

## Original Article

## A Model of Intrauterine Growth Restriction Induced by Bilateral Uterine Vessel Clamping in Pregnant Rats

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### ABSTRACT

**Background:** Intrauterine growth restriction (IUGR) is considered to be a major risk for cerebral palsy and disability after birth. However, IUGR lacks effective treatment strategies. We aimed to establish a rat model of bilateral uterine artery ischemia-reperfusion as a surgical model of IUGR.

**Methods:** At the 17th day of pregnancy, six Sprague-Dawley (SD) rats were randomly divided into sham (NOR) and growth restriction (GR) groups. In the GR groups, four small artery clamps were used to occlude the uterine vessels near the lower and upper ends of the bilateral horns for 25 min; then, the clamps were removed, and the horns were placed back into the abdomen cavity. In the sham-operated animals, the uterine horns were exposed for 25 min without artery clamping. After labour and delivery, the death rates and weights during the first 8 days of newborn rats were recorded. A pathological examination was performed with hematoxylin-eosin staining (HE) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling staining (TUNEL) on 4-day-old and 8-day-old brain tissues of the newborn rats.

**Results:** The mortality within the first 24 h of rats in the GR group were increased (66.7% vs 0.0%), and their daily weights within the first 8 postnatal days were decreased. In addition, the karyopyknosis percentages of 4-day-old and 8-day-old rats were increased (4-day-old: 45% vs 11%; 8-day-old: 34.7% vs 5.3%), and the numbers of TUNEL-positive cells were increased (4-day-old: 110 vs 12.7; 8-day-old: 146.7 vs 4).

**Conclusions:** This model of foetal IUGR produced a decrease in weight, an increase in the death rate within 24 h and an increase in apoptosis in newborn rats. The establishment of the model was successful. (Funded by the National Natural Science Foundation of China, the Major State Basic Research Development Program, and the Grants from Science and Technology Bureau of Sichuan Province.)

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Intrauterine growth restriction (IUGR) is a common disease during the perinatal period. The specific definition of IUGR is a foetus whose estimated weight is below the 10<sup>th</sup> percentile or beyond two standard deviations for its gestational age and a neonatal birth weight below 2,500 g after full-term pregnancy (1, 2). Based on current epidemiological

analysis and clinical evidence, the morbidity of IUGR is approximately 5% to 12% in the perinatal period, and IUGR likely accounts for 10%-15% of pregnancy complications (3-5). IUGR has adverse effects on early life. IUGR is reported to be the second leading cause of neonatal death after premature delivery. Moreover, IUGR is the leading cause of

cerebral palsy and birth defects (6, 7). IUGR can also increase the incidence of cardiovascular disease, endocrine disease, cognitive impairment and mental disorders in adulthood, which burdens society and the family (8).

Pathological factors can contribute to IUGR. It has been shown that the mechanism of IUGR is generally related to inadequate placental blood flow (9), which can lead to placental insufficiency and reduction of oxygen delivery to the foetus. Clinical studies have demonstrated that neonatal brain injury frequently occurs in conjunction with prenatal hypoxic-ischemic insults (10). The primary cause of neonatal hypoxic-ischemic encephalopathy (HIE) is related to foetal intrauterine anoxia that occurs before delivery, which is the likely the leading cause of brain damage over the long term. Meanwhile, asphyxia neonatorum frequently causes HIE. Animal models of asphyxia in newborns, extremely preterm infants and preterm infants are usually established to investigate hypoxic-ischemic brain injury. In contrast, it is relatively infrequent that a model of IUGR is established to study the outcomes of foetal intrauterine anoxia.

