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ORIGINAL ARTICLE

Characterization of early changes in fetoplacental hemodynamics in a diet-induced rabbit model of IUGR

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Intrauterine growth restriction (IUGR) is associated with adverse perinatal outcomes and late-onset diseases in offspring. Eating disorders, voluntary caloric restriction and maternal undernutrition can all induce IUGR but a relevant model is required to measure all its possible consequences. In this work, pregnant rabbits were used as an IUGR model. Control females ($n = 4$) received *ad libitum* diet throughout pregnancy, whereas underfed females ($n = 5$) were restricted to 50% of their daily requirements. Offspring size was measured by ultrasonography and *in vivo* at birth. Hemodynamic features of the umbilical cords and middle cerebral arteries (systolic peak velocity, end diastolic velocity, pulsatility index and resistance index) were characterized by Doppler ultrasonography. At day 21, maternal underfeeding resulted in a significant reduction of fetal size (occipito-nasal length). At birth, the size of kits from the underfed group was significantly lower (lower crown-rump length, biparietal and transversal thoracic diameters) and a reduced weight with respect to the control group. Feed restriction altered blood flow perfusion compared with does fed *ad libitum* (significant higher systolic peak, time-averaged mean velocities and lower end diastolic velocity). Fetuses affected by IUGR presented with compensative brain-sparing effects when compared with the control group. In conclusion, the present study supports using rabbits and the underfeeding approach as a valuable model for IUGR studies. These results may help to characterize IUGR alterations due to nutrient restriction of mothers in future research.

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Key words: brain sparing, Doppler ultrasound, intrauterine growth restriction, pregnancy, rabbits

Introduction

Nutritional imbalances in developed societies are traditionally linked to excessive food intake, but currently the incidence of eating disorders and voluntary caloric restriction for aesthetical reasons are increasing.[1] These circumstances, besides the traditional high prevalence of maternal undernutrition in developing countries,[2] make research on the effects of undernutrition in the reproductive outputs of the exposed individuals necessary. Maternal undernutrition is considered a major cause of intrauterine growth restriction (IUGR).[3] Other maternal causes of IUGR described in the bibliography are hypertensive disorders of pregnancy, diabetic vasculopathy, chronic renal disease, collagen vascular disease and thrombophilia.[4]

IUGR is defined as the failure of the fetus to reach its genetically established growth rate. It is annually related to 800,000 neonatal deaths worldwide[5] and can be classified as 'symmetrical' or 'asymmetrical', with different degrees of severity. Symmetrical IUGR is characterized by a uniform reduction in size of the fetus and its organs and is associated with genetic and infectious factors. Asymmetrical IUGR, however, is characterized by a reduction in size of some organs, while other organs remain normal. It is mainly related with insufficient nutritional delivery to the fetus by maternal undernutrition or placental insufficiency. Predisposing factors that alter the fetal growth trajectory are a detrimental maternal nutritional status and inadequate diet during pregnancy.[6,7] Moreover, maternal undernutrition may affect placental growth and function, thus decreasing nutrient availability to the fetus and as a result affecting neonatal size.[8,9]

Furthermore, a clear relationship between IUGR infants and metabolic, cardiovascular and neurological pathologies in adulthood has been documented.[10,11] The association between inadequate intrauterine nutrition with the occurrence of IUGR and disease risk at postnatal life gave basis to the concept of the Developmental Origins of Health and Disease, formerly known as fetal programming or the Barker hypothesis.[12] This concept predicts that specific situations experienced in early life may increase the risks of suffering late-onset diseases.[13] However, it is unclear how the fetus responds to this prenatal environment to increase its chances of survival.

There is a serious necessity to address the potential long-term effects of undernutrition on the offspring via preventative

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procedures and focused treatments. Seeing as experimental studies on humans are limited due to ethical constraints, translational research based on the use of animal models have become imperative. This is verified by the fact that around three quarters of IUGR experiments have been performed in rats and mice.[14] However, in the recent years, the rabbit has been established as one of the most amenable animal model for reproductive studies.[15] In brief, ovulation in rabbits is induced by coitus, resulting in a precise pregnancy and embryonic age. Implantation starts on day 6–7, completing the chorioallantoid placentation around day 9.[16] After 31 days of pregnancy, females are able to deliver more than eight fetuses, hence enabling a high number of samples per female when compared with other mammal models, reinforcing the 3 R concept on Animal Experimentation.[17] The placental structure of the rabbit is hemodichorial (two cellular layers of chorion between the maternal and the fetal blood) and so it is closer to the human structure than that of other laboratory animals, like rodents (hemotrichorial). Moreover, the similar development of the brain in rabbits and humans (white matter maturation process starts during the intrauterine period[18]) favours the use of rabbits as an useful model to study perinatal processes of brain development.[19] Furthermore, the fetal size in rabbits, significantly larger than in rodents, allows accurate fetal measurements performed with common veterinary ultrasound equipments.[20]

