Original Research *Highlight article*

Strategies for intra-amniotic administration of fetal therapy in a rabbit model of intrauterine growth restriction

Mari Kinoshita[1,2], Fàtima Crispi[1,2,3], Carla Loreiro[1,2], Eduard Gratacós[1,2,3], Míriam Illa[1] and Mònica Zamora[1,2]

[1]BCNatal | Fetal Medicine Research Center (Hospital Clínic and Hospital Sant Joan de Déu), University of Barcelona, Barcelona 08028, Spain; [2]Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona 08036, Spain; [3]Centre for Biomedical Research on Rare Diseases (CIBER-ER), Madrid 28029, Spain

Corresponding author: Mari Kinoshita. Email: kinomari625@gmail.com; Mònica Zamora. Email: mzamorav@clinic.cat

Impact statement

This is an exploratory study attempting to establish a therapeutic strategy in an IUGR rabbit model. An optimal strategy that is both feasible and beneficial to IUGR fetuses would allow a detailed assessment of the therapeutic effects in the animal model, bringing us a step closer to potential application in this human pregnancy complication that has no effective therapy to date. By focusing on the methodological aspect of fetal administration, our study illustrates the actual surgical procedures and the many factors to consider during fetal intervention in a specific animal model. Our work points to the often unmet need for studies to rigorously report methodological details for subsequent reproduction and also highlights the challenges of selecting an animal model from among various animal species and model characteristics. This methodological approach will be of high value in the field and will provide useful input for planning future experimental study designs.

Abstract

Intrauterine growth restriction affects up to 10% of all pregnancies, leading to fetal programming with detrimental consequences for lifelong health. However, no therapeutic strategies have so far been effective to ameliorate these consequences. Our previous study has demonstrated that a single dose of nutrients administered into the amniotic cavity, bypassing the often dysfunctional placenta via intra-amniotic administration, improved survival at birth but not birthweight in an intrauterine growth restriction rabbit model. The aim of this study was to further develop an effective strategy for intra-amniotic fetal therapy in an animal model. Intrauterine growth restriction was induced by selective ligation of uteroplacental vessels on one uterine horn of pregnant rabbits at gestational day 25, and fetuses were delivered by cesarean section on GD30. During the five days of intrauterine growth restriction development, three different methods of intra-amniotic administration were used: continuous intra-amniotic infusion by osmotic pump, multiple intra-amniotic injections, and single fetal intraperitoneal injection. Technical feasibility, capability to systematically reach the fetus, and survival and birthweight of the derived offspring were evaluated for each technique. Continuous intra-amniotic infusion by osmotic pump was not feasible owing to the high occurrence of catheter displacement and amnion rupture, while methods using two intra-amniotic injections and one fetal intraperitoneal injection were technically feasible but compromised fetal survival. Taking into account all the numerous factors affecting intra-amniotic fetal therapy in the intrauterine growth restriction rabbit model, we conclude that an optimal therapeutic strategy with low technical failure and positive fetal impact on both survival and birthweight still needs to be found.

Keywords: Fetal growth restriction, FGR, prenatal intervention, nutritional therapy, transamniotic, surgical model

Experimental Biology and Medicine **2021; 246: 1668–1679. DOI: 10.1177/15353702211003508**

Introduction

Intrauterine growth restriction (IUGR) is defined as suboptimal fetal growth that results in an estimated fetal weight below the 10th percentile for gestational age and affects up to 10% of pregnancies.[1] The most common underlying mechanism of IUGR is placental insufficiency, where dysfunction of the placenta leads to a chronic reduction of nutrients and oxygen to the fetus.[2–4] IUGR represents one of the major causes of perinatal morbidities and mortality, but also of later neurodevelopmental and cardiovascular problems.[5–8] Despite our better understanding of lifelong consequences due to fetal programming, there is no established therapy to improve IUGR and ameliorate its effects.[8,9]

In the developed world, placental insufficiency is the primary cause of IUGR not attributable to genetic factors.[5] Regarding this etiology, some methods used for IUGR induction in research are uterine artery ligation in rats, guinea pigs, rabbits, and sheep; uterine artery or placenta embolization in sheep; carunclectomy in sheep; uteroplacental vessel ligation in rats, guinea pigs, and rabbits; and progressive uterine artery occlusion in guinea pigs.[10–13] Similarly, our group has reported an IUGR induction in a rabbit model by selective surgical ligation of uteroplacental vessels in the mother in late pregnancy.[14] In this animal model, the reduced placental supply of both oxygen and nutrients resembles human placental insufficiency and results in lower birthweight and survival. Additionally, the rabbit is large enough to allow procedural manipulation of the fetus but small enough to be more cost-effective with a shorter gestation period and larger litters than those of pigs and sheep.