Several animal models have been developed to study the mechanisms of IUGR. The frequently used species are rats, sheep, pigs, rabbits or lambs (11). Because rat has several advantages: short gestation, large litters and large enough for complex surgical intervention, so most of these studies have been performed in rats (12, 13). Experimental IUGR model induced by uterine vessel ligation was first described by Wigglesworth (14), other common models include maternal diabetes, stress, hypoxia, nutrient restriction, protein restriction (11, 12, 15). Compared with exposing pregnant animals to surgical interventions, it is inconvenient to alter maternal nutritional and hypoxic conditions during pregnancy. Additionally, surgical interventions include bilateral or unilateral uterine artery ligation, which lead to ischemia by decreasing placental flow and thus affect both placenta and fetus. But it is reported that uterine artery ligation shows several disadvantages. The surgery reproducibly lead to IUGR, however, followed by high maternal mortality, high fetal mortality and resorption (14, 16, 17). It was reported that in some cases all the fetuses within the ligated horn die and

had been partially resorbed (14). In contrary, bilateral uterine artery occlusion can offset these disadvantages to some extent. Transient occlusion of bilateral uterine vessels avoided high fetal mortality and resorption, obtained high surviving little size, and provided a possibility of investigating long-term effects of IUGR during postnatal brain development (18).

Transient occlusion of bilateral uterine vessels can cause ischemia-reperfusion and may lead to inadequacy of placental blood flow. This operation, which has been shown to have systemic effects on intrauterine factors, such as umbilical cord factors, placental factors and maternal factors in the foetus, can cause chronic placental insufficiency and foetal intrauterine anoxia. It has been commonly used as an IUGR model to understand the biological processes associated with foetal growth restriction (19-22).

As a consequence of the problems mentioned above, we made modification of occlusion time on the basis of previous study (18) according to preliminary experiments. In the current study, we set up an IUGR model in pregnant Sprague-Dawley (SD) rats, produced by transient bilateral uterine vessel occlusion, to measure foetal weights and mortality. We also investigated pathological changes in 4- and 8-day-old rat brains. Our data showed that bilateral uterine vessel ligation is an effective method to induce IUGR in a rat model.

## MATERIALS AND METHODS

### Animals

SD rats capable of bearing offspring were obtained from the Medical Animal Centre of Sichuan Province (Chengdu, Sichuan, CN). Rats were maintained on 12 h light/dark cycles and allowed free access to food and water. At oestrus, the males and females were housed for mating in a proportion of 1:2. If sperm was observed under a microscope from vaginal smears or plugs and a mixture of semen and vaginal secretions were found with the naked eye on the next morning (Figure 1), conception was determined, and the date was recorded as the first day of gestation. Then, the pregnant rats were housed in individual cages until the 17<sup>th</sup> day of gestation. Meanwhile, pregnancy weight parameters were recorded to determine whether conception was successful. All

animal procedures were approved by the Sichuan University Committee on Animal Research.

### Experimental Design

Six SD rats were randomly divided into a Sham group (NOR group) and an IUGR group (GR group) on gestation day 17. The IUGR animal model was constructed as previously described (19). Pregnant SD rats were anesthetized by intraperitoneal injection of 1% pentobarbital (40 mg/kg). After conventional sterilization, the uterus and its vasculature were removed from the abdominal cavity through a 3-4 cm incision in the abdomen. Bilateral uterine vessels were occluded for 25 min by arterial clamps followed by removal of the clamps to permit reperfusion (Figure 2). In the NOR group, a sham operation was performed that consisted of surgery without vasculature clamping. During the operation, the uterus and its vasculature were covered by gauze drenched with 37 °C normal saline to maintain body temperature. Moreover, the body and uterine surface temperatures were appropriately maintained with an infrared light. Then, the uterus artery pulse was re-established after removal of the arterial clamps. The uterus and its vasculature were returned to the abdomen to allow natural delivery of the pups. Penicillin (8,000 U) was injected into the abdominal cavity daily until the pups were delivered.

