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The Use of Domestic Animals as Biomedical Models

# The use of ruminant models in biomedical perinatal research

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**Abstract**

Animal models are of critical importance in biomedical research. Although rodents and lagomorphs are the most commonly used species, larger species are required, especially when surgical approaches or new medical devices have to be evaluated. In particular, in the field of perinatal medicine, they are critical for the evaluation of new pharmacologic treatments and the development of new invasive procedures in fetuses. In some areas, such as developmental genetics, reproductive biotechnologies and metabolic programming, the contribution of ruminants is essential. The current report focuses on some of the most outstanding examples of great biomedical advances carried out with ruminant models in the field of perinatal research. Experiments recently carried in our research unit using ruminants are also briefly described.


*Keywords:* Biomedical research; Animal models; Ruminants; Perinatal

**Contents**



## 1. Introduction

As a consequence of obvious ethical considerations concerning what is feasible or not in the field of human biomedical research, animal models are of critical im-

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portance both for medical doctors, veterinarians and scientists. Many species have been and are currently used as animal models. Rodents and lagomorphs are the most popular, essentially because of their low cost, handling, and rearing facilities, limited ethical impact, and the availability of a wide range of genetic research tools in these species. Nevertheless, the physiological mechanisms observed in these species might be very different from those of humans, especially in the perinatal field, because of their large number of fetuses and short gestation length.

Large animal species are required when surgical approaches or new medical devices have to be evaluated. The adult pig is widely used in these situations, as well as ruminants, such as the sheep and the goat. The pig, however, is polycotous and fetal size is small compared with that of human, making it a less suitable model compared with small ruminants for research on the fetoplacental unit. Concerning physiologic, anatomic, and genetic considerations, large primates could be considered as the "gold standard" animal model because of their important similarities to humans. Their use for biomedical research, however, is greatly limited by their behavioral and social organization, raising important ethical questions and their high cost.

A very large number of experiments using ruminants as animal models have been published. A rapid bibliometric analysis performed using the PubMed database from 1969 to 2012 retrieved 1194 literature reviews using the Mesh keywords "ruminant and animal model". Six hundred forty-one references were available only for the year 2011 using the same Mesh research and covering all types of publications. The sheep is the most widely used ruminant model, and no less than 7461 publications can be found using the Mesh keywords "sheep and animal model". As a consequence, it appears neither possible nor suitable to carry out an exhaustive overview of all the research possibilities offered by the different ruminant models. The current report focuses on the most outstanding examples of great biomedical advances carried out with ruminant as models in the field of perinatal research (i.e., during embryonic, fetal, and placental development), which is our field of expertise.

We chose to develop a limited number of examples of ruminant model-based approaches covering the major fields of biomedical research: organ development and fetal physiology, evaluation and development of new pharmacologic treatments, new invasive procedures in fetuses, gestational imaging, and evaluation of the mechanism and consequences of fetal programming. The experiments that appeared to best illustrate each perinatal research topic are presented and put into their clinical context.

## 2. Reproductive technologies

The contribution of ruminants to embryo production and embryo manipulation is immense: since the first embryo transfer in cattle in 1950 [1,2], the industry has developed and benefited from the possibility to perform nonsurgical embryo transfer, to synchronize cycles between donors and recipients, and to freeze embryos, reaching more than 700 000 embryos produced worldwide in 2010 [3]. Sheep were also the first ever mammalian species to be successfully born after nuclear transfer of embryonic [4] or somatic cells [5]. In the current context, the use of artificial reproductive technologies (ART) is developing very rapidly (European Union being the leader with 458 759 treatment cycles reported in 32 European countries in 2006, initiating approximately 54% of all reported ART cycles worldwide in 2010 [6], whereas 1% of all births were derived from ART in the United States in 2010; http://www.cdc.gov/art/PDF/NationalActionPlan.pdf), the experience gained from ruminants is unvaluable. In particular, they had a unique role for pinpointing the importance of embryo culture conditions on long-term development, with the development of the large offspring syndrome or abnormal offspring syndrome [7–9] in association with some embryo culture conditions (i.e., the addition of human serum to culture medium and coculture of embryos) [10,11]. Studies on ruminants have subsequently been a landmark for evidencing the role of epigenetic modifications in the early embryo in modifying the phenotype of a developing individual [12–15]. Such striking pathologies have not been observed in humans after ART, maybe because in vitro maturation of oocytes is so far rarely performed routinely and because embryos are usually transferred at the two- to four-cell stage. A controversial increase in pathologies, such as the Beckwith-Wiedeman or the Simpson-Golabi syndromes, which are due to imprinting perturbations, has, however, been associated with ART [16]. Moreover, increased adiposity has been observed in adolescents born after intra cytoplasmic sperm injection [17].

