group (Figs 3 and 4). However, in the IUGR group, these variables remained unchanged during gestation (Figs 3 and 4). Conversely, there were no significant differences between the control and IUGR groups in maternal and fetal heart rates during gestation (data not shown).

Notably, the analysis of chorionic plate artery remodelling at term showed similar internal diameters in the two groups. However, the IUGR group showed a marked increase in the vascular wall thickness (~30%, $P < 0.05$), and therefore greater external diameter (~80%, $P < 0.01$) than the controls (Table 1), with a reduction of ~30% in the relative luminal to total vessel area ($P < 0.01$; Fig. 5).

### Fetal organs and placental weight at near term

At near-term (60–63 days), IUGR fetuses were on average 30% smaller than control fetuses ($P < 0.01$), with 9 of the remaining 11 fetuses of the IUGR group with body weights below the confidence interval of the control group ($n = 15$; CI 76–89 g; Fig. 6*A*). This was associated with a marked decrease in the placental weight of the IUGR fetuses ($P < 0.05$; Fig. 6*B*) and placental efficiency (estimated as fetal weight/placental weight) ($P < 0.05$; Fig. 6*C*) in the IUGR group. IUGR fetal heart, lung, liver and kidney weights were significanty lower than those of control fetuses ($P < 0.05$), but brain weight was comparable between groups (Fig. 7*A* and *B*). Furthermore, the ratio between brain and liver weights was higher in the IUGR group ($P < 0.001$; Fig. 7*C*), evidence of an asymmetric IUGR.

## Discussion

This work established a novel procedure that gradually induced IUGR at mid-gestation in guinea pigs, allowing real time characterization of the *in vivo* effects of reduced utero-placental perfusion. Implanting ameroid constrictors at mid-gestation induced a reduction in fetal growth potential, reflected in lower abdominal circumference growth, with relatively sustained growth of the biparietal diameter. Additionally, fetuses from sows with ameroid constrictors displayed increased placental vascular resistance as early as 10 days after constrictor placement, which continued throughout gestation, along with increased thickness of chorionic plate artery walls at term compared to control animals. These changes were associated with a reduction in fetal and placental weight, as well as a diminished fetal to placental ratio (placental efficiency) at term. Moreover, significant reductions in the weights of the heart, lungs, liver and kidneys were observed in IUGR fetuses. Finally, the brain to liver weight ratio reflected asymmetric fetal growth, which correlates with *in vivo* changes in BPD and abdominal growth. These results support the use of this new method to alter the fetal growth trajectory early in gestation, resulting in asymmetrical IUGR, in a small animal model that easily tolerates handling and ultrasound examinations in a conscious state.

Intrauterine growth restriction represents an important factor for fetal and neonatal morbidity and mortality (Halliday, 2009; Marsal, 2009), with long-term effects on physiology (Gluckman *et al.* 2008). Considering that fetal growth depends on a sustained nutrient supply throughout gestation, which is subject to placental and maternal physiology, diverse approaches have been used to alter nutrient supply and subsequent fetal development and growth (Nathanielsz, 2006; Valsamakis *et al.* 2006;