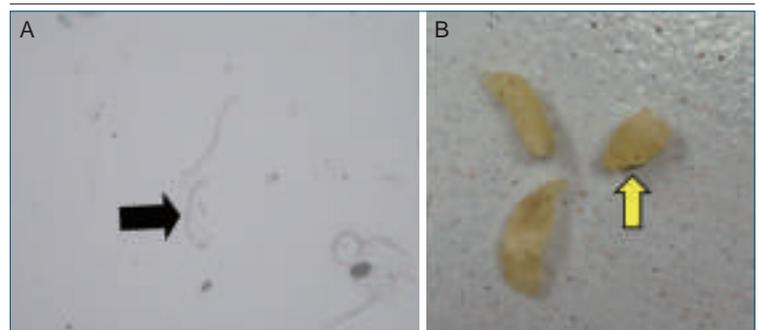
### Neonatal Weight and Calculation of Neonatal Mortality

The day of birth was defined as postnatal day 1 (PND 1). The weight of newborns was recorded daily until PND 8. Neonatal mortality was calculated as the number of viable newborns recorded within 24 h divided by the number of fetuses counted at the time of surgery. Data are presented as percentages.

The criterion for success in this model was designed by a significant weight reduction of 15% to 30% in the fetuses in the GR group as compared to those in the NOR group (13, 23).

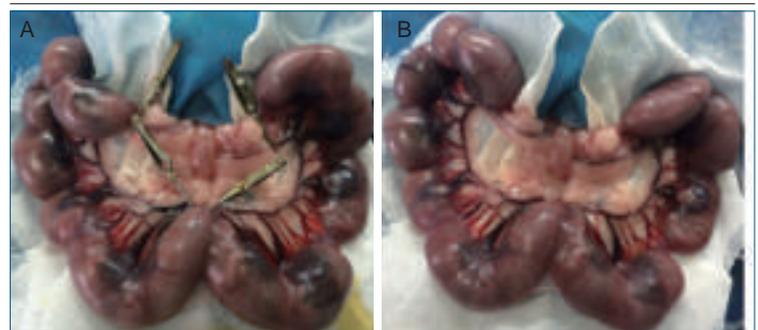
### Tissue Preparation and Evaluation of Brain Pathological Changes

On PND 4 and PND 8, one surviving fetus of each individual rat was anaesthetized with diethyl ether inhalation and transcardially perfused



**Figure 1. Signs of Pregnancy.**

A. Sperm under a microscope; B. Vaginal plugs.



**Figure 2. Establishment of the IUGR Model.**

A. Occluded uterine arteries with small artery clamps; B. Removal of the clamps to reperfuse the uterine arteries.

with saline solution, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.4. Newborns were sacrificed by decapitation on ice, and the brains were removed, dissected and post-fixed in 4% paraformaldehyde for 24 h at 4 °C. The brains were then embedded in paraffin, and serial sections were obtained every 4 mm through the target area and stained with haematoxylin and eosin (HE). The stained sections were examined microscopically for pathological changes.

Pathological changes were evaluated according to macroscopic observations, such as the cell arrangement and structure, by a pathologist blinded to the research. We mainly focused on the bilateral hippocampus. The severity of pathological changes in the hippocampus is presented as the ratio of neurons with karyopyknosis, which was defined as the percentage of karyopyknotic cells counted within the field of view. The hippocampus was examined at 400x magnification, and the ratio of neurons with karyopyknosis was calculated for five fields of view per section and then averaged.

**Table 1. Comparisons of Neonatal Rat Mortality within 24 h between the NOR and GR Groups.**

Group	Number of pregnancies	Number of foetuses	Number of non-viable foetuses	Neonatal mortality
NOR	3	30	0	0%
GR	3	21	14	66.7% (14/21)*

\*represents P<0.05 compared to the NOR group.