Most methods used to induce IUGR are based on nutrient restriction or surgical modification.[21,22] Some authors propose the ligature of uteroplacental vessels as the most precise method, since restrict both nutrient and oxygen supply; however, that procedure is performed on the last few days of pregnancy and is associated with high mortality.[22] As an alternative, underfeeding regimes are an easier way to induce IUGR in fetuses and are also useful for understanding the pathology of IUGR and its connection to inadequate dietary habits. The availability of a reliable animal model to study fetoplacental hemodynamics in IUGR pregnancies would be highly beneficial in order to develop diagnostic, preventive and therapeutic strategies in humans and animals. Our hypothesis is that the rabbit is a suitable model for the study of the IUGR syndrome caused by maternal undernutrition.

Therefore, the present work aimed to evaluate the suitability of the rabbit as a maternal undernutrition IUGR model. Suitability will be determined by ultrasonography and direct measurements of effects of such perturbations on the development of the offspring and characterizing fetal hemodynamic features at 70% pregnancy.

Methods

Animals and husbandry

The experiment involved a total of nine multiparous pregnant New Zealand × California rabbits (*Oryctolagus cuniculus*) in optimal body conditions and with similar parity. Animals were maintained in individual flat-deck cages under approved animal husbandry conditions with a constant photoperiod of 16 h of light per day, a temperature of 18–22°C and a relative humidity of 60–75%, since these are the normal husbandry conditions for rabbits.[23] The research was performed under a Project License approved by the UPM Committee of Ethics in Animal Research, in agreement with the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 about the protection of animals used in research.

Rabbits were inseminated using fresh diluted semen (commercial extender, MA 24; Ovejero, León, Spain). Each dose contained at least 25 million spermatozoa in 0.5 ml of diluent (Magapor S.L., Zaragoza, Spain). Ovulation was induced with gonadoreline at the time of mating (20 μg/doe, i.m.; Inducel-GnRH; Ovejero). Pregnancies were diagnosed by ultrasonography at day 9 after artificial insemination (Sonosite S-Series; SonoSite Inc., Bothell, WA, USA).

Underfeeding model

At day 9 of pregnancy (this is 30% of the pregnancy length in rabbits, with a total pregnancy period of 31 days), when the trophoblast begins to tap maternal blood vessels,[24] nine pregnant rabbits were randomly distributed in two different groups adjusted for similar weight and therefore feed intake consumption. Females were fed the same standard diet (16% crude protein, 37% crude fiber, 3.7% fat and 2400 kcal/kg of digestible energy) fulfilling either their daily maintenance requirements for pregnancy (control group, $n = 4$) or only 50% of that requirements (underfed group, $n = 5$). The diet of each female was adjusted to its feed intake, estimated 2 weeks before the experiment began. Every day, the individual feed ration was weighed and given to the rabbits in their cages. In order to determine changes in maternal body weight, does were weighed on the day of the artificial insemination and after delivery. After insemination, until the end of the trial, maternal feed consumption from the control group was estimated weekly to evaluate a possible increase of the intake during pregnancy.

Conceptuses study

Ultrasound evaluations were performed in all rabbits on the 21st day of pregnancy (≈70% of the total pregnancy length). At this stage, fetal viability is critical since a significant change in the uteroplacental blood flow takes place.[25] This is, when the irrigation of the placenta becomes more abundant than that of the uterus, in order to cover the needs of the developing conceptuses. Moreover, at this moment, organogenesis is assumed to be achieved.[22] Rabbits were shaved in the abdominal area and manually restrained in dorsal recumbence, without anesthesia to avoid any effect on the heart rate or blood flow during the observations. All the females had been handled and restrained before the experimental phase, which diminished animal stress. The same blind operator scanned all rabbits using a Vivid-I ultrasound machine (General Electric, Fairfield, CT, USA)

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equipped with a multifrequency (8–12 MHz) lineal array probe. Scans were recorded using the 'cine-loop' option; this allowed animals to be released quickly, minimizing the restraining time. A complete scan exploration did not last >20 min/female.