## 3. Implantation and immunology of pregnancy

Although the placentation is very different in ruminants versus primates (i.e., late implantation vs. early implantation; epitheliochorial vs. hemochorial placen-

tation in primates and ruminants, respectively). However, the short gestation length and the specificities of the murine placenta make it a less than ideal model for studying immunologic processes during pregnancy [18]. The role of the immunosuppressive interferon tau, of trophodectoderm origin, in the maternal recognition of pregnancy was initially shown in sheep [19]. In early gestation, through the use of uterine ligatures, the systemic and paracrine regulation of events taking place in the pregnant horn can be studied. Thus, it was shown that the dynamic changes of lymphocyte populations CD45R+ and T-γδ in the endometrium during gestation are not affected by the local embryonic secretions [20]. Female lambs treated with a synthetic analogue of progesterone in the first 2 mo after birth lack endometrial glands as adults (uterine glands knockout or UGKO phenotype) and this affects embryo elongation and subsequent survival [21]. Using this model, it was shown that progesterone treatment can restore functional endometrial glands and delay the rejection of allogenic tissues placed in the uterus of nonpregnant UGKO or control ewes [22,23]. The availability of genomic tools as well as the possibility to produce embryos in vitro has also enabled the study of endometrial response to embryos of different origin in cattle. Hence, it was shown that the uterus responds differently to in vivo- or in vitro-produced embryos of different developmental potential [24,25], which may be of major interest for understanding which parameters are important for implantation in humans [26,27].

## 4. Organ development and fetoplacental physiology

Because of its size and developmental rate close to that of the human fetus, the sheep fetus is a unique tool to study fetal physiology. Many of what was initially known on placental blood flow and amniotic fluid regulation was first demonstrated in sheep or goats [28]. In the UK, early work by Comline and Silver, who developed fetal catheterism in cows, sheep, and horses as a means to finely observe and challenge fetal development [29–32], as well as the work of Thorburn and Basset in Australia [33–35], led the path to the understanding of the physiological regulation of the growth and function of many organs, including adrenal, pancreas, and lung. The small ruminant model is still considered as a key to the understanding of fetal and placental physiology in fields as varied as cardiac development [36], lung physiology [37], skeletal development [38], endocrinology [39], and behavior [40].

Examples of the contribution of physiological studies on ruminants to biomedical research are numerous. Among the most important, the pregnant sheep model has been historically used for research on the initiation of parturition and fetal maturation [41–43]. The understanding of the role of the glucocorticoids has led to their use in human pregnancies at high risk for preterm delivery, which has been a major step in the prevention of respiratory distress syndrome (RDS) and other morbidities in the preterm newborn. The antenatal glucocorticoid treatments were first evaluated by Liggins in the sheep in 1969 [44]. Thanks to these very promising observations with premature deliveries of fetal lambs infused with glucocorticoids, in which pulmonary maturation appeared greatly improved, Liggins ran the first clinical controlled trial of antenatal glucocorticoid treatment in pregnant women, which was published in 1972 [45]. The rate and severity of RDS were demonstrated to be decreased in premature newborns of mothers who had received antenatal courses of glucocorticoids. Subsequently, this led to extensive research about glucocorticoids and other hormones as regulators of lung development. The maturational effects of antenatal glucocorticoids were established in the lung, but also in several other organ systems, such as the brain and the digestive tract [46,47].

