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Cauterization of Meso-ovarian Vessels, a New Model of Intrauterine Growth Restriction in Rats

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ABSTRACT

Intrauterine growth restriction (IUGR) remains an important cause of perinatal morbidity and mortality. Both IUGR and low birth weight have been identified as risk factors for increased incidence of cardiovascular disease, dyslipemia, and other diseases in the adulthood.

Several animal models have been developed to study the underlying mechanisms of IUGR and its later consequences, with utero-placental ischemia by uterine artery ligation (UAL) being the most frequently used in rats. The relevance of this model lies in the fact that it induces altered placental perfusion, the main cause of IUGR in humans in Western countries. However, there is also controversy over the grade and homogeneity of IUGR obtained.

In this study, we propose a new animal model of IUGR related to placental ischemia through the cauterization of meso-ovarian vessels. We aimed to test the feasibility of meso-ovarian vessel cauterization (CMO), and to compare it with uterine artery ligation (UAL). The CMO group had similar incidence of perinatal mortality, percentage of IUGR, and evolution of body weight during early extrauterine life to the UAL group, indicating that both methods are similarly efficient for inducing IUGR. Moreover, both of them affect the ratio of fetal to placental weight, and the weight of vital organs, supporting the hypothesis of a fetal compensatory response or “brain- and heart-sparing effect”. Both operative models suffer approximately 50% perinatal mortality, implying that they are both more efficient in the production of IUGR when C-section is performed. On the other hand, CMO was significantly faster to perform than UAL and seemed to produce a more uniform ischemia throughout the uterus than the UAL method, resulting in a more homogeneous group of IUGR pups.

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1. Introduction

Intrauterine growth restriction (IUGR) remains a common and important cause of perinatal and neonatal morbidity and mortality [1]. The period of *in utero* development is sensitive to nutritional constraints that are postulated to permanently alter pivotal homeostatic processes throughout life. These changes, or fetal programming, may confer some advantages to the offspring [2], but

can also predispose an individual fetus to diseases after birth and in adulthood [3,4]. IUGR and low birth weight have been identified as risk factors for increased incidence of cardiovascular disease, dyslipemia and diabetes [4–7].

Several approaches have been developed in animal models to study the underlying mechanisms of IUGR, such as exposure to alcohol [8], antineoplastic drugs [9], synthetic thromboxane A₂ analogues [10], nutritional constraints [11,12], hypoxic stress [13], or surgical manipulation [14–19]. This last group, based on the induction of uterine ischemia, is highly relevant because it might mimic what happens in altered placental perfusion, the main mechanism involved in IUGR in humans in developed countries [7,20].

Wigglesworth showed that uterine artery ligation (UAL) during the last third of gestation in rats produces IUGR [14]. In this model, ischemia is produced by a single localized ligation of the uterine

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arteries, inducing different grades of IUGR, depending on the position of the fetus with respect to the ligation [14,21]. Although it has been widely used to study the consequences of IUGR [22–26], this model has some limitations, such as a high incidence of perinatal mortality and fetal demise and a large variation in fetal weight depending on the fetal position [14,23,24]. Moreover, a recent study has suggested that UAL and subsequent decreased placental flow is not an appropriate or reproducible rodent model of IUGR [27].

The aim of the present study was to test the efficacy and feasibility of a new model of IUGR in rats through cauterization of the meso-ovarian vessels (CMO). This included measurement of the weight of the placenta and the fetus/placenta weight ratio, anthropometric measurements of the newborns, analysis of the CMO-induced effects on the weight of the main fetal organs and description of the weight trajectory until day 16 of age.

Our goal was to obtain a new animal model of IUGR, also induced by decreased placental blood flow, but with a more uniform distribution of utero-placental ischemia resulting in a more homogeneous population of fetal weights.

2. Methods

2.1. Animals

Pregnant female Wistar rats (250–300 g) from Harlan Laboratories were housed in individual cages, maintained in 12-h light/dark cycles, and allowed free access to a standard rodent diet and water. Animal care and the conduct of all experiments were approved by the Animal Ethics Committee of the University of Barcelona.

2.2. Outline of the experiments

See Table 1.