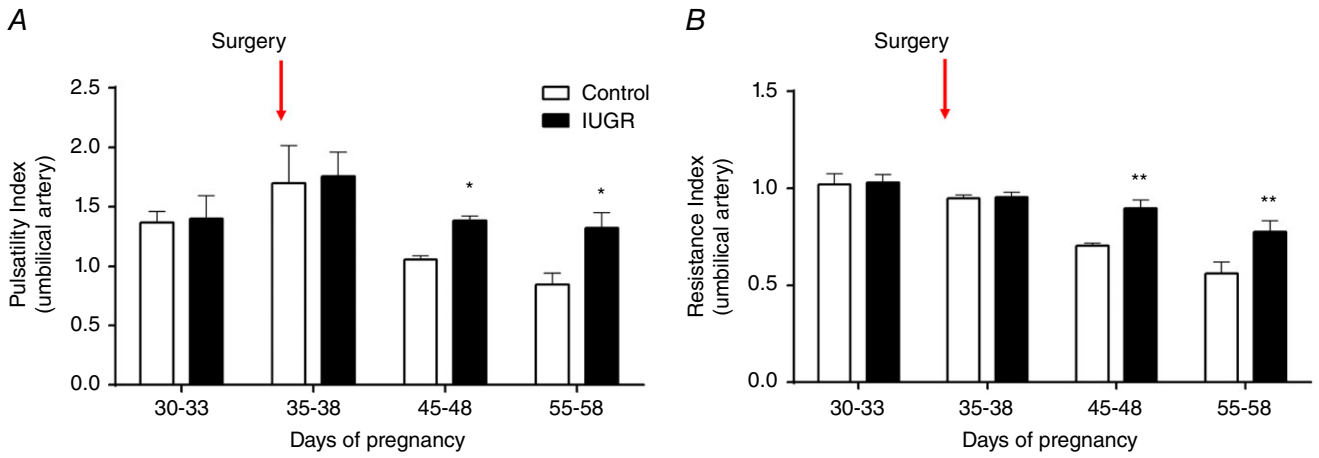


**Figure 4. Resistance index and pulsatility index in the umbilical artery during gestation**
Umbilical artery pulsatility (*A*) and resistance (*B*) index derived from Doppler waveforms acquired prior to and after the surgery or control (open bars) and IUGR (filled bars) fetuses. *$P < 0.05$, **$P < 0.01$ *vs.* control, two-way ANOVA.

**Table 1. Chorionic plate artery morphology at term**

| | Control | IUGR |
|---|---|---|
| Internal diameter ($\mu$m) | 69.8 ± 6.9 | 75.7 ± 7.6 |
| External diameter ($\mu$m) | 179.6 ± 20.8 | 230.7 ± 23.9* |
| Wall thickness ($\mu$m) | 49.9 ± 7.0 | 81.17 ± 11.0** |

Values expressed as means ± SEM; 4 placentae for each group were analysed considering at least 5 different sections in every case. *$P < 0.05$, **$P < 0.01$ *vs.* control, Mann–Whitney test.

Hendrix & Berghella, 2008). The aetiologies of most IUGR cases are unknown, but they share placental dysfunction as a common characteristic (Valsamakis *et al.* 2006; Hendrix & Berghella, 2008). With the aim of addressing mechanisms involved in the IUGR pathogenesis, several animal models have been developed, mainly with sheep, rat, rabbit and guinea pig, where placental dysfunction is induced through a reduction in placental perfusion (Nathanielsz, 2006; Carter, 2007; Swanson & David, 2015). One of the most common methods used with small animals consists of reducing the utero-placental blood flow by the acute and permanent ligation of the uterine arteries, relatively late in gestation (Nathanielsz, 2006; Carter, 2007; Turner & Trudinger, 2009; Swanson & David, 2015). Here, we present an alternative technique to reduce utero-placental blood flow by gradual bilateral occlusion of uterine arteries at mid-gestation, using ameroid constrictors, instead of an abrupt interruption of uterine blood flow later in gestation.

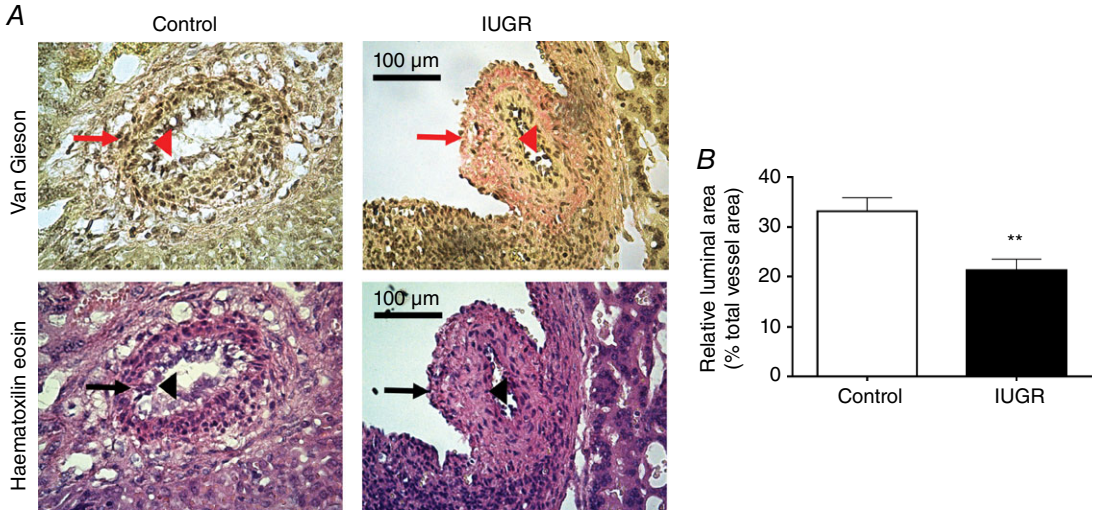


**Figure 5. Remodelling of placental chorionic arteries of control and IUGR fetuses**
*A*, representative micrograph of chorionic plate arteries in placentae of control and IUGR fetuses at term, stained with van Gieson's stain (upper panel) and haematoxilin–eosin (lower panel). Vessel wall thickness, and internal and external diameter were delimited considering the tissue indicated between the arrow and the arrowhead. *B*, relative luminal area derived from the determination of internal and external diameter obtained in *A*. **$P < 0.01$ *vs.* control, Mann–Whitney test.