**Table 3. Analysis of Correlation Parameters between the Karyopyknosis Ratio and TUNEL-Positive Cells.**

Time point	Correlation coefficient (r)	P value
PND 4	0.883	0.020
PND 8	0.835	0.039

**Table 2. Body Weight Changes in the NOR and GR Groups in the First 8 Postnatal Days.**

Group	Weight (g)							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
NOR	6.4±0.59 (n=30)	7.1±0.8 (n=30)	8.1±1.0 (n=30)	9.5±1.6 (n=30)	10.9±1.9 (n=27)	12.7±2.1 (n=27)	14.6±2.4 (n=27)	16.8±3 (n=27)
GR	5.5±0.8* (n=7)	5.6±1.0* (n=7)	6.1±1.2* (n=7)	7.3±1.7* (n=7)	7.8±2.4* (n=4)	8.8±2.6* (n=4)	9.9±2.5* (n=4)	11.1±2.9* (n=4)
GR/NOR	85.9%	78.9%	75.3%	76.8%	71.6%	69.3%	67.8%	66.1%

Data are expressed as mean ± SEM. \*represents P<0.05 compared to the NOR group.

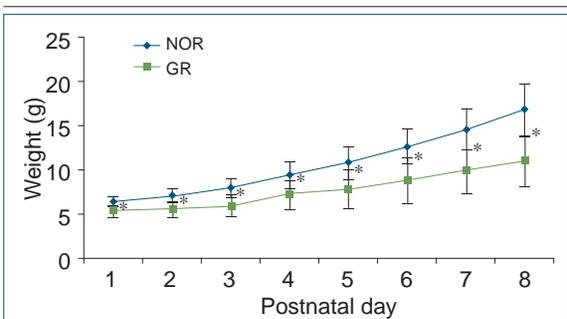
**TUNEL Staining by Immunofluorescence Assay**

Cell apoptosis was detected using a terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL) kit (Promega, Madison, WI), according to the manufacturer's protocol. Briefly, thin sections measuring 4 µm were conventionally deparaffinized. After washing with normal saline and PBS, sections were fixed in 4% paraformaldehyde and permeabilized with approximately 100 µl proteinase K (20 µg/ml) for 16 min at room temperature (RT), followed by incubation with a fluorescent-conjugated nucleotide mixture that reacted with terminal deoxynucleotidyl transferase for 60 min at 37 °C in a damp box. The reaction was terminated by incubation in 2x SSC buffer for 15 min at 37 °C, and the sections were then incubated with 4',6-diamidino-2-phenylindole (DAPI). Each of the steps was followed by three 5-min rinses in PBS.

Tissue sections were observed with fluorescent microscopy (LEICA RM 2016). Green fluorescence expression in the rat nucleus represented TUNEL-positive cells. Images were collected at 200x magnification, and the number of TUNEL-positive cells in the hippocampus was counted by an OLYMPUS Standard Cell Sens for five fields of view per section and then averaged.

**Statistical Analysis**

Statistical analyses were conducted using SPSS Statistical Software version 18.0. Except for da-