2.3. Anesthesia and surgery

All the interventions were performed on day 17 of gestation. Under general anesthesia with isoflurane 2.5%, the dams underwent midline laparotomy in order to expose the uterine horns and their vascularization. The surgical technique corresponding to the assigned experimental group was performed. Dams with more than 14 or fewer than six fetuses were excluded before surgery to provide a more

homogeneous population. Upon completion of the procedure, the uterine horns were replaced in the abdominal cavity and the incision closed by suturing the abdominal wall layers.

- (i) *Control group – Sham operations*: both uterine horns and their vessels were exposed and examined, with no further manipulation.
- (ii) *UAL group*: the uterine arteries were bilaterally ligated near the cervical end of the arterial arcade with a 3-0 silk suture, according to a modified version of the method reported by Wigglesworth [14].
- (iii) *CMO group*: two meso-ovarian vessels located in the borders between the upper and medium and the medium and lower thirds of the corresponding horn were cauterized. An electric monopolar scalpel-shaped system (COAG-ULADOR 970/970B; position 4) was used for this purpose. See Fig. 1.

CMO and UAL were considered as intervention groups.

2.3.1. Procedure 1: study of placental and fetal weights after caesarean section

In order to study the placental weights and the fetus/placenta weight ratio, six mothers (two per group) were allocated to undergo surgical intervention on day 17 of gestation. All the placentas and fetuses were extracted by caesarean section on day 22 and subsequently weighted (Accuracy: 0.001 g; PB 153-L Mettler Toledo®). These fetuses were sacrificed after the caesarean section by anesthetic overdose.

2.3.2. Procedure 2: induction of growth restriction

Forty pregnant dams were randomized to one of the three experimental groups. After surgical intervention on day 17, the animals were allowed to deliver spontaneously (see Fig. 2 for the number of animals at each step).

Newborn animals were defined as growth restricted when their birth weight (BW) was <2SD of the mean BW of controls. This criterion is a close representation of the clinical significance of small for gestational age [14,15] and has been adopted in other animal models [17,18].

2.3.3. Procedure 3: repercussion of IUGR on organ weight

An independent group of seven dams (three of the UAL group, three of CMO and one of the control group) were operated on day 17, allowed to recover and to give birth spontaneously. All pups in the control group and in the intervention groups were sacrificed at day 2. Weights for brain, heart, thymus, liver and kidneys were obtained with a highly accurate digital scale (Accuracy: 0.001 g; PB 153-L Mettler Toledo®).

2.3.4. Procedure 4: follow-up

To assess changes in the weight in the offspring, a third experiment was carried out. From the 40 animals allowed to deliver spontaneously in procedure 2, 29 reached the end of pregnancy (7 from the control group, 11 from the UAL group and 11 from the CMO group). The newborn pups were counted and their body weights recorded on their second day of life. (Accuracy: 0.1 g; PB 3001-L Mettler Toledo®). To ensure sufficient and homogeneous milk supply throughout the litter, numbers were adjusted at day 2 to a maximum of four pups per mother, excluding the bigger and the smaller ones in both the control and intervention groups. Pups in each group were monitored and body weights determined every day until day 16.

2.4. Statistical analysis

Quantitative values are given as means with their standard deviation (mean ± SD). All statistics were analyzed using non-parametric tests with the package SPSS 14 for Windows. Differences in growth parameters, in organ weight at day 2 and in fetus/placenta ratio between groups were evaluated with the *Mann-Whitney U* and *Kruskal-Wallis tests* and weight evolution over time was compared with two-way analysis of variance (ANOVA). Correlation between parameters was assessed with the *Spearman coefficient*. Statistical significance was assigned to *P* values of <0.05.

3. Results

3.1. Procedure 1: study of placental and fetal weights after caesarean section

The analysis of the fetuses obtained by caesarean section on day 22 of gestation revealed significant differences between the weight of control and UAL fetuses ($P < 0.001$), and also between control and CMO fetuses ($P < 0.001$), with the pups in the intervention groups being significantly smaller. There were no IUGR fetuses in the control group, whilst there were six in the UAL group and seven in the CMO group. The difference in weight between intervention groups was not statistically significant. There were also significant

Table 1
Outline of the experiments.