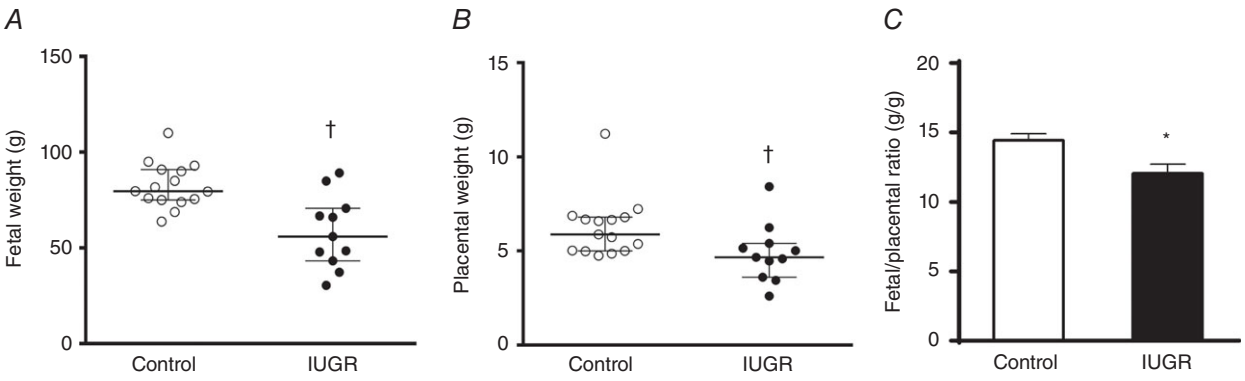


**Figure 6. Placental and fetal weight at near term**
Fetal (*A*) and placental (*B*) in control (open circles) and IUGR (filled circles) fetuses at 63 days of gestation. *C*, fetal to placental weight ratio as index of placental efficiency in Control (open bar) and IUGR (filled bar) groups. †$P < 0.05$, *$P < 0.05$ *vs.* control, Mann–Whitney test.

Ameroid constrictors have been used for surgical correction of extra-hepatic post-systemic shunts in dogs with a substantial improvement in post-operative recovery compared to ligation procedures (Mehl *et al.* 2005). This surgical tool has also been applied to obtain gradual and chronic occlusion in experimental models of coronary ischaemia in pigs (Harada *et al.* 1996) and hindlimb ischaemia in rats (Tang *et al.* 2005). In these cases, there was a progressive decrease in blood flow during the first week after implantation, with better post-surgical recovery. In the current work, examining the potential for this intervention in generating a physiologically relevant IUGR model, the placement of ameroid constrictors at mid-gestation resulted in an increase in the pulsatility and resistance indexes in umbilical arteries at 7–10 days post-surgery. The latter suggests progressive adaptation of utero-placental vasculature during this period, along with compensatory perfusion by cranial anastomosis of the ovarian arteries. Considering the role of oxygen in controlling placental vascular tone (Hampl & Jakoubek, 2009), the maintained vascular resistance after placing the constrictors is likely to have resulted from reduced oxygen availability rather than altered nutrient supply (Tchirikov *et al.* 2011; Lopez-Tello *et al.* 2015). Conversely, the intervention decreased abdominal growth trajectory, which was later evidenced by a marked reduction in fetal liver weight at term. At the same time, brain and skull growth was relatively normal, which is characteristic of asymmetric IUGR. The definition of IUGR as a fetal body weight below the 10th centile represents a clinical tool to better predict which subjects are at high risk of neonatal morbidity and mortality. However, IUGR is in essence defined by any reduction in the fetal growth rate or trajectory (Figueras & Gardosi, 2011). In this context, the possibility of monitoring fetal growth, as well as *in vivo* placental vascular function in conscious small animals, represents an exceptional opportunity to assess the effects of impaired fetal growth and the mechanisms contributing to the development of placental dysfunction and IUGR. In fact, the proposed intervention reduced fetal body weight at term by 80%. This effect was not associated with specific fetal localization in the uterus or with litter size.