Several studies have suggested therapeutic administration into the amniotic cavity, i.e. intra-amniotic (IA) therapy, as a potential solution that bypasses the often dysfunctional placenta in human IUGR. "Transamniotic fetal feeding (TAFF)" of nutrients or IA administration of growth factors have been reported since the early 1990s, using daily injections to ovine fetuses or continuous infusions to rabbit fetuses using small osmotic pumps or catheters that pass through maternal subcutaneous tunnels.[15–19] These studies showed improved fetal growth and intestinal absorption, but were limited in their reported outcomes owing to the characteristics of the animal models chosen. Different animal models of IUGR reproduce varying aspects of human IUGR; the abovementioned studies failed to evaluate the therapeutic effect on outcomes such as fetal survival because the selected IUGR models did not show lower survival compared with control animals. Since the agent administered into the amniotic cavity is swallowed and absorbed by the fetus,[15,20,21] this therapeutic strategy of IA administration has also been applied to treat other fetal conditions besides IUGR.[22,23]

A previous study from our research group has demonstrated that a single IA injection of nutritional solution to our surgical IUGR rabbit model improved survival at birth but not birthweight.[24] Therefore, we hypothesized that supplying sufficient nutrients directly to the fetus—by increasing the dose and optimizing the therapy—could promote not only survival but also attenuate the adverse effects of IUGR such as lower birthweight. The main purpose of this study is to compare three different methods of IA administration that enable an increase of direct nutrient supply to the fetus: continuous IA infusion by osmotic pump, multiple IA injections, and single fetal intraperitoneal (IP) injection, and to develop an effective strategy for fetal nutritional therapy in an IUGR rabbit model.

Materials and methods

Animals

A total of 42 multiparous New Zealand White pregnant rabbits were obtained from a certified breeder (Granja San Bernardo, Navarra, Spain). For breeding, a female rabbit was kept in the same cage as a male rabbit for one day, and pregnancy was later confirmed by palpation before the female rabbit was sent from the breeder to our animal facility during the third week of gestation. The specific day of mating was considered day 0 of pregnancy. Upon arrival at the animal facility at one week before surgery, animals were housed individually under reversed 12 h/12-h light cycle with free access to normal diet. All procedures involving animals were performed under the guidelines and regulations to protect animal welfare, and ethical approval was obtained from the Ethical Committee for Animal Research (459/16) of the University of Barcelona.

Study design: Experimental model to develop three strategies for IA fetal therapy

Three different strategies for IA fetal therapy were explored and compared in our animal model (Figure 1): continuous IA infusion by osmotic pump, multiple IA injections, and single fetal intraperitoneal (IP) injection. The experimental model was divided into two stages: Phase I, where we evaluated the technical feasibility of the strategies in control fetuses without administration of nutrients, and Phase II, where we evaluated the therapeutic effects in both control and IUGR fetuses after administration of nutrients. Only those strategies that were successfully implemented in Phase I were employed in Phase II.

Phase I. Twenty-four pregnant rabbits were included in Phase I. Surgery was performed on gestational day 25 (GD25) recapitulating the previously described surgery that we have performed to induce IUGR,[14] but in this case without the ligation of uteroplacental vessels (see Phase II for a brief explanation of the surgery performed), and neonates were delivered by cesarean section on GD30. Fetuses were immediately wiped dry and weighed while kept warm, and survival was calculated as the proportion of fetuses alive at birth out of the total number of fetuses found in each horn of the bicornuate uterus. In this phase, filtered phosphate-buffered saline (PBS) was prepared in the laboratory and used as the vehicle solution. To lower the risk of the solution causing fetal hypothermia, PBS was taken out from the stored refrigerator and kept in warm water for at least an hour before fetal administration by IA injections.

Phase II. Eighteen pregnant rabbits were included in Phase II. The surgery to induce IUGR was performed on GD25 as previously described with minor modifications.[14] In short, pregnant rabbits underwent general anesthesia by subcutaneous administration of ketamine 35 mg/kg and xylazine 5 mg/kg, followed by inhaled oxygen given at 2 L/min and intravenous infusion of ketamine and xylazine (5 mg/kg/h and 1.5 mg/kg/h, respectively). After a midline abdominal laparotomy, both horns of the bicornuate duplex uterus were exposed. Fetuses were identified by their position, with the fetus at the ovarian end considered to be the first fetus of the respective horn. One horn was