Procedure 1: caesarean section at day 22		
Group	Mothers (n = 6)	Fetuses (n = 53)
Control	2	23
UAL	2	16
CMO	2	14
Procedure 3: spontaneous vaginal delivery. Pups sacrificed at day 2 for organ weight measurement. Number of IUGR fetuses in brackets		
Group	Mothers (n = 7)	Pups (n = 43)
Control	1	10
UAL	3	20 (5)
CMO	3	13 (5)
Procedures 2 and 4: spontaneous vaginal delivery. Weight at day 2, followed up until day 16. Only four pups per mother were followed up (see text)		
Group	Mothers (n = 29)	Pups (n = 158)
Control	7	65 (followed up: 28)
UAL	11	45 (followed up: 44)
CMO	11	48 (followed up: 44)

After surgical intervention on day 17 of gestation, the fetuses were either extracted by c-section on day 22 of gestation (procedure 1) or the dams were allowed to deliver spontaneously (procedures 2, 3 and 4). In procedure 1, the fetuses were weighted immediately after being extracted by c-section. In procedure 2, the pups obtained from dams in the 3 groups (7 controls, 11 CMO, 11 UAL) were weighted on day 2 in order to validate the capacity of the interventions to induce IUGR. In procedure 3, all the pups from 7 dams (1 control, 3 CMO, 3 UAL) were sacrificed on day 2 and dissected in order to analyze the differences in organ weight between them. In procedure 4, the litters from dams in procedure 2 (7 control, 11 CMO, 11 UAL) were reduced to a maximum of 4 pups per dam and their body weights were recorded daily up until day 16.

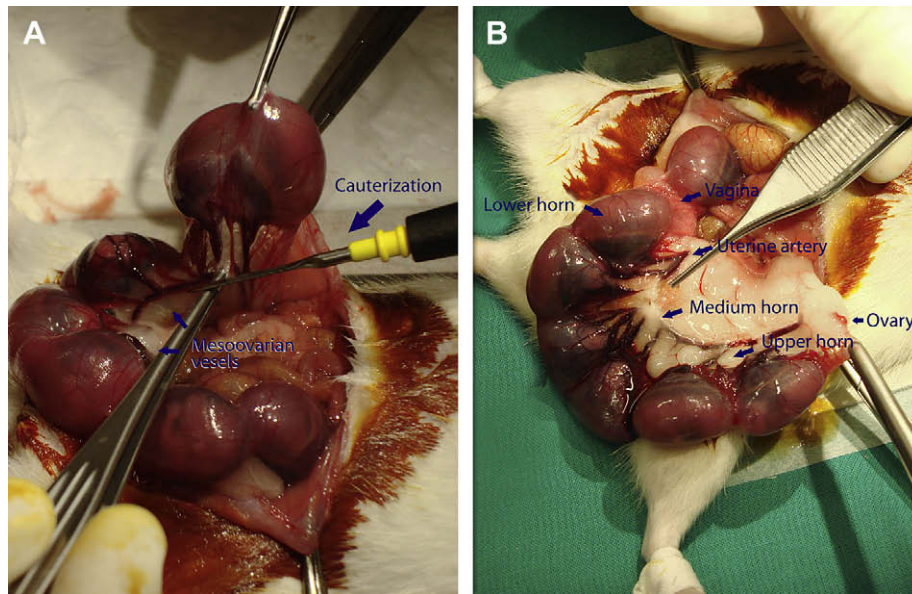


Fig. 1. CMO procedure. Two meso-ovarian vessels located in the borders between the upper and medium and the medium and lower thirds of the corresponding horn were cauterized. A: site of cauterization. B: distribution of the uterine vasculature after cauterization. Note the reduction of meso-ovarian vascularization immediately after performing the technique.

differences between the weight of control and UAL placentas ($P < 0.001$), and between control and CMO placentas ($P < 0.005$). The fetus/placenta weight ratio showed significant differences between control and CMO groups ($P < 0.05$), but not between control and UAL (Table 2).

3.2. Procedure 2: induction of growth restriction

Three of the mothers in the UAL and three in the CMO groups suffered from chorioamnionitis and were excluded from the study. 158 pups were born alive; 65 in the control group, 45 in UAL and 48

in CMO (Fig. 2). All of the pups were born between days 21 and 23 of gestation. No difference in the time of delivery among the three groups was found (control: 21.84 ± 0.56 ; UAL: 21.63 ± 0.76 ; CMO: 21.80 ± 0.45 ; $P = 0.16$).