To visualize the uterine horns and fetuses on the transverse, frontal and sagittal planes, rabbits were scanned by placing the transducer on one flank of the rabbits and moving it to the opposite flank. Four fetuses were selected at random in each female to minimize individual effects. Hence, 16 fetuses were analyzed in the control group and 20 in the underfed group, but the four fetuses measured per mother were averaged to result in one data point. Measurements were obtained with built-in electronic calipers on the cine-loop once the complete examination was recorded. Since the size of the fetus was too large for viewing the entire body-length at this pregnancy stage, measurements included the thoracic diameter, the occipito-nasal length and the biparietal diameter (Fig. 1).

Immediately after birth, all the kits were classified as newborns or stillborns. Only newborn were measured. Using a slide caliper, we also determined biparietal diameter (from one parietal eminence to the other), thoracic diameter (adjusted between underarms) and crown-rump length (maximum distance from crown to tail basis; see Fig. 2).

Doppler evaluation of fetal hemodynamics

At the 21 day of pregnancy, blood flow parameters from the umbilical cord arteries (UCAs; Fig. 3a) and from the middle cerebral artery (MCA; Fig. 3b) were determined in the same fetus in which the body size was ultrasonically evaluated. Briefly, after identifying the vessels with color Doppler (UCAs were found at the free-floating umbilical cord proximal to the placental insertion whereas MCA were located after the Circle of Willis identification), the sample pulsed Doppler gate was placed over the vessels. Then, the waveforms of three consecutive cardiac cycles in each vessel were recorded, disregarding views with insonation angles between 20–50°. Measurements were obtained once the entire examination was recorded and included resistance index (RI), pulsatility index (PI), systolic peak velocity (SPV), end diastolic velocity (EDV) and time-averaged mean velocity (MV), measured at UCA and MCA (Fig. 3). Finally, the cerebroplacental ratios (i.e. the ratios between MCA and UCA values) for RI, PI, SPV, EDV and MV were also calculated.

Data analysis

Statistical analysis was performed with the Statistical Analysis System Software (SAS, 1990). Groups were compared using an analysis of variance (ANOVA) with the treatment (control or underfeeding diet) as the main source of variation and the number of fetuses per doe as a covariate. Mortality rate was assessed with a χ^2 test. If significant main effects were detected, a Student's *t*-test was used to compare averages among groups ($P < 0.05$). For assessing morphometric data, the four fetuses measured per mother were averaged to result in one data point. The relationships between morphometric changes and hemodynamic parameters were measured by Pearson correlation procedures. Regarding maternal body weight, statistical analysis was performed with an ANOVA and the weight at the beginning of the trial as a covariate. Feed intake during pregnancy in the control group was assessed by a repeated measure analysis with time as the main effect. All data were reported as means ± S.E.M. and probabilities were considered significant at $P < 0.05$.

Results

Maternal status

Regarding maternal body weight, no differences were found at the day of the artificial insemination (4.30 ± 0.008 *v.* 4.34 ± 0.010 kg, in control and underfed groups, respectively) nor after delivery (4.44 ± 0.037 *v.* 4.42 ± 0.081 kg). Similarly, feed intake did not change significantly along pregnancy in the control group (216.16 ± 10.60 g/day in the 1st week; 236.33 ± 18.08 g/day in the 2nd week; 218.85 ± 22.14 g/day in the 3rd week; 176.31 ± 22.14 g/day in the 4th week of pregnancy; $P = 0.22$).