Glucocorticoids acutely change the structure in fetal lung by thinning the mesenchyma and decreasing alveolar septation [48,49]. The clinical result is that lung compliance and gas exchange are improved [50], with a reduction in the risk of RDS by about 50% with a single course of treatment. Glucocorticoid administration to pregnant women at high risk for preterm delivery is now recommended worldwide. The effects of antenatal and early postnatal glucocorticoid treatment were evaluated in rodents, rabbits, and other small animals with short gestational duration. The developmental period at which the saccular lung begins to alveolarize, however, is markedly different in these species when compared with sheep or humans and thus the effects on the lungs could not be demonstrated as such.

Intrauterine fetal growth restriction, which markedly affects the head circumference of the newborn, is the major side effect of glucocorticoids during pregnancy. This side effect was first observed in sheep: in fetal sheep, a single maternal dose of betamethasone given at about 70% gestation decreases fetal weight by about 11%, which represents 3 days of growth arrest [48]. A similar growth arrest in humans would decrease fetal weight by about 4.8%, which is not easily detectable.

These side effects were confirmed in humans in the late 90s and led to more caution in the use of glucocorticoids during pregnancy.

The pregnant sheep is considered an essential model for the evaluation of the effects of antenatal glucocorticoids because of its gestational length and several fetal characteristics, such as weight at term, weight gain during the third part of the pregnancy, and alveolar maturation. It is still widely used for the evaluation of several end points, such as the impact of repeated courses or new pharmaceutical forms of glucocorticoids on fetal and newborn development [51–53].

Another of the countless examples of the importance of small ruminants is related to gonadal development. The timing of gonadal development during fetal life is close to humans in ruminants, with meiosis and folliculogenesis taking place during gestation as opposed to in rodent where folliculogenesis begins after birth [54,55]. This is important for the study of the effects of excess endogenous steroids [56,57], endocrine disrupters [58,59], and maternal nutrition [60] on reproductive function [61].

## 5. New invasive and noninvasive procedures during pregnancy

The pregnant sheep was previously described as a very suitable animal model for the evaluation of fetal invasive procedures [62,63]. This model is particularly adapted for such research protocols because of the size of the fetus and the length of the gestation which enables a long-term in utero follow-up. The absence of uterine contractions during procedures represents another advantage of this model.

Because of its similarity with the human fetus in size and anatomy, the ovine fetus has been the animal model of choice for developing new techniques in fetal surgery [64,65]. This model was widely used both for reproducing several developmental abnormalities frequently observed in human fetuses and evaluate new in utero therapeutic approaches. Some of the most demonstrative examples, which gained recent validation as valuable therapies in human fetuses, are certainly the surgical treatment of myelomeningocele defects, a current central nervous system disorder leading to severe nervous damage in the newborn, and the so-called PLUG therapy which consists of the placement of an intratracheal balloon in fetuses presenting with a congenital diaphragmatic hernia [66,67].

Our group has been involved in evaluating the management of monochorionic twins. In humans, monochorionic twin pregnancies can be complicated by the malformation of a twin, twin reversed arterial perfusion sequence, or twin-to-twin transfusion syndrome. In these cases, selective termination of a twin can be the only option for saving the other fetus [68]. Because of placental vascular anastomoses between the twins, it is not possible to use a fetal intravascular injection of potassium chloride solution or lidocaine [69]. As a consequence, a selective occlusion of the umbilical cord of the terminated twin is usually performed. Techniques, such as bipolar forceps, laser coagulation, and cord ligation under echoguidance or fetoscopy have been developed [69]. However, they require the introduction of instruments with a diameter of 2 to 5 mm within the amniotic cavity, thus increasing the risk for rupturing the membranes and inducing premature labor.