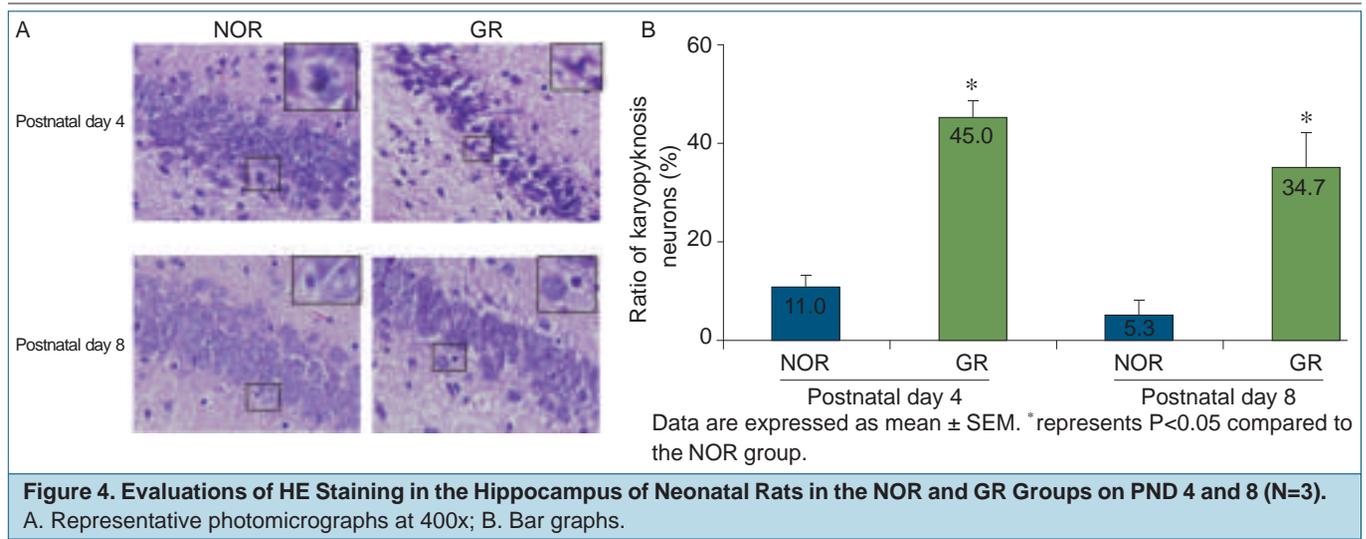
**Figure 3. Neonatal Weight of the NOR and GR Groups within the First 8 Postnatal Days.**

ta on neonatal mortality, data are presented as means ± SEM. Comparisons between two groups were analysed using Student's t test, while data regarding neonatal mortality were analysed with a Chi-squared test with continuity correction. Correlation analysis was performed with a Pearson's correlation coefficient test. The significance level was defined at P<0.05.