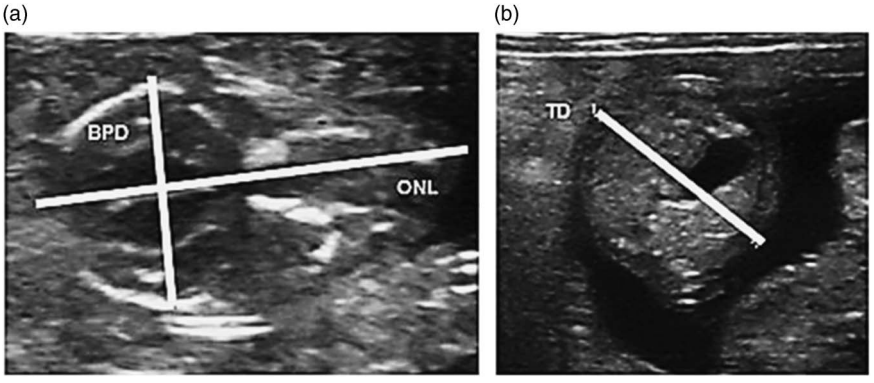

Fig. 1. Ultrasound image of the head (*a*) and the thorax (*b*) of a rabbit fetus at day 21 of pregnancy. ONL, occipito-nasal length; BPD, biparietal diameter; TD, thoracic diameter.

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Morphometry of fetuses and offspring

Table 1 depicts the morphometric data of fetuses and kits. On day 21 of pregnancy maternal underfeeding was related to a reduction in fetal size, specifically in a shorter occipito-nasal length ($P < 0.05$), while biparietal and thoracic diameters were similar in control and underfed fetuses. At birth, a total of 43 newborns from control group and 53 in the underfed group were included in the study. Kits from the underfed group showed the lowest size (in terms of crown-rump length, biparietal and thoracic diameters; $P < 0.05$) and a decreased weight when compared with that of the control group ($P < 0.05$). Time of delivery was not affected by the feed restriction (all females delivered on day 31 of pregnancy). No difference was obtained between groups in the mortality rate at birth (1 out of 44 and 3 out of 56 in control and underfed groups, respectively; $P > 0.05$).

Doppler evaluation of fetoplacental hemodynamics

At the UCAs, significantly higher SPVs and time-averaged mean velocities ($P = 0.02$ and $P = 0.01$, respectively) were

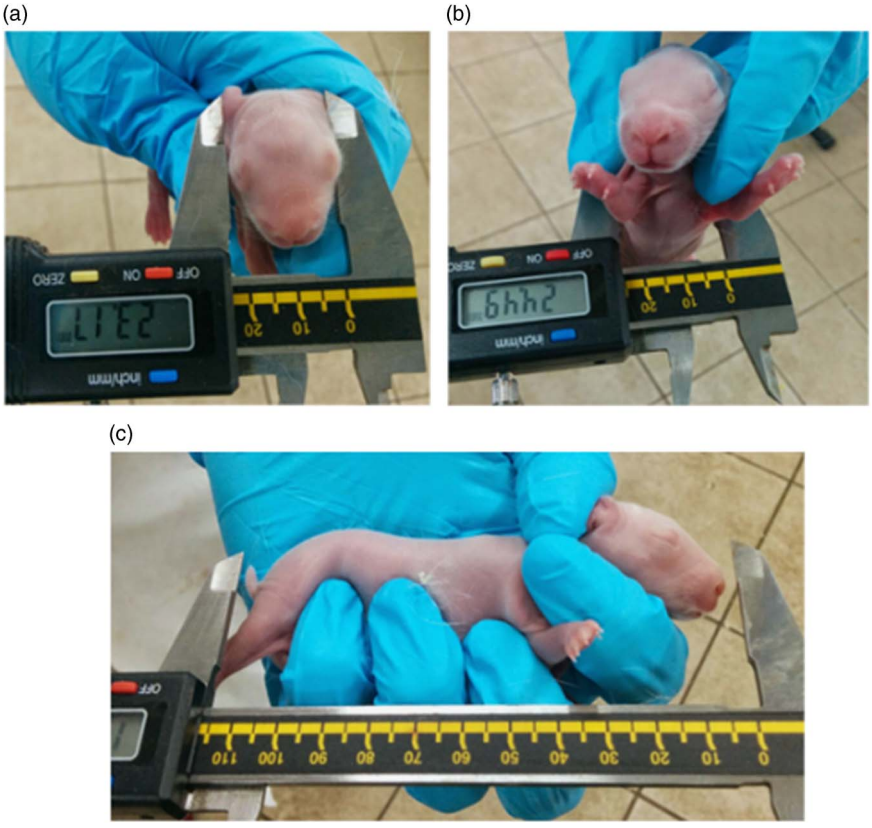

Fig. 2. Measurements obtained at birth: (*a*) biparietal diameter, (*b*) transversal thoracic diameter and (*c*) crown-rump length.