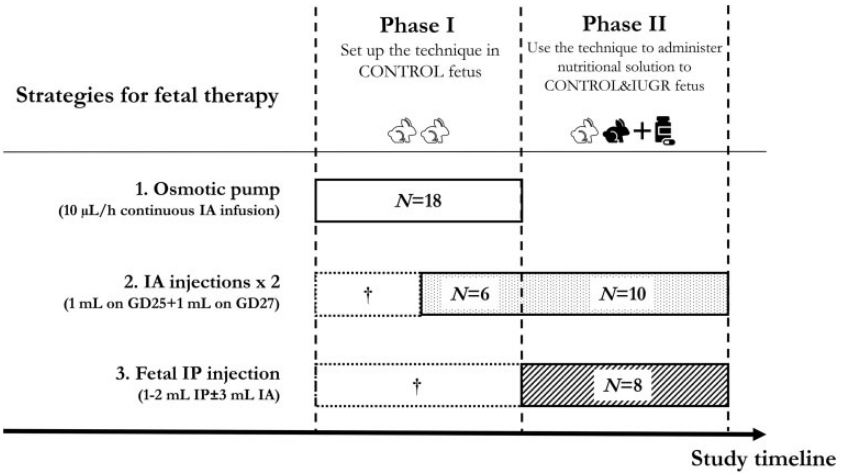

Figure 1. Experimental design: Exploring three different strategies for fetal therapy. The diagram presents the general study design including IUGR induction in the experimental animals, administration of nutritional solution, and sample size (number of pregnant rabbits) for each of the three methods. Different stages for the development of fetal therapy were designed: Phase I evaluated the feasibility of the technical procedures and Phase II evaluated the therapeutic effect of the treatment using the established techniques. Phase I: Strategies using osmotic pump or two IA injections were first evaluated for technical feasibility in control fetuses without nutritional solution. The dotted bar marked with † represents our previous experience with IA injections,[24] which is why we shortened Phase I in this study. Only the treatment with two IA injections passed on to the next stage. Phase II: A strategy using two IA injections was then evaluated for therapeutic effect in control and IUGR fetuses with nutritional solution. Also, as an extension of the two-injection technique, one IP injection was evaluated for therapeutic effect in Phase II. As in the treatment with two IA injections, we abbreviated Phase I of the treatment with one IP injection owing to our previous experience with ultrasound-guided fetal injections in the experimental setting. IA: intra-amniotic; GD: gestational day; IP: intraperitoneal; IUGR: intrauterine growth restriction.

randomly assigned as the case horn (IUGR), and the contralateral horn as the control horn (control). In the control horn, no ligation was performed. In the case horn, we ligated 40–50% of the uteroplacental vessels (i.e. the vessels connecting the branches of uterine arteries and veins to the vessels of each placenta) of every gestational sac with silk sutures (3–0) to induce IUGR. After confirming the anatomical structure of the vessels that is unique to each pregnant rabbit, sutures for ligation were placed prior to the most distal branches closest to the gestational sacs. The sacs that were outside the abdominal cavity were constantly rinsed with warm Ringer's lactate solution to prevent fetal hypothermia. The abdomen was then closed at each layer with a single continuous suture (2–0 for fascia, 3–0 for skin). We administered postoperative analgesia for 48 h (buprenorphine 0.05 mg/kg/12 h), and the maternal condition was followed daily. Five days later, neonates were delivered by cesarean section on GD30 under the same anesthetic protocol as the previous surgery. Fetuses were immediately wiped dry and weighed while kept warm, and survival was calculated as the proportion of fetuses alive at birth out of the total number of fetuses found in each horn of the bicornuate uterus.

In this phase, both nutritional and sham solutions were used. They were prepared under sterile conditions by the Pharmaceutical Unit at Hospital San Joan de Déu (Barcelona, Spain). The nutritional solution contained the same proportion (per 30 mL) of nutrients (glucose, amino acids) as previously published:[24] glucose 3.2 g, amino acids 1.1 g (L-isoleucine 8 g/L, L-leucine 13 g/L, L-lysine monoacetate 12 g/L, L-lysine 8.5 g/L, L-methionine 3.1 g/L, L-phenylalanine 3.8 g/L, L-threonine 4.4 g/L, L-tryptophan 2.0 g/L, L-valine 9 g/L, L-arginine 7.5 g/L, L-histidine 4.8 g/L, glycine 4.2 g/L, L-alanine 9.3 g/L, L-proline 9.7 g/L, L-serine 7.7 g/L, taurine 0.4 g/L, N-acetyl-L-tyrosine 5.2 g/L, L-tyrosine 4.2 g/L, N-acetyl-L-cysteine 0.7 g/L, L-cysteine 0.5 g/L, L-malic acid 2.6 g/L), potassium 0.3 mEq, calcium 0.7 mEq, magnesium 0.2 mEq, chloride 0.1 mEq, phosphate 0.2 mmol, acetate 0.7 mEq, carnitine 3.7 mg, heparin 15.3 IU (osmolarity 974 mOsm/L, pH 6.0–6.1). The sham solution contained the same concentration of electrolytes without the glucose, amino acids, carnitine, and acetate. To lower the risk of the solutions causing fetal hypothermia, they were taken out from the stored refrigerator and kept in warm water for at least an hour before fetal administration by IA or IP injections.

Strategies for IA fetal therapy

Strategy 1: Continuous IA infusion by osmotic pump. To administer solution continuously throughout the five days of IUGR development in our rabbit model (Figure 2), small osmotic pumps (ALZET model 2ML1, DURECT Corporation, Cupertino, CA) were loaded with the appropriate solution using a syringe and a blunt-tipped filling tube supplied with the pump on the day before their insertion, and primed overnight in saline at 37°C. Each pump was filled with 2 mL of PBS under sterile condition, and the solution was released at a regular rate of 10 μL/h. Bromodeoxyuridine (BrdU) (B5002, Sigma-Aldrich, Saint Louis, MO) was added to the solution (at a final concentration of 10 mg/mL) as a label to later trace fetal absorption from the IA administration (Figure 3(a)).