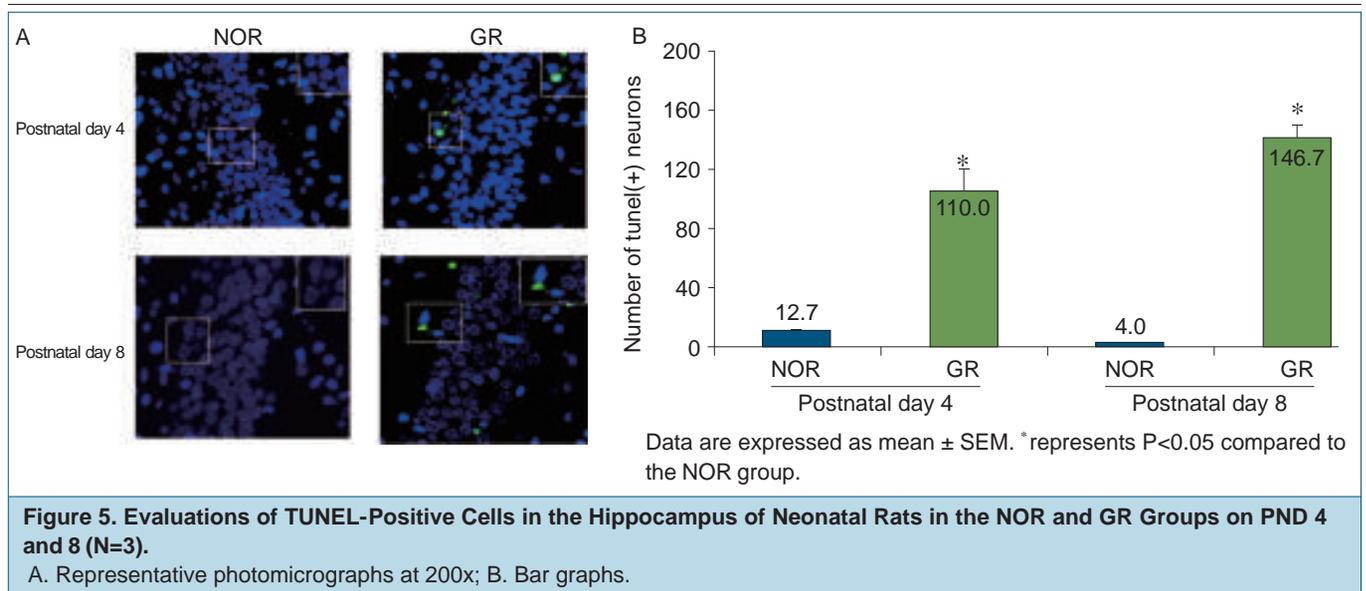
**RESULTS**

**Neonatal Mortality and Weight**

No deaths occurred in the pregnant rat group. A statistically significant increase in neonatal mortality was observed in the GR group (66.7%) compared to that in the NOR group (0%; Table 1). Pups were more vulnerable to resorption in the GR group than in the NOR group. The neonatal weight within the first 8 postnatal days sig-



**Figure 4. Evaluations of HE Staining in the Hippocampus of Neonatal Rats in the NOR and GR Groups on PND 4 and 8 (N=3).**  
A. Representative photomicrographs at 400x; B. Bar graphs.



**Figure 5. Evaluations of TUNEL-Positive Cells in the Hippocampus of Neonatal Rats in the NOR and GR Groups on PND 4 and 8 (N=3).**  
A. Representative photomicrographs at 200x; B. Bar graphs.

nificantly reduced after IUGR establishment compared to that in NOR group, reflecting a mean weight reduction. Weight reduction was 14.1%- 33.9% , about 20% was more readily achieved. However, catchup growth was not observed. The neonatal weight within the first 8 postnatal days and comparisons between the two groups were presented in Table 2 and Figure 3.

### Histological Assessment of the Hippocampus

Observation under a microscope showed that each subfield of the hippocampus was normal and clearly visible. In each subfield, nerve fibres and glial cells presented a normal distribution, were arranged loosely and were clearly visible.

No significant morphological changes were found in the hippocampus of PND 4 and PND 8 rats in the NOR group. When newborns in the GR group were compared with vehicle controls, abnormal changes were observed in the GR group; the number of hippocampal neurons was decreased, the volume of the nucleus became smaller, and obvious karyopyknosis was observed. The ratio of neurons with karyopyknosis was significantly higher in the GR group than in the vehicle controls (P<0.05; Figure 4).

### IUGR Caused Neuronal Apoptosis

The results of the TUNEL assay demonstrated that the nuclei of the apoptotic cells expressed

green fluorescence, and few scattered apoptotic nuclei were visible in the NOR group. The number of apoptotic cells in the GR group was significantly higher than in the control group on PND 4 and PND 8 ( $P < 0.05$ ; Figure 5).

### Positive Correlation between Karyopyknosis and Apoptosis

Table 3 shows that the karyopyknosis ratio was significantly correlated with neuronal apoptosis on PND 4 and PND 8 ( $P = 0.02$  and  $P = 0.039$ , respectively). A higher karyopyknosis ratio indicates increased neuronal apoptosis, which suggests that morphological changes should be identified by multiple indexes.

## DISCUSSION

Uterine artery ligation has long been used in large animal models such as sheep, rabbits or larger rodents to produce IUGR (11). According to previous studies (19, 21, 22, 24), we chose a commonly used uterine ischemia-reperfusion operation to establish an IUGR model based on preliminary experimental data; bilateral uterine vessels were occluded for 25 min followed by removal of the clamps to permit reperfusion. After intrauterine ischemia, hypoxia and reperfusion, the placenta was hypoxic, and its blood flow was inadequate, which led to placental insufficiency and reduced oxygen delivery to the foetus, followed by IUGR of the foetal rats. The detrimental effects of the uterine ischemia-reperfusion operation have been demonstrated in several experimental models when uterine vessels were clamped transiently rather than ligated permanently. Success in the model was confirmed by showing a mean weight reduction of 14.1% to 33.9% in GR group compared with the neonatal weight of the NOR group, although a neonatal weight reduction of 14.1% on PND 1 did not seem satisfactory owing to less surviving fetus. It was receivable that the results of the present study showed that this method of transient uterine vessel clamping significantly retarded newborn growth and caused morphological changes, which could be diagnosed as IUGR, compared to rats in the NOR group. Therefore, the animal model was indeed effective for simulating IUGR.