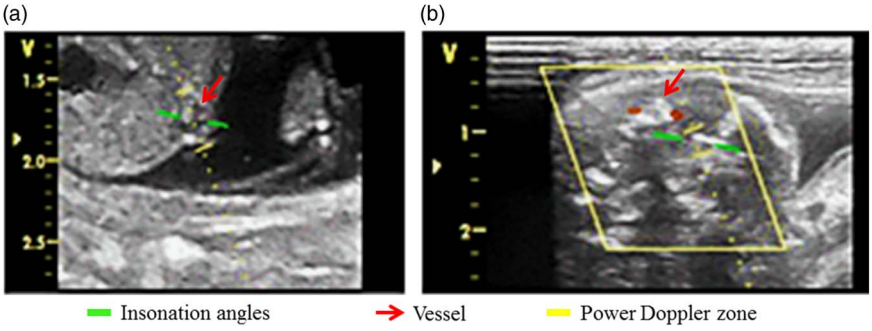

Fig. 3. Doppler image of UCA (*a*); and MCA (*b*) of a rabbit fetus at day 21 of pregnancy. UCA, umbilical cord artery; MCA, middle cerebral artery.

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Table 1. *Offspring development of rabbit does in the control group and underfed group at day 21 of pregnancy and at birth*

	Control group	Underfed group
Fetal parameters	$n = 16$	$n = 20$
Occipito-nasal length (cm)	1.92 ± 0.07^{a}	1.60 ± 0.05^{b}
Biparietal diameter (cm)	1.02 ± 0.02	1.0 ± 0.02
Thoracic diameter (cm)	1.34 ± 0.04	1.32 ± 0.03
Kits parameters	$n = 43$	$n = 53$
Mortality rate (%)	2.43 (1/44)	5.35 (3/56)
Crown-rump length (cm)	10.75 ± 0.14^{a}	10.21 ± 0.12^{b}
Biparietal diameter (cm)	2.26 ± 0.02^{a}	2.03 ± 0.02^{b}
Thoracic diameter (cm)	2.37 ± 0.03^{a}	2.15 ± 0.03^{b}
Weight (g)	53.36 ± 1.70^{a}	48.0 ± 1.51^{b}

[a,b]Different superscripts within a row indicate significant differences among groups ($P < 0.05$).

observed in the underfed fetuses when compared with the control group (Table 2). Whereas, fetuses from the underfeed group showed a decreased EDV ($P = 0.05$). Indexes of pulsatility ($P = 0.07$) and resistance ($P = 0.06$) tended to be higher in the underfed fetuses, without being statistically significant.

There were no significant differences in the hemodynamic parameters at the MCA between both groups at any analyzed variable. Finally, the cerebroplacental ratio for MV was significantly lower in the underfed fetuses ($P = 0.02$).

Relationships between morphometric changes and hemodynamic parameters

Correlations between fetal size and hemodynamic features obtained by the Pearson procedure showed significant differences between the two nutritional regimes. In the control and underfed groups, the smallest fetuses, with smaller occipito-nasal length, had a lower EDV at UCA ($r = 0.671$, $P = 0.03$). There were no other significant effects in the control group; conversely, in the smallest fetuses of the underfed group, those affected by a more severe IUGR, a lower occipito-nasal length was also related to lower pulsatility and RIs ($r = 0.502$, $P = 0.03$; $r = 0.673$, $P = 0.02$, respectively), SPV and MV ($r = 0.763$ and $r = 0.698$, respectively; $P = 0.006$ for both) at the UCA. Moreover, restricted IUGR fetuses who had a smaller thoracic diameter showed increased cerebroplacental ratios for SPV and MV ($r = 0.524$, $P = 0.01$ and $r = 0674$, $P = 0.004$, respectively). Finally, a smaller biparietal diameter was related to an increased cerebroplacental ratio for EDV ($r = 0.394$, $P = 0.04$).