The weight of pregnant dams at day 17 of gestation correlated significantly with the number of fetuses per mother ($P < 0.001$), but not with the number of IUGR pups per litter (data not shown). No correlation was found between the number of fetuses and the weight of the pups on day 2 of life ($r = -0.11$; $P = 0.05$).

Control group (Sham operated): 65 pups were born to mothers included in this group (Table 3). All the weights of the pups were

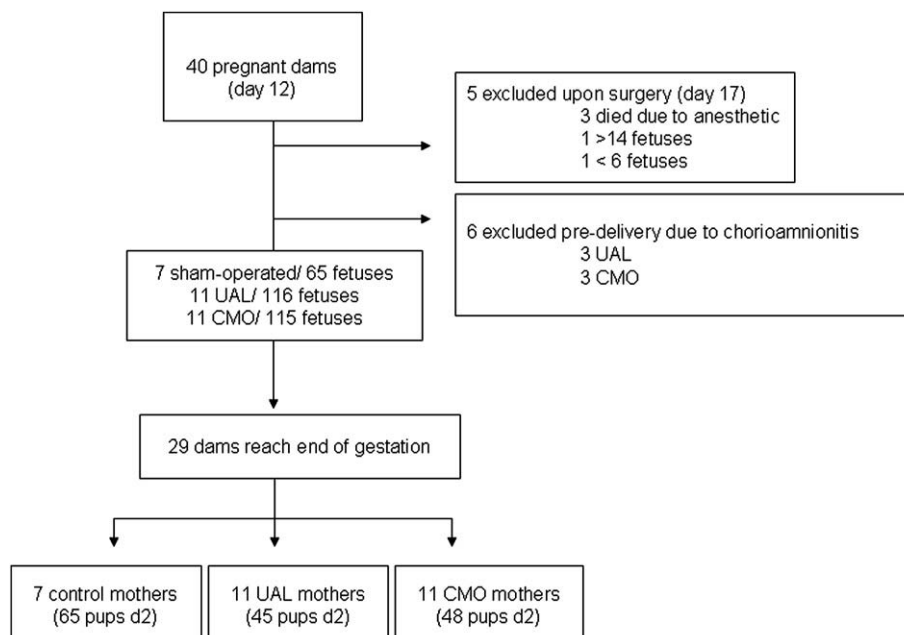


Fig. 2. Flow diagram of the animals used in the procedures 2 and 4. d2 stands for postnatal day 2.

Table 2
Caesarean section at day 22: fetal and placental weights.

	Control	UAL	CMO
Fetal weight (g)	5.39 ± 0.45	4.57 ± 1.03*	4.93 ± 0.57*
Placental weight (g)	0.77 ± 0.23	0.55 ± 0.15*	0.57 ± 0.14*
Fetus/placenta	7.01 ± 1.6	8.3 ± 2.7	8.64 ± 2.1*

Results are expressed as mean and SD. * $P < 0.05$ when compared with control.

within ± 2 SD of the mean. None of the mothers suffered surgery complications or infection. The average time of surgery was 13.6 ± 4.3 min. There were no IUGR pups (weight 2 SD below the mean of the control group; 4.98 g) in this group.

UAL group: 116 fetuses were identified during surgery but only 45 were alive on day 2 after birth (Table 3). Four pups had weights that were more than 2 SD above the mean weight for the control group. Nine live pups (20%) were IUGR. The average time of surgery in this group was 19.6 ± 4.9 min.

CMO group: Out of 115 fetuses counted on day 17 of gestation 48 were alive on day 2 (Table 3). Eleven live pups (23%) were growth restricted. The average time of surgery in the CMO group was 14.3 ± 2.4 min.

The mean weight of the pups at day 2 in both the intervention groups (UAL and CMO) was lower, without statistically significant differences between them. The range of pups' weights was nevertheless different, with fetuses over 2 SD in the UAL group but not in the CMO group, in which the range of weights was narrower. The homogeneity of the pups' weights in each group was evaluated using the Pearson's coefficient of variation ($CVx (\%) = 100 \times SD/\text{mean}$). The values of CVx are 20% in the UAL group, 11% in the controls and 15% in the CMO group showing that between UAL and CMO, the CMO technique renders a more homogeneous population. In both interventional groups the distribution of weights is skewed towards the lower values, so that the mean and the median are different (Fig. 3 and Table 3)

3.3. Procedure 3: reperfusion of IUGR on organ weight

3.3.1. Analyzing all pups together (see Table 4)

In the analysis of organ weight from all the animals in the intervention groups (including those with weight over 2 SD), the differences between the intervention groups and controls in the weights of the kidney (only in CMO) and the thymus reached statistical significance ($P < 0.05$), being smaller in the interventional groups. Table 4 shows the weights of the organs of the control, UAL and CMO groups of pups sacrificed on day 2.