It is well recognised that this period, gestation day 17, represents the onset of rapid fetal

growth and corresponds to the beginning of the third trimester in human pregnancy (25). It was on gestation day 17 that surgery operation (uterine artery ligation bilaterally or unilaterally) was conducted in previous models (18, 23). According to comparative time schedule for CNS development in humans and rats, the rat central nervous system is immature at birth. The central nervous system maturity of the rat on PND 4 and PND 8 corresponds developmentally to the preterm and term human infant, respectively (26, 27). On the ground of above, we chose gestation day 17 as surgical time point and PND 4 and PND 8 as indices measurement.

The design of our operation procedure was modified from previous models (18) after preliminary experiments, mainly in time point of occlusion time. The occlusion for 25 min avoided high fetal mortality and resorption, obtained high little size, and provided a possibility of investigating long-term effects of IUGR during postnatal brain development. The results of our preliminary experiment showed that uterine vessel clamping for 30 min resulted in an extremely low viability rate in neonates, and neonatal mortality within 24 h was approximately 80% which resulted in few available experimental animals. In addition to causing newborn death, uterine artery ligation results in a high abortion rate of foetuses (at least 30%) (3, 14). Under the current experimental conditions, the authors did not record the abortion rate of the foetuses. Uterine vessel clamping for 25 min narrowly met the requirement to conduct the experiment. Neonatal mortality within 24 h was approximately 66.7%. In consideration of this point, we occluded vessels of the two horns of the uterus for only 25 min.

Additionally, in our preliminary experiment, we found that the viability of newborns delivered by caesarean section was lower than that of pups delivered vaginally. No deaths of newborns occurred because neonates survived by breastfeeding at the time of birth. Therefore, in the present study, the method of vaginal delivery was chosen to obtain newborn rats.

As IUGR is associated with uterine artery clamping, our experiment showed that neonatal mortality in the GR group was significantly higher than that in the NOR group. Our data revealed significantly decreased weights for neonates in the GR group, which was accompanied

by growth and development delays and early catch-up growth (22) that were related to the lack of comprehensive nutritional supplements in our study. We speculated that the intrauterine operation influenced the gastrointestinal physiology of the fetuses, which resulted in malabsorption followed by nutrient deficiencies. Finally, this commonly used surgical technique was performed to obtain a model of IUGR conditions. It not only influenced weight and viability parameters but also affected internal organ development, and previous studies have reported effects on the brain (28), liver (28) and skin (29). The brain is sensitive and has poor tolerance to hypoxia. Therefore, we focused on brain tissue.

HE staining of brain samples demonstrated that IUGR led to an irregular arrangement of cells, loss of cells, obvious karyopyknosis and dissolution of the nucleus, which were all morphological changes resulting from brain injury due to IUGR. We also examined cell apoptosis to further identify the effect of IUGR on the brain. As expected, significant differences were observed. TUNEL-positive cells were observed much more frequently in rats with IUGR. Neuronal apopto-

sis has been suggested to be related to dysfunction (30, 31). If a series of behavioural tests were performed during adolescence, mental deficiencies would be found. Evidence provided by HE staining was less accurate; however, a TUNEL assay is commonly recognized as the “gold standard” to evaluate apoptosis. We also demonstrated a positive correlation between karyopyknosis and neuronal apoptosis, which showed that data regarding both were consistent.

## CONCLUSIONS

The IUGR induced by bilateral uterine vessel clamping for 25 min increased neonatal mortality with 24 h, contributed to reduced newborn daily weight, increased karyopyknosis and apoptosis and led to brain injury. Our experiment showed that uterine artery ligation was indeed effective for inducing IUGR.

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All authors have no conflicts of interest to declare for this work.

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