Discussion

The results of the present study show that underfeeding pregnant rabbits, at a level that does not affect maternal body weight, can be considered a useful model for IUGR studies. The level of restriction affected fetal development by reducing

Table 2. *Fetal hemodynamic parameters obtained from rabbit does in the control group and underfed group at day 21 of pregnancy*

	Control group ($n = 16$)	Underfed group ($n = 20$)
Resistance index		
UCA	0.79 ± 0.01	0.85 ± 0.01
MCA	0.71 ± 0.02	0.74 ± 0.02
Cerebroplacental ratio	0.90 ± 0.03	0.86 ± 0.03
Pulsatility index		
UCA	1.33 ± 0.04	1.42 ± 0.03
MCA	1.10 ± 0.04	1.20 ± 0.07
Cerebroplacental ratio	0.83 ± 0.04	0.84 ± 0.05
Systolic peak velocity		
UCA (cm/s)	26.71 ± 2.29^{a}	37.18 ± 3.33^{b}
MCA (cm/s)	18.41 ± 1.41	19.00 ± 1.19
Cerebroplacental ratio	0.70 ± 0.08	0.51 ± 0.05
End diastolic velocity		
UCA (cm/s)	5.40 ± 0.39^{a}	5.22 ± 0.35^{b}
MCA (cm/s)	5.39 ± 0.32	4.90 ± 0.41
Cerebroplacental ratio	1.07 ± 0.09	0.94 ± 0.09
Time-averaged mean velocity		
UCA (cm/s)	16.05 ± 1.26^{a}	22.40 ± 1.73^{b}
MCA (cm/s)	12.80 ± 0.99	11.60 ± 0.52
Cerebroplacental ratio	0.79 ± 0.09^{a}	0.53 ± 0.05^{b}

UCA, umbilical cord artery; MCA, middle cerebral artery.
[a,b]Different superscripts within a row indicate significant differences among groups ($P < 0.05$).

the prenatal growth of the offspring and therefore their size and weight at birth, without being associated to a higher mortality rate. Our data also indicate that IUGR fetuses develop compensative 'brain-sparing effect' for preserving adequate blood supply to the brain, with this effect being compromised in fetuses with severe IUGR. Thus, these data are similar to that previously reported in humans at 70% pregnancy.[26] Our study also confirms the adequacy of the use of ultrasound scanning for the assessment of fetal characteristics, reinforcing previous studies in rabbits.[20] This finding gives a way for future translational studies in this model since, in humans, ultrasound technology is widely used in prenatal care to estimate gestational age, to assess fetal growth and to determine physical abnormalities.[27] Moreover, fetal biometry during pregnancy is a confirmed method to predict probable later adverse perinatal outcomes.[28,29]

In the current study, ultrasonography showed fetal morphometric changes that were induced by maternal nutritional restriction at 70% pregnancy. Underfed fetuses showed a smaller occipito-nasal length than fetuses in the control group, without any other major changes. The occipito-nasal length is a more

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accurate indirect measure for fetal development than other parameters since the skull remains in a good examinable position[30] and the hyperechogenic limit of the bones enables an easier measurement. From the morphometric changes, the limited impact of maternal restriction on embryo growth, together with the fact that rabbit and other species including human fetuses show a faster body growth toward the end of the pregnancy can be explained.[31] In the early pregnancy, embryo–fetal needs are scarce, with maternal metabolism being anabolic in this period[32] to ensure enough energetic reserves. Moreover, the does in our study were at optimal body weight at the beginning of the pregnancy and could have partially satisfied the low fetal growth requirements during the two first thirds of pregnancy without needing to mobilize their own body reserves.

The last third of pregnancy is characterized by maximum fetal growth rate and, therefore, due to the need for a higher amount of nutrients; maternal metabolism becomes catabolic,[33] and nutritional restriction during this phase of pregnancy normally has a severe impact on fetal growth, which caused the differences found at birth. In our study, feed intake was not significantly altered during the pregnancy in the control group, neither the maternal weight; this was consistent with other studies in rabbits with different levels of restriction showing no differences in maternal weight at the end of the trials.[34,35] Hence, we can hypothesize that despite the fetuses requiring a higher level of resources near pregnancy term, the mother may have invested her limited resources for her own metabolism, subsequent lactation and in her future reproduction instead of her litter growth or placental development.[36] There was not a higher mortality rate among restricted newborns, demonstrating that the maternal 'decision' of not dedicating more energy to their litter was right, in the view of survival rate. Probably a more severe restriction would induce changes in weight of the mothers, but further research is necessary to explore this issue and to determine if it would be a limiting factor in the translational value of the rabbit model.