Regarding the ratios of organ weight/fetus weight, there were no differences between controls and intervention groups, but the "brain to fetus weight ratio" had a tendency to be increased in the CMO group when compared to controls, although this tendency did not reach statistical significance ($P = 0.07$), possibly due to small number of pups in the control group. No differences in the brain to

fetus weight ratio were detected between the control and UAL groups ($P = 0.8$).

3.3.2. Analyzing only IUGR pups in the interventional groups (see Table 5)

When only the IUGR animals were analyzed, there were significant differences in the weight of the thymus ($P < 0.01$), liver ($P < 0.05$) and kidney ($P < 0.05$) in the CMO group when compared to controls. The differences between UAL animals and controls regarding organ weight did not reach statistical significance, except for the thymus ($P < 0.05$). No significant differences were observed between intervention group IUGR animals and controls in the weights of the heart ($P = 0.355$) or brain ($P = 0.231$). With regards to the ratios of organ weight/pup weight, a significant difference between the CMO group and controls was found specifically for the ratio of brain weight/pup weight ($P < 0.05$), but not between the UAL group and controls (Table 5).

3.4. Procedure 4: growth restriction and follow-up

The weight trajectory from day 2 to day 16 was similar in both interventional groups (UAL and CMO) and both were statistically different when compared with the control group ($P = 0.0001$ and $P = 0.001$, respectively). No catch-up growth was observed in neither of the interventional groups (Fig. 4) up to day 16.

4. Discussion

The ligation of the uterine artery causes a complete obstruction of the blood supply to the fetuses located in the most caudal portion of the uterine horn, resulting in death and partial absorption of about 30–85% of the fetuses [14,21,24,27].

In order to obtain a more homogeneous distribution of utero-placental ischemia, we developed the present model, based on electrical cauterization of the meso-ovarian vessels (CMO) along the uterine horns of pregnant rats. Technically, no sophisticated materials are needed and surgery is faster than with uterine artery ligation (UAL).

In both UAL and CMO models, IUGR is related to lower placental weight compared to controls, as shown by the results of the caesarean sections on day 22. This supports the hypothesis that alterations in placental circulation with decreased placental mass induce intrauterine growth restriction in the fetus, reviving the old concept that the fetus/placenta coefficient is a good indicator of fetal restriction [28].

The body weights of the newborn rats after CMO were significantly lower than those in the control group ($P < 0.0001$) and the percentages of growth restricted animals in the CMO (23%) and UAL (20%) groups were similar, indicating that both methods are equally efficient for inducing IUGR. In both interventional models the distribution of weights is skewed towards the lower values, so that the mean and the median are different. These new reported data provide unique information which can rarely be found in the

Table 3
Comparison of CMO and UAL models with the control group.

Model	Mothers	Fetus		Litter		Weight				IUGR		
		n	n	n/mother	n	Mortality	Mean	SD	Median	Range	n	%
(i) Control	7	65	9.2	65	0.0	6.50	0.76	6.22	5.07	7.91	0	0
(ii) UAL	11	116	10.5	45*	61.28*	6.04*	1.20*	5.89	3.64	9.64	9*	20
(iii) CMO	11	115	10.4	48*	58.27*	5.87*	0.93*	5.7	3.30	7.29	11*	23

Mean weight, mortality and IUGR percentage. Weight in grams; n/mother: number of fetus per mother; * $P < 0.05$ when compared with control.