Radiofrequency (RF) is a mini-invasive thermo-ablative procedure. It has been recently evaluated as potentially more appropriate for selective termination of pregnancy. Encouraging results of RF procedures applied for selective termination of acardiac twins in pregnant women were published since 2002 [70,71]. The interest of RF in reducing the risk of rupture of the membranes was confirmed. However, despite the fact that the umbilical cord blood flow was successfully stopped in the 29 described procedures, the delay between the beginning of the procedure and the complete occlusion of the umbilical cord could not be precisely defined in humans.

Radiofrequency efficacy for umbilical cord occlusion was further evaluated experimentally in vitro [72] and in vivo in fetal sheep (Fig. 1) [73]. It was shown that the target temperature (100 °C in these experiments) was reached 180 sec after the beginning of the procedure in vitro and 154 sec in vivo. The in vitro findings showed that the vascular occlusion, evaluated by monitoring the cord perfusion pressure, occurred only when the target temperature was reached.

The absence of immediate cord occlusion was considered as a possible risk of exsanguination of the surviving twin into its terminated cotwin before the end of the procedure. This critical end point led to increased cautiousness for the use of RF in human pregnancies. It also called for a long-term follow-up of the neurodevelopment of the surviving twins in which the procedure had already been applied [74].

In this example, the cord diameter and vascular fluxes of fetal sheep at 90 days' gestation were similar to those of human fetuses at 26-wk gestation, which is the usual stage for such procedures.

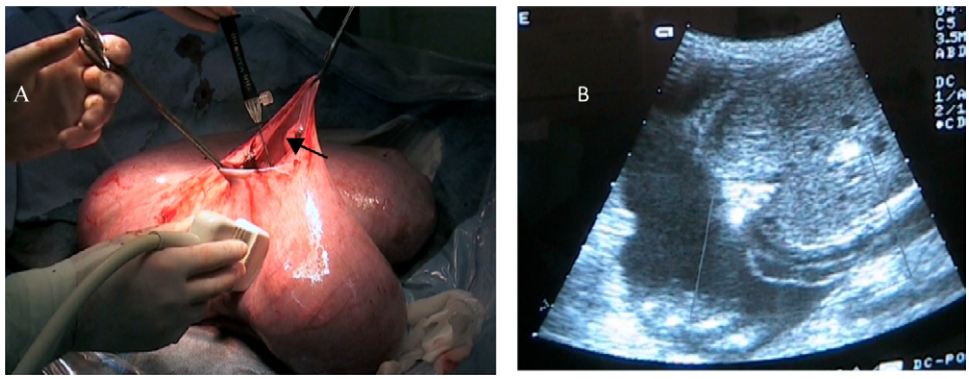


Fig. 1. Surgical procedure for radiofrequency (RF) occlusion of the umbilical cord in sheep. (A) Under general anesthesia, a hysterotomy is performed, fetus and cord are exposed, and RF electrode (arrow) is visually inserted into the cord. (B) Ultrasonographic visualization of the RF electrode inserted into the cord.

Noninvasive methods to visualize the fetus also need to be either developed or validated in human-size animals. Advances in ultrasound imaging now enable noninvasive 3-D volume and Doppler signal quantification using automatic acquisitions. The power Doppler angiography (PDA) is a new tool to study blood flow within an organ or volume of interest (VOI). This noninvasive technique is safe during pregnancy, as contrast agents or exposure to radiation are not needed [75].

In humans, impaired placental perfusion can lead to severe diseases, such as intrauterine growth retardation and pre-eclampsia. These pathologies represent up to 30% of maternal and fetal perinatal morbidity and can lead to fetal and maternal death. The diagnosis is currently performed only at a late stage of the disease, when the termination of pregnancy is the only treatment available, usually leading to severe prematurity. The evaluation of the uteroplacental perfusion might be of great clinical interest but is not directly feasible with the current imaging techniques.