The results obtained after the evaluation of the fetal blood flow by Doppler ultrasound in the present study reinforce the use of rabbits as a model for IUGR studies. Fetuses showed a high flow velocity at the umbilical arteries, resembling values in humans at the second trimester of pregnancy.[37–40] We focused our research on the umbilical artery since previous studies have shown that the measurement of insonation at umbilical arteries is easy to obtain and offers reliable data on blood exchange with the placenta than insonation of other vessels.[41] Hence, a high blood flow induces high PI and RI values, like in humans.[39]

In IUGR pregnancies, the increase in the resistance at the small arteries and arterioles of the villi, decreases the diastolic flow, which raises the MV at the UCAs.[40–42] Concomitantly, in the present study we observed that fetuses exposed to maternal underfeeding evidenced a lower EDV, higher systolic peak and time-averaged mean velocities, with a trend for higher indexes of pulsatility and resistance when compared with those of the control group. These alterations, usually detected in IUGR, are related to a lower supply of oxygen and nutrients to the fetus as a consequence of a placental ischemia.[42] Placental ischemia is considered the ultimate cause for IUGR and is associated with high perinatal morbility and mortality.[43] It could be hypothesized that fetuses in the underfed group were in the first stages of IUGR and demanded more blood flow to try to increase blood exchange rate,[43] since the nutrient content and oxygen supply were reduced. Consequently, the differences found in the current study could indicate a lower blood perfusion in fetuses from underfed mothers caused by the nutritional restriction.

Fetuses at IUGR risk, under circumstances such as poor nutrition or inadequate placental supply, develop a redistribution of blood circulation to increase the blood supply to the fetal brain. This phenomenon is known as 'the compensatory brain-sparing effect'[44] and its purpose is to assure adequate brain development and function, since a failure of this causes a broad spectrum of adverse neurological outcomes, thus compromising postnatal vitality and survival of the neonate.[45,46] In human medicine, the occurrence of brain sparing consecutive to IUGR has been identified by assessing Doppler ratios.[44] The cerebroplacental ratios quantify the redistribution of the cardiac output by dividing Doppler index from cerebral and fetoplacental vascularity. In this scenario, umbilical arteries represent the right ventricular afterload, whereas, the MCA reflects the left ventricular afterload.[47]

The brain-sparing effect was manifest in the underfed fetuses of the current study (significantly lower cerebroplacental ratio and a trend for higher PI); this is evidence of a redistribution of the cardiac output to the cerebral circulation (Table 2). Our study also identified the weakness and even the failure of this compensatory mechanism in those fetuses suffering a more severe IUGR, those with smaller biparietal and thoracic diameters, as evidenced by the Pearson correlations.

Our results indicate the reliability and usefulness of the rabbit model and of the Doppler procedure for the study of IUGR. However, we cannot leave aside some limitations of this study. The first limitation would be a systematic assessment of the changes in IUGRs throughout the entire pregnancy. However, we preferred limited scanning sessions since repeated examinations of the pregnant does could have caused stress-induced abortion, even in trained animals, especially during the last third of pregnancy, when the decidua, the maternal side of the placenta is thinner and less attached.[48] A second limitation would be the small sample size used in this study, which could have reduced the power of the study and masked significant differences. Finally, more information about the UCA diameters could have been relevant, there can be errors when measuring vessel diameter, in particular for vessels with a high pulsating blood flow.[49] In our case those measurements were more complicated since no anesthetic drugs were used, since they could interference with maternal uteroplacental blood flow.

In conclusion, our data indicate that rabbit fetuses exposed to maternal underfeeding are prone to show a disrupted developmental trajectory and, due to this restriction, to

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undergo IUGR. IUGR fetuses were able to develop compensative brain-sparing effects in a similar way to that previously described for humans. Thus, this study supports the rabbit as a reliable translational model for studies on IUGR associated with feed restriction and associated diseases. The use of the Doppler technique to explore umbilical arteries, as well as the estimation of the cerebroplacental ratios led to the early detection of fetal blood flow alterations and should be taken in consideration as a primary diagnosis tool. The information herein could be of interest to researchers studying the vascular adaptation and/or deterioration processes and help in exploring possible therapies in experimental models of IUGR.

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Conflicts of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (European Union Directive about the protection of animals used in experimentation and the Spanish policy for animal protection RD53/2013) and has been approved by the institutional committee (Polytechnic University of Madrid), which meets the requirements of the European Union for scientific procedure establishment, under project license of the UPM Scientific Ethic Committee.

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