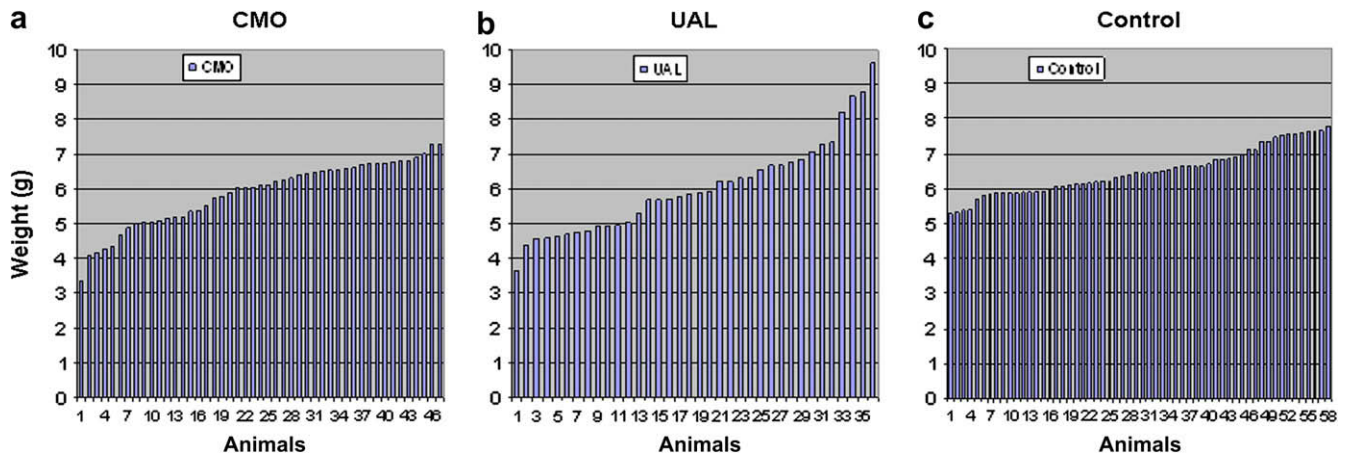


Fig. 3. Histograms of the birth weight distribution in the three groups: (a) CMO; (b) UAL; (c) control.

literature on such animal models, suggesting the importance of expressing the results in mean and median for further investigations. However, the pups in the CMO group were more homogeneous than in the UAL. All pups in the former group had body weights below the mean + 2 SD of the control group range, which was not the case in UAL. None of the intervention models had an impact on the timing of spontaneous delivery, and the population of manipulated pups was the same gestational age as the control one.

We propose that the CMO method produces a more uniform ischemia throughout the uterus and therefore affects all implanted fetuses more homogeneously than the UAL method, in which the degree of restriction depends on the particular site of implantation relative to the ligation [14,21]. According to the data obtained in procedure 1, no weight gradient can be observed between the fetuses in the CMO group. The animals which were under the cauterization zone were smaller or miscarried but there were no animals more than 2 SD above the mean in this group and the variation between the weight of the animals is smaller than in the UAL group. We are currently investigating the possibility of testing this hypothesis experimentally by introducing a mark on the fetuses at the moment of the intervention or by delivering the pups by caesarean section.

Although the perinatal mortality in the experimental groups was high, there were no differences in this between the UAL and CMO groups. Similar mortality rates have been reported by other authors [23,24] and this may reflect the incompatibility with life of severely growth restricted fetuses. The high perinatal mortality after the intervention implies that both models would be more efficient for the induction of IUGR when pups are delivered by a cesarean section.

Table 4

Organ weight on postnatal day 2: thymus, liver, kidney, heart and brain weights; all the animals of the interventional groups included.

	Organ weight			Organ/animal weight ratio		
	Control	UAL	CMO	Control	UAL	CMO
Thymus	18.7 ± 1.8	18.8 ± 1.4Δ	14.1 ± 1.8*	3.4 ± 1.5	2.5 ± 0.5	2.6 ± 1.2
Liver	266.8 ± 40	265.3 ± 49	235.8 ± 45	43.6 ± 7.7	42.1 ± 8.3	44.8 ± 13
Kidney	32.6 ± 6	39 ± 13	27 ± 5*	5.2 ± 0.9	6.3 ± 3	5.7 ± 0.9
Heart	38.7 ± 9	43.9 ± 14	35.9 ± 7.5	13.8 ± 1.7	6.8 ± 1.8	6.9 ± 1.4
Brain	246.7 ± 20	257.8 ± 44	212.7 ± 36	40.2 ± 2.8	40.6 ± 6	41.3 ± 9.1

Weight is expressed in mg. * $P < 0.05$ (CMO compared to control). Δ $P < 0.05$ (UAL compared to control).