Preliminary reports suggest that impaired uteroplacental perfusion could be diagnosed in humans using the PDA technology [76–79] and could thus enable the implementation of preventive treatments. With the PDA, the perfusion within the VOI is not defined in terms of mL per min or vascular resistance indexes, but three quantified indexes are used. The vascularization index (VI) is the ratio of color voxels to gray voxels, given as a percentage. The flow index (FI) represents the mean power Doppler signal intensity value. The vascularization flow index (VFI) which is calculated as VFI = VI × FI/100. Flow index and VFI are expressed as absolute values between 0 and 100. These quantified PDA indexes are representative of the number and intensity of color voxels within a VOI. The relationship between PDA indexes and blood flow was recently studied in vitro using a nylon particle-based blood mimicking liquid within different ultrasound test tanks [79,80]. In these models, VI, FI, and VFI were significantly affected by volume flow, vessel number, and the concentration of nylon particles. The indexes were also affected by variations in machine settings. These results were confirmed in an in vitro human placenta perfusion setting [81]. While relevant information was provided with in vitro models, the in vivo correlation between PDA indexes and blood flow within the uteroplacental unit had not been demonstrated.

Our experimental study in vivo in a pregnant sheep model confirmed a significant correlation between real blood perfusion and quantitative 3-D Doppler indexes measured within the uteroplacental unit [82]. The pregnant sheep model was chosen because of its large size, allowing 3-D ultrasound acquisitions with the same equipment and probe as in pregnant women, and the operative facilities in this model.

A sensor measuring blood flow and a controllable vascular occlusion system were placed around the common uterine artery, while all the other arterial supplies were ligated (Fig. 2). Several occlusion levels were applied and 3-D Doppler acquisitions of placentomes were concomitantly realized, using standardized settings. Each placentome was analyzed with the VOCAL software version 4 (GE Medical Systems, Zipf, Austria) (Fig. 3). High correlations between PDA indexes and blood flow were confirmed [82]. Reproducibility was excellent both for intra- and interobserver, with intraclass correlation coefficients of at least 0.799.

These results confirmed the potential interest of 3-D Doppler ultrasound for the assessment of placental vas-