Numerous articles on state-of-the-art IUGR models show that mice and rats are the most commonly used small animals (Nathanielsz, 2006; Swanson & David, 2015). While fewer studies have used guinea pigs, pregnancy in this species presents remarkably similar features to that of human pregnancy at the physiological level, which validates the guinea pig as a highly suitable model to mimic the effect of short- and long-term altered fetal growth in humans. Unlike other rodents, the guinea pig has a relatively long gestation, offering a wider window of time for chronic perturbation and adaptive fetal development to occur. Guinea pigs have a high maternal to fetal gradient of serum cortisol, which decreases at term by the down-regulation of placental 11$\beta$-hydroxysteroid dehydrogenase 2 (11-$\beta$-HSD2), which is similar in humans (Dalle & Delost, 1979; Sampath-Kumar *et al.* 1998). Furthermore, human and guinea pig fetuses are born with more adipose tissue deposits than other species (Widdowson, 1950), with comparable weight gain and fat deposition during the last third of gestation (Engle & Lemons, 1986). Adult sows present other similarities to humans, such as vitamin C requirements (Yu & Schellhorn, 2013), cholesterol metabolism (Fernandez, 2001), maternal serum progesterone levels (Mitchell & Taggart, 2009), cardiac function during gestation (Taggart *et al.* 2014) and spontaneous obesity development with age (Michel & Bonnet, 2012). These characteristics strengthen the advantages of guinea pigs over other rodents for studying conditions of altered fetal growth and development.

Indeed guinea pigs have been extensively used for *in utero* and postnatal IUGR metabolic studies (Detmer *et al.* 1991; Persson & Jansson 1992; Dunn *et al.* 2010; McKendry *et al.* 2010; Nguyen *et al.* 2010; Al-Hasan *et al.*

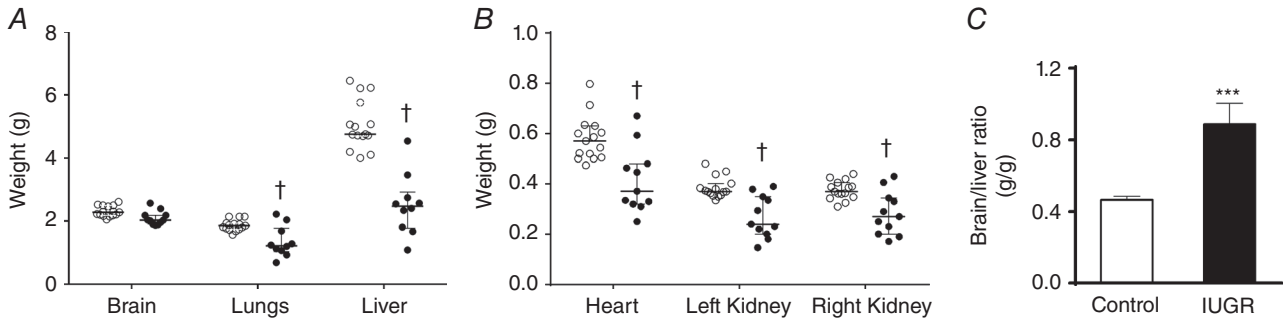


**Figure 7. Fetal organs weight at near term**
Weights of fetal organs (brain, lungs and liver in *A* and heart, left kidney and right kidney in *B*) of control (open circles) and IUGR (filled circles) fetuses at 63 days of gestation. *C*, fetal brain to liver weight ratio as index of fetal growth symmetry in the control (open bar) and IUGR (filled bar) groups. $^{\dagger}P < 0.05$, $^{*}P < 0.05$ *vs.* control, Mann–Whitney test.

2014; Palliser *et al.* 2014). Compared to other rodents, guinea pigs present a haemomonochorial placenta with extensive trophoblast invasion, along with a relatively long gestation that allows for evaluating therapeutic agents in a wider time window (Carter, 2007; Swanson & David, 2015). Fetal guinea pig renal, skeletal muscle and cerebral development is comparable to that of the human fetus (Carter, 2007), which in later life may affect cardiovascular and behavioural functions. Therefore, the timing of constrictor implantation to induce placental insufficiency at approximately mid-gestation (∼32 days, term 68 days) is important since it fits with the period of maximal fetal differentiation and growth (Widdowson *et al.* 1972; Tilley *et al.* 2007). Using progressive occlusion during this period and achieving total obliteration of the vessel over time has enabled us to induce a substantial decrease in fetal body and kidney weights, as well as the weights of other organs. In conclusion, we have developed a new surgical intervention in a small animal model to substantially mimic human IUGR, which opens new possibilities to enhance the translational significance of animal research and for the study of, and the search for better platforms to investigate, new therapeutic approaches to prevent and treat IUGR.

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## Additional information

### Competing interests

The authors have no competing interests.

### Author contributions

E.A.H., M.F., T.R.H.R., P.C. and B.J.K. conceived and designed the experiments. E.A.H., R.A., F.D.L., C.H., M.F., P.C. and B.J.K. collected, analysed and interpreted the experimental data. E.A.H., T.R.H.R., R.U. and B.J.K. drafted the article, and all authors revised it critically. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

### Funding

This work was funded by grant nos 1130801, 1120928 and 115119 from the National Fund for Scientific and Technological Development (FONDECYT-Chile).

### Acknowledgements

We are very grateful to Marta Gonzalez and René Vergara for their excellent technical assistance.