Our data on the weight of organs from pups sacrificed on the second day of life is similar to that found in an earlier report by Wigglesworth [14]. When compared to controls, the IUGR pups obtained by CMO intervention showed a significant reduction in the absolute weight of the thymus, liver and kidney, but not in that of the heart or brain. When organ weights are considered relative to body weight, the changes in thymus, liver, kidneys, and heart are not statistically significant whereas the relative weight of the brain is significantly increased ($P = 0.031$). This is consistent with the proposed pathophysiology of severe fetal growth restriction. Placental disease leads to a placental respiratory failure and fetal hypoxemia, that, in turn, triggers compensatory hemodynamic changes including blood flow redistribution towards essential fetal organs (brain and heart) at the expense of other systems (e.g. kidney, liver and thymus). This fetal compensatory response results in increased blood flow to the brain and is also called the “brain-sparing effect” [29].

In this study, induction of IUGR after CMO on day 17 of pregnancy leads to a persistent growth restriction without any sign of ‘catch-up’ in body weight at least until day 16 of extrauterine life. These results are comparable to those of Huizinga et al., obtained with another model of vascular alteration (UAL) [19]. As suggested in the literature, the timing of IUGR or other environmental changes could be critical for regulation of postnatal growth, and the limitations of substrate availability from day 17 of pregnancy could lead to a deregulation of postnatal growth in which no catch-up in body weight is observed [16,30]. If the IUGR-inducing intervention is performed some days later (day 19), as in the experiments by Simmons et al., “catch-up” growth in weight is observed after two months [31]. The possibility of a similar delayed “catch-up” growth in our model cannot be ruled out, and will need further investigation.

Table 5

Organ weight on postnatal day 2: thymus, liver, kidney, heart and brain weights; only IUGR animals in the interventional groups included.

	Organ weight			Organ/animal weight ratio		
	Control	UAL	CMO	Control	UAL	CMO
Thymus	18.7 ± 8	11.9 ± 4Δ	7.9 ± 5**	3.4 ± 1.5	2.9 ± 1	1.8 ± 1.1
Liver	266.8 ± 40	238 ± 60	204.9 ± 45*	43.6 ± 7.7	53.2 ± 12	46.6 ± 10
Kidney	32.6 ± 6	25.8 ± 9	24.5 ± 3*	5.2 ± 0.9	6 ± 1.3	5.6 ± 0.7
Heart	38.7 ± 9	35.1 ± 3	34 ± 4	13.8 ± 1.7	8 ± 1.4	7.8 ± 0.7
Brain	246.7 ± 20	227.5 ± 55	230.6 ± 50	40.2 ± 2.8	40 ± 10.2	52 ± 9.3*

Weight is expressed in mg. * $P < 0.05$ (CMO compared to control), ** $P < 0.01$ (CMO compared to control). Δ $P < 0.05$ (UAL compared to control).

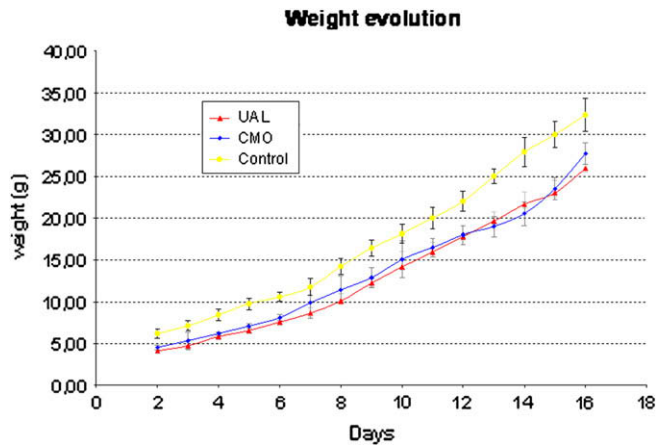


Fig. 4. Changes in weight from day 2 to day 16 of extrauterine life.

In summary, CMO has a comparable degree of perinatal mortality but its effect on the fetus/placenta weight ratio is more remarkable than in the UAL model. A brain-sparing effect can also be observed in both models. The main advantages of CMO are the high homogeneity of the offspring regarding birth weight and that CMO is a technically faster surgical technique than UAL. We conclude that CMO is a feasible technique to study IUGR and that it provides a useful alternative to the UAL method.

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