For Phase I trials (18 pregnant rabbits), we implanted the pumps for IA infusion on GD25, exactly as if we were performing the surgery for IUGR induction but using an abdominal retractor to widely open the incision, and without the actual ligation of uteroplacental vessels. The optimal method for the IA infusion setup was thoroughly explored by repeated trials adjusting various factors such as the shape of the catheter tip, the insertion method of the

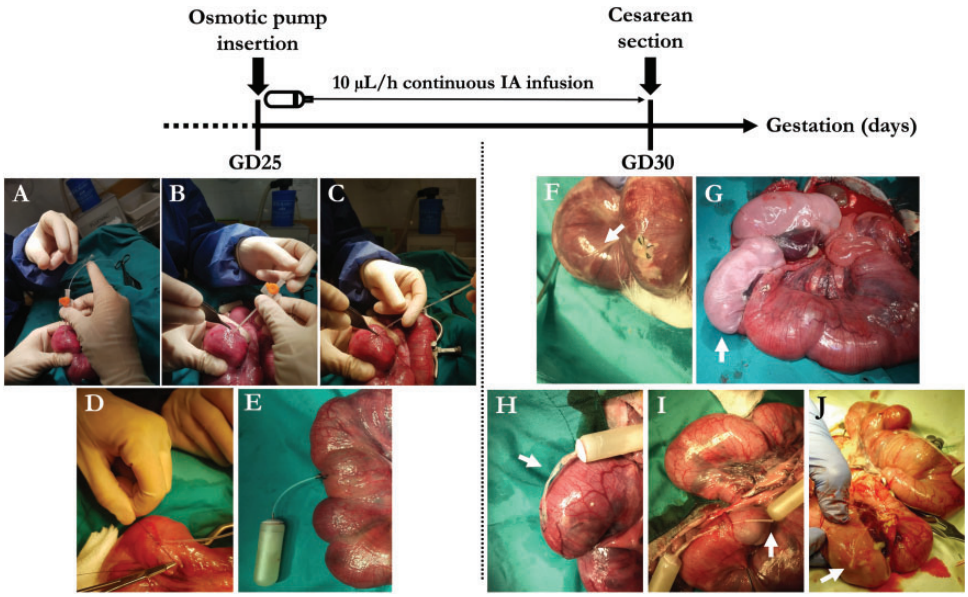

Figure 2. Illustrative images of Strategy 1: Continuous IA infusion by osmotic pump. The diagram presents the procedural flow. (a–e) During surgery on GD25: catheter is inserted into the amniotic cavity using a 14-gauge needle with external guiding sheath (a, b), the guiding sheath is removed (c), and the insertion point is closed with a suture (d). (e) After completing the procedures for IA administration and before returning the uterus into the maternal abdominal cavity with catheters and pumps. (f–j) During cesarean section on GD30: technical failures included the catheter being completely pulled out (f, white arrow), the amnion completely missing around the fetus (g, white arrow), and when the catheter could be fixed in place (h, white arrow) but the tip was outside the amniotic cavity (i, white arrow). The technique was successful when the catheter tip was positioned under the amnion and confirmed to be inside the amniotic cavity (j, white arrow). GD: gestational day. (A color version of this figure is available in the online journal.)

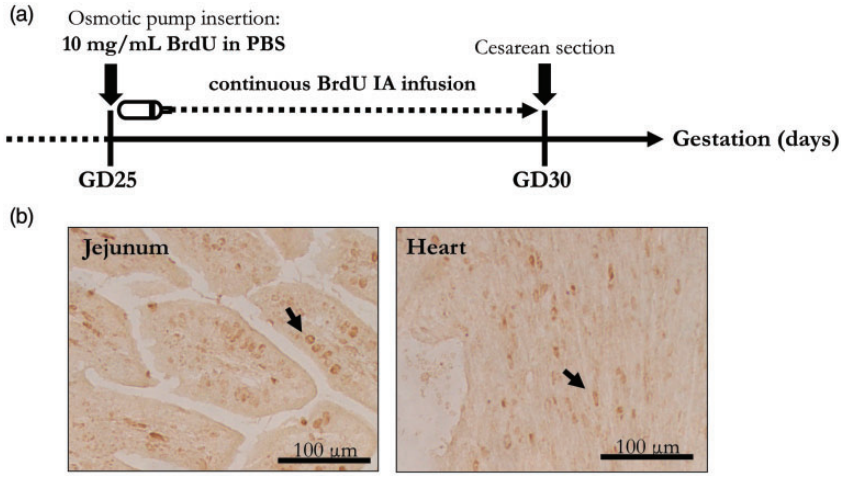

Figure 3. Continuous IA infusion by an osmotic pump reaches the fetus and its internal organs. (a) The diagram presents the procedural flow. BrdU solution was continuously infused from the osmotic pump into the amniotic cavity until the fetus was delivered. (b) Cryosections of jejunum and heart immunostained against BrdU were captured at ×20 magnification. Black arrows point to one of the many BrdU-positive nuclei stained in brown. The fetus from which the tissues for the pictures were obtained did not complete the therapy as planned: the catheter connected to the osmotic pump was completely pulled out on GD30, so it was unclear when the last BrdU entered the amniotic cavity. Nonetheless, BrdU had entered the amniotic cavity while the catheter was in place, and was then swallowed and absorbed by the fetus and passed into the fetal circulation. BrdU: bromodeoxyuridine; PBS: phosphate buffered saline; IA: intra-amniotic; GD: gestational day. (A color version of this figure is available in the online journal.)