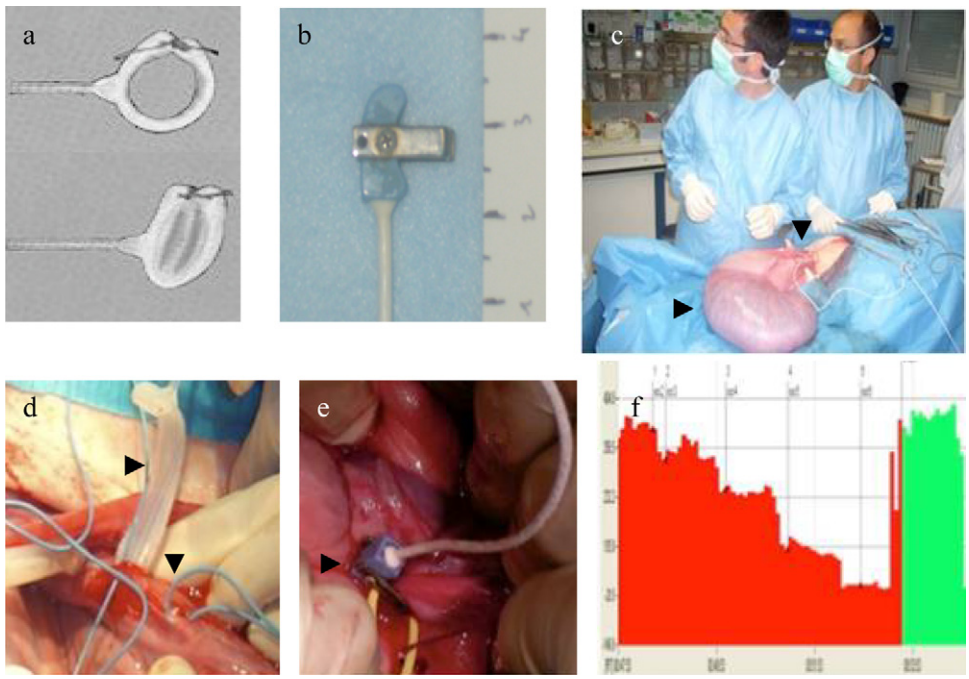


Fig. 2. Modulation and measurement of the blood flow within the uterine artery. (A) In vivo Metric 6 mm occluder (OC6, In Vivo Metric, Healdsburg, CA, USA) before (top) and after (bottom) filling the balloon that provides occlusion. (B) Invasive quantitative flow sensor (Transonic, S serial, 4 mm, with CRA10 connector, Emka) used for real time assessment of the blood flow (expressed in mL per min) within a vessel. (C) Exposition of the pregnant horn (arrowhead) after median laparotomy and dissection of the common uterine artery (arrowhead). (D) Placement of the occluder (arrowhead) around the common uterine artery (arrowhead) of the pregnant horn at 2 cm far from the primary split. All the other uterine arterial supplies were concomitantly ligated. (E) Placement of the flow sensor (arrowhead) 1 cm downstream from the vascular occluder. (F) Flow data as recorded with IOX 4 software (Emka Technologies USA, Falls Church, VA, USA) with increasing occlusion levels, from 0 to 100%.

cular insufficiencies both in clinical cases and in a research setting. Such invasive experiments, calling for a surgical approach of the uterine vascular supplies during an ongoing pregnancy, could be performed only in animal models presenting a pelvic vasculature anatomy close to that of pregnant women, and a large enough size, such as the sheep.

## 6. Study of the mechanisms and consequences of the developmental origins of health and diseases

Dietary restriction during pregnancy can lead to various consequences on offspring growth and development, such as a higher risk of developing a metabolic syndrome in humans [83–86]. These observations were confirmed in rodents and sheep models, leading to extensive work on the fetal and postnatal consequences of maternal feed restriction [87–91]. Most of the work is currently performed in rodents, however, when changes in the nutritional behavior, such as overfeeding, are observed in kids born to dams that were underfed during gestation. Despite their epitheliochorial placentation which is different from that of humans, ruminant models are important to study fetal programming as they have long pregnancies, making it possible for chronic fetoplacental adaptations to take place [92]. Moreover, surgical removal of endometrial caruncles, which are the endometrial counterpart of the fetal cotyledon during placentation, is a physiological way to induce intrauterine growth retardation because of placental insufficiency [93], whereas approaches developed in rodents, such as uterine ligation are less effective and induce confounding factors, such as inflammatory processes [94,95]. Single and twin pregnancies can be easily obtained, and many studies indicate the difference in response between singleton and twin pregnancies [96–100], thus indicating that these must be analyzed separately and shedding some concern upon polycotous species. As discussed above, fetal growth, placental function, and uterine vascularization can also easily be monitored with conventional ultrasound equipment [101]. Moreover, genomic tools are becoming increasingly available in these