catheter into the amniotic cavity (by tiny uterine incision or puncture without incision), catheter length inside and outside the gestational sac, catheter fixation, closure of amnion, and closure of uterine wall (Table 1). Upon reaching the 10th trial, a catheter (ALZET catheter polyethylene tube 0007750, DURECT Corporation, Cupertino, CA) without flange tip and cut to 6–7 cm was inserted into a gestational sac under guidance of a 14-gauge intravenous catheter (393230, Becton, Dickinson, and Company, Franklin Lakes, NJ) without hysterotomy, leaving 5 cm of length outside, and was fixed to the uterine wall by a 4–0 silk purse-string suture at the insertion point and by a 2–0 silk z-suture a few cm from that point (Figure 2(a) to (d)). A z-suture is a continuous suture that is passed through the tissue four times in a zigzag-shaped path, thus creating two adjacent loops that facilitate fixation of the surrounded point. Past reports of successful pump insertion were taken into account.[25,26] A total of four pumps with the

Table 1. Methodological factors to consider during the insertion of osmotic pump in a rabbit model.

Methodological factors	Detailed components
Catheter (connected to mini-osmotic pump)	
Catheter type	Material and its characteristics; total length
Catheter tip	With/without flange
Insertion method[a]	Incision with surgical blade/scissors or puncture with 14-gauge needle
Insertion point	With/without "base" sutures[b] (6–0 or 2–0); include/exclude catheter in the sutures[c]
Length inside amniotic cavity	Direction inside cavity (toward fetal head or along fetal spine); distance to fetus
Length outside uterine wall	Distance until connection with mini-osmotic pump
Catheter fixation[a]	Suture type (use "base" suture or not) and thickness (6–0 or 2–0)
Amnion	
Insertion method[a]	Cut/puncture separately per layer or all together
Closure of amnion[a]	Separately per layer or all together; suture type (continuous, purse-string, z-suture, or no suture) and thickness (6–0)
Uterine wall	
Insertion method[a]	Cut/puncture separately per layer or all together
Closure of uterus[a]	Separately per layer or all together; suture type (continuous, purse-string, or z-suture) and thickness (6–0, 4–0, or 2–0)

[a]Factors that seem to most affect the technical failures (i.e. catheter displacement, amnion rupture).
[b]"Base" sutures were two sutures made in the beginning of procedure near the insertion point to hold all membranes together (including the uterine wall).
[c]Including the catheter inside the sutures for closing the membranes (along with the uterine wall) affects both the catheter fixation and closure of amnion and uterine wall.

same solution were set up in a pregnant rabbit, each pump connected to a catheter inserted into the amniotic cavity of a fetus (Figure 2(e)), and the pumps were returned into the abdominal cavity of the mother rabbit. Four was determined as the maximum number of pumps to be inserted per pregnant rabbit owing to the effect of anesthesia on the fetal and maternal condition under surgery as well as the difficulty of gently returning the uterus with the catheters and pumps into the maternal abdominal cavity. The four fetuses chosen for inserting the catheters were those in the first and third gestational sacs of each uterine horn, counting from the ovarian end. During cesarean section on GD30, the integrity of the amnion as well as the final positions of the pumps and the catheter tips were confirmed before fetal delivery (Figure 2(f) to (j)). Technical success was defined as the catheter tip being properly inside the amniotic cavity with the osmotic pump connected to the catheter. The pumps were then collected, and the remaining solution aspirated to confirm that its amount was as expected after five days of continuous infusion.

Strategy 2: Two IA injections on GD25 and GD27. Following our experience with IA injections in our previous study,[24] we manually administered to all fetuses in each pregnant rabbit either sterile PBS in Phase I (six pregnant rabbits), or nutritional or sham solution after the ligation of the uteroplacental vessels in one horn during the surgery to induce IUGR in Phase II (10 pregnant rabbits) (Figure 1). Surgery under general anesthesia was performed similarly to that carried out in Strategy 1. Solutions were administrated into the amniotic cavity as a single dose of 1 mL using a 1 mL syringe and 25-gauge needle (303175, Becton, Dickinson, and Company, Franklin Lakes, NJ) to puncture the uterine wall without hysterotomy on GD25, and the injection was repeated two days later on GD27 (Figure 4(a)). Each IA injection was manually administered while using the other hand to gently stabilize the position of the gestational sac within the uterus. The second surgery required for IA injection on GD27 was basically the same as the first surgery except that no ligation of uteroplacental vessels was performed. Evans blue (EB) dye (E2129, Sigma-Aldrich, Saint Louis, MO) (0.5%) was added to the solution in Phase I to later trace fetal absorption from the IA administration (Figure 4(a)).

Strategy 3: One fetal IP injection on GD25. Based on our experience with ultrasound-guided fetal injections in the experimental setting, we started investigating a single fetal IP injection therapy (Figure 1) directly in Phase II (eight rabbits). The nutritional or sham solution, as appropriate, was administered to all fetuses as a single dose of 2 mL or 4 mL using a 5-mL syringe and 25-gauge needle to puncture the uterine wall and fetus without hysterotomy, but only during the surgery on GD25 after IUGR induction (Figure 5(a)). Surgery under general anesthesia was performed similarly to that carried out in Strategies 1 and 2. Since the injections were given directly into the fetus, BrdU and EB dye were not added for tracing. IP injection was investigated as an alternative target route of administration to improve fetal absorption. IP injection only (2 mL) and a combination of both routes, i.e. IP (1 mL) followed by IA (3 mL) before withdrawing the injection needle from the gestational sac (IP+IA), were explored. The injections for this strategy were performed under ultrasound guidance: the Vevo 3100 Imaging System (FUJIFILM VisualSonics, Toronto, Canada) with a 24 MHz linear probe and an adjustable syringe holder was used to precisely inject the needle into the fetal IP cavity, to avoid in particular the level of the axial plane through the liver and to distinguish between the IP and IA space (Figure 5(d)). We applied abundant warm gel and placed the probe directly on the uterine wall for visual guidance while controlling the syringe holder to move the needle (Figure 5(b) and (c)).

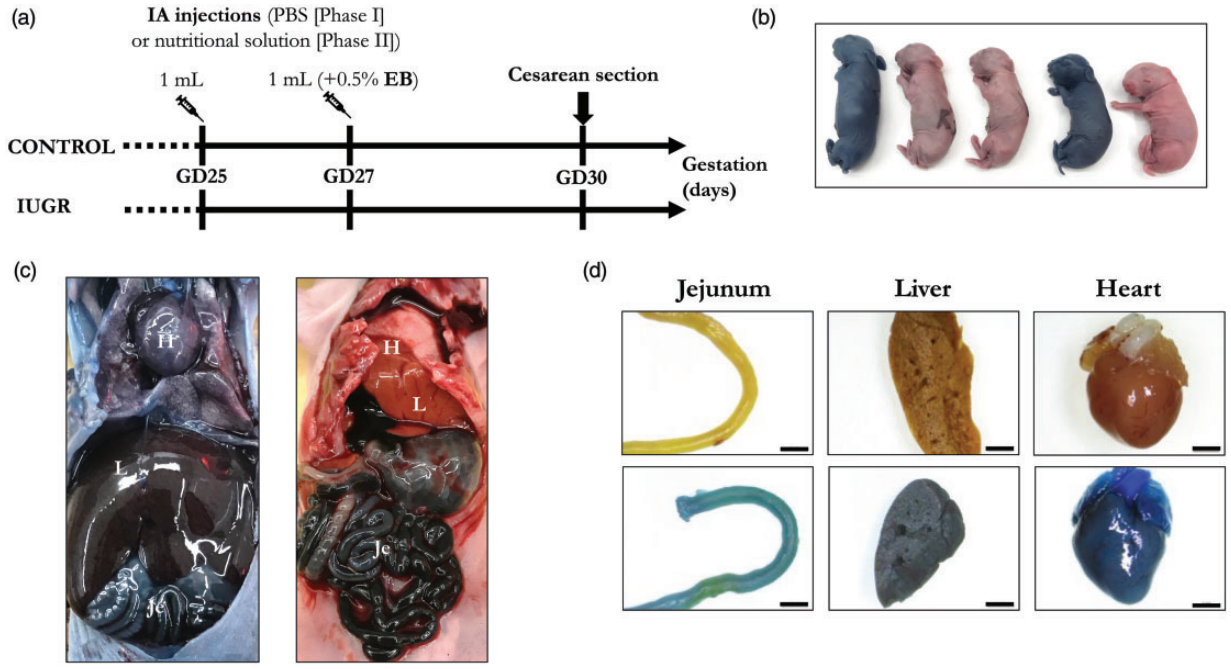

Figure 4. Illustrative images of Strategy 2: Two IA injections and tracing of administered solution. (a) The diagram presents the procedural flow: in Phase I, PBS was administered as the first injection into the amniotic cavity of control fetuses on GD25, and PBS with EB dye was administered as the second injection on GD27; in Phase II, sham or nutritional solution was administered into the amniotic cavity of both control and IUGR fetuses on GD25 and 27. (b) Phase I fetuses immediately after birth on GD30, showing apparent difference in skin color from blue to pale blue to pink. The variability between fetuses is apparent: all fetuses in the picture had received the two-injection therapy in the same way. (c) Macroscopic staining of internal organs (H: heart; L: liver; Je: jejunum): for the three fetuses in (b) that were not completely blue, only the gastrointestinal tract was stained blue (right picture) in contrast to the fetuses that were completely blue inside and outside (left picture). (d) During Phase I, jejunum, liver, and heart from a fetus that had not received any injections (upper row) are compared with those of a completely blue-skinned fetus (lower row). The scale bar on the lower right of each image represents 2 mm. IA: intra-amniotic; EB: Evans blue; GD: gestational day; IUGR: intrauterine growth restriction. (A color version of this figure is available in the online journal.)