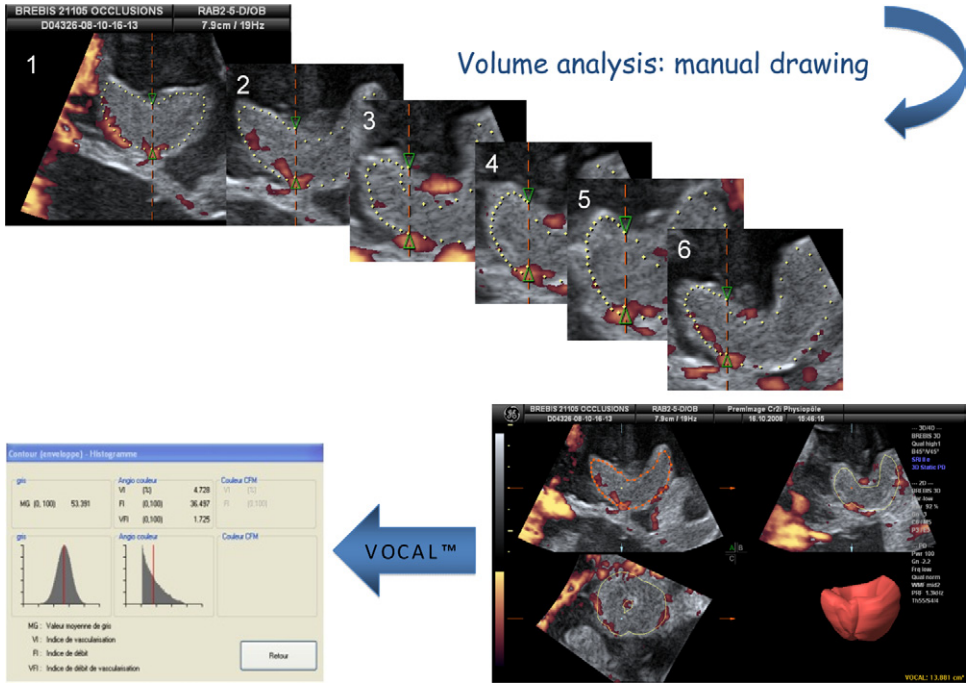


Fig. 3. Placentome analysis using the VOCAL software version 4 (GE Medical systems, Zipf, Austria). Each placentome was rebuilt three-dimensionally. Plane A of each acquired volume was used as a work pattern. A rotational multiplanar technique was applied. The contour of the maternal side of each placentome was outlined (yellow dotted line) after rotating the volume every 30°, 15°, or 9° (30° rotation angle in this example). Three quantitative 3-D Doppler indexes were automatically generated by the histogram function of the VOCAL (i.e., vascularization index [VI], flow index [FI], and vascularization flow index [VFI]).

species and enable extensive exploration of gene expression in various tissues.

As for fetoplacental physiology, reports on the effects of impaired placental function or abnormal maternal food intake on fetal adaptation and subsequent postnatal obesity and metabolic disturbances, using ruminants and other animals as models, have been summarized elsewhere [102]. Here we provide one example from our group, dealing with the programming of nutritional behavior, where the ruminant model was used.

Our group recently studied the effects of nutritional restriction of gestating goats on the physiology and feeding behavior of their offspring. Food intake regulation is mainly controlled by the hypothalamus. In sheep and goats, as in humans, the maturation of this central organ of regulation occurs during late gestation [103], concomitantly with the acceleration of brain development [104], in contrast to rodents where it occurs after birth. Therefore, observed effects of maternal undernutrition of food intake in rats needed to be confirmed in a ruminant model, closer to humans in terms of hypothalamic maturation. Underfed or control pregnant Alpine or Saanen goats were used. The underfed goats received from 50% to 80% of the amount of a total mixed ration given to control goats during the last third of pregnancy, which affected their own metabolism and feeding behavior [105]. Twin and triplet offspring were studied postnatally. At birth, only male kids were growth retarded but rapidly caught up, and there were no changes in feeding behavior, morphology, or metabolism before weaning at 5 wk of age [106]. In contrast, female offspring from the restricted group had a higher cortisol response to adrenocorticotropic hormone challenge at 14 mo of age and remained lighter despite a higher daily feed intake and a tendency to eat more rapidly up to 2 yr of age [107], thus underlining the importance of studying long-term effects of fetal perturbations.

## 7. Conclusions

As a consequence of indisputable ethical considerations in humans, the animal models are of critical importance for biomedical research and especially in the field of perinatal medicine. Much scientific progress, past, present, and future, in human medicine would have been and still are impossible to obtain without the use of animal models.

In pregnant women, in most of the cases, clinical trials to evaluate new biomedical approaches can and should be performed, only after first steps validations of safety and potential efficiency in a suitable animal model. Furthermore, the models allow an easier and faster understanding of pathologies and consequences of treatments or stresses during pregnancy. They provide simplified and controlled observations, without the vagaries that can occur in clinical trials in human.

As developed above, ruminant animal models are of critical importance for directing clinical investigators toward effects which may occur and be clinically important in many fields of perinatal biomedical research. These considerations call for a policy to maintain and develop research structures on ruminant models, in terms of (1) multidisciplinary human skills, involving medical doctors, veterinarians, and scientists, (2) livestock capacities of animal models, and (3) advanced research equipment, at a time when research on farm animals is seriously threatened both in the United States and in Europe [108].

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