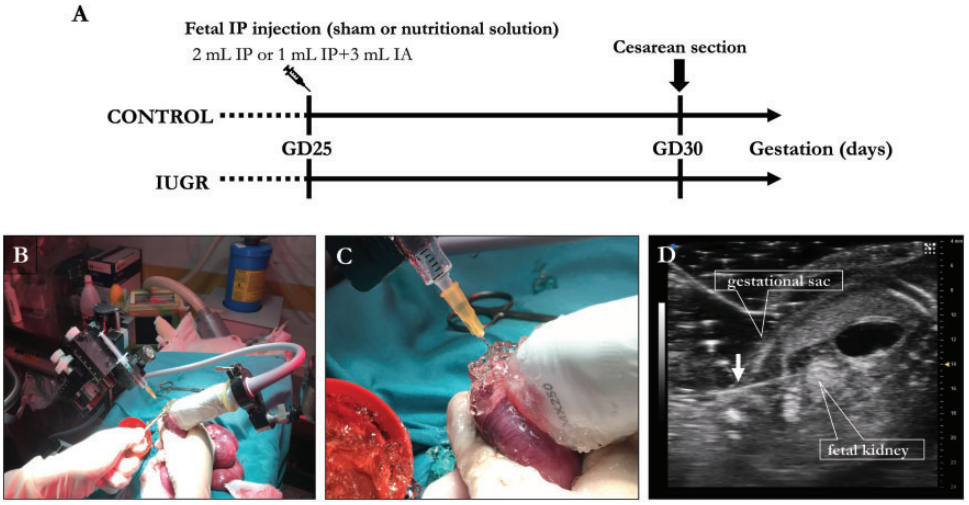

Figure 5. Illustrative images of Strategy 3: One fetal IP injection. (a) The diagram presents the procedural flow. (b) The fetus within the uterus was gently handled to set its position under the ultrasound probe. Abundant warm gel was applied to the uterine wall while determining the target point of injection under ultrasound view of the fetal IP cavity, and the syringe holder was set in place. (c) The syringe holder was manipulated to move the syringe at a precise angle toward the target point, and IP injection was administered while the fetus was held in place. (d) Representative echography image during fetal IP injection: the white arrow points to where the needle punctures the gestational sac to pass through the amniotic cavity and into the fetal IP space. IP: intraperitoneal; GD: gestational day; IUGR: intrauterine growth restriction. (A color version of this figure is available in the online journal.)

Sample processing and evaluation of fetal absorption

Sample processing. After delivery, all alive neonates were weighed and sacrificed by decapitation. The heart, jejunum, and liver were collected, and photographs were taken for macroscopic anatomy alongside visual inspection for the blue staining by EB dye where tracing was used. The sampled organs were subsequently washed in PBS enriched with 2% heparin and fixed with 10% formalin for 48–72 h at 4°C. The organs were then treated with 30% sucrose and embedded in O.C.T. (optimal cutting

temperature) compound and stored at −80°C. Cryomedia-embedded tissues were cut into 10 µm transversal sections and prepared for histological evaluation.

Immunohistochemistry: BrdU labeling and detection. BrdU staining was used to detect whether the solution administered by osmotic pumps had reached fetal organs at the cellular level in the heart, jejunum (Figure 3(a)), and liver. Tissue sections on slides were defrosted at room temperature (RT) for over 30 min, then heated for 3 min in citrate buffer (C1909, C7254, Sigma-Aldrich, Saint Louis, MO) (pH 6.0) for antigen retrieval. After DNA hydrolysis (30 min incubation with 1 M hydrogen chloride at 37°C, followed by 10 min at RT with 0.1 M sodium borate), sections were washed for 10 min with PBS + 0.025% Triton X-100 (X100, Sigma-Aldrich, Saint Louis, MO), then with PBS + 0.1% Tween 20 (170–6531, Bio-Rad Laboratories, Hercules, CA) (PBS-T, over 1 min), and blocked with PBS-T + 5% goat serum (G9023, Sigma-Aldrich, Saint Louis, MO) +1% bovine serum albumin (A9647, Sigma-Aldrich, Saint Louis, MO) for 1 h at RT. The tissue sections were incubated with mouse anti-BrdU (B8434, Sigma-Aldrich, Saint Louis, MO) (primary antibody, diluted to 1:500 in PBS-T) overnight at 4°C, washed with PBS-T, then internal peroxidase was blocked by 30 min incubation with 0.3% hydrogen peroxide and washed with PBS. Finally, Mouse ABC Detection Kit (ab64259, Abcam, Cambridge, UK) was used to detect BrdU with 3, 3′-diaminobenzidine (DAB).

Statistical analysis

All data analyses were performed using Stata 14.1 IC (StataCorp LLC, College Station, TX). Statistical significance was defined as two-tailed values of $P \leq 0.05$. Normality of the quantitative variables was assessed by quantile–quantile (Q–Q) plot and Shapiro–Wilk test. The experimental groups were compared using the two-way analysis of variance (ANOVA) with *post hoc* tests (Tukey–Kramer) of pairwise comparisons. Proportions were compared using the Chi-squared test and corrected by Bonferroni's method for multiple comparisons.

Results

Continuous IA infusion with osmotic pump is associated with numerous technical difficulties in a rabbit model

A total of 11 trials (18 rabbits) was dedicated to establishing the technical procedure of IA administration by osmotic pump (Figure 2). Each methodological factor listed in Table 1 was scrutinized for the optimal choice of its components, giving substantial combinations of factors to consider. At first, we created a flange at the catheter tip to help prevent the catheter from being pulled out, inserted the catheter from a tiny incision cut in the uterine wall, chorion, and amnion between two base sutures (i.e. sutures made at the beginning of the procedure near the insertion point to hold all the membranes together), closed all layers together, and fixed the catheter using 6–0 continuous suture. However, owing to the problem of the catheters being pulled out by GD30, after two trials we changed to 2–0 suture for fixing the catheter to the uterine wall. Although catheter fixation seemed to improve, we continued to have issues during the following four trials with the displacement of catheter tips and amnion rupture, following which we eventually discarded the use of base sutures and blindly punctured the uterine wall with a 14-gauge needle to guide the catheter (with no flange) into the amniotic cavity. In an attempt to better stabilize the catheter position, from the 10th trial onwards, we closed the uterine wall at the insertion point with 4–0 purse-string suture and fixed the catheter again by 2–0 silk z-suture at few cm away from that point.

However, 11 trials did not allow us to resolve all the problems and achieve the optimal methodology. Four trials resulted in a high mortality (>50–60%) of the fetuses with catheters inserted into the amniotic cavity and connected to osmotic pumps. Although it was difficult to confirm the cause during the exploratory stage of the strategy, the high mortality did not persist as we adjusted the methodological factors with each trial. Results obtained from the final two trials after consideration of each methodological factor (Table 1) during the preceding nine trials are presented in Table 2. The critical issues that persisted until the final trials were the dislocation of the catheter tips and the fragility of the amnion. Though the tips were properly inserted into the amniotic cavity on GD25, as confirmed by the backflow of the amniotic fluid inside the catheter before connecting to the osmotic pump, by GD30 they were found to be unsuccessful in keeping their infusion system intact: two-thirds of the inserted catheters were either completely pulled out from the uterus or still fixed to the uterine wall but with their tips outside the amniotic cavity; close to half the fetuses had a torn amnion and of these some lacked their amnion completely (Figure 2(f) to (i)). In the end, despite the low mortality (<20%) of the fetuses with catheters inserted into the amniotic cavity and connected to osmotic pumps, the technical challenges led to this strategy being labeled unfeasible for developing an applicable therapy administration to treat IUGR in our rabbit model.

Continuous IA infusion with osmotic pump reaches the fetus and its internal organs

Immunohistochemistry against BrdU revealed positive brown nuclei in tissue sections of the heart and the jejunum sampled after continuous IA infusion during Phase I (Figure 3(b)). This confirmed that the solution administered by osmotic pump through the connected catheter into the amniotic cavity had entered the fetal gut through swallowing, been absorbed in the small intestine and taken up into the bloodstream, and had eventually reached the proliferating cells in the fetal heart.

Administration of nutrients by two IA injections leads to high mortality in the IUGR fetuses

Since the technique of manually administering IA injections was easily established in Phase I (following our previous study)[24] without any apparent detrimental effect on

Table 2. Summary of the three different strategies for fetal therapy in our IUGR rabbit model.

Fetal therapy	Experimental stage (*N*)[a]	Technical outcome	Survival, % (*n*)[b]	Birthweight,[c] g mean (SD)	Pros	Cons
Osmotic pump[d] (IA)	Phase I[e] (4)	58% of fetuses had intact amnion, 33% of catheters had tips inside amniotic cavity on GD30	83 (12)	43.41 (7.30)	• Continuous infusion throughout 5-day period of IUGR development. • Low mortality. • Multiple administration during 5-day period of IUGR development. • Easy and stable administration technique.	• Low technical success. • Limited number of pumps per animal. • Additional surgery affecting both maternal and fetal conditions. • Limited doses during 5-day period. • High mortality in IUGR fetuses.
	Phase II (0)	–	–	–		
Two injections (IA)	Phase I[f] (6)	100% executed by manual injections	65 (31)	36.09 (5.28)		
	Phase II (10)	–	Control+sham: 80 (30)	31.99 (7.82)		
			Control+tx: 71 (17)	33.75 (5.39)		
			IUGR+sham: 38* (21)	32.38 (4.43)		
			IUGR+tx: 24*# (41)	32.93 (5.59)		
One injection (IP)[g]	Phase I (0)	–	–	–	• More direct administration of nutrients to fetus via IP. • Feasible administration technique.	• More complicated technique involving precise target within fetus. • Risk of fetal bleeding, particularly from liver. • Limited dose only at start of 5-day period. • High mortality.
	Phase II (8)	IP only: 100% executed under US guidance	Control+sham: 86 (7)	39.32 (2.88)		
			Control+tx: 0* (7)	–		
			IUGR+sham: 14* (14)	28.21 (–)[h]		
			IUGR+tx: 0* (10)	–		
		IP+IA: 20% required second puncture to complete administration under US guidance	Control+sham: 50 (8)	38.80 (8.54)		
			Control+tx: 50 (6)	36.25 (2.11)		
			IUGR+sham: 25 (8)	33.75 (–)[h]		
			IUGR+tx: 40 (10)	33.38 (2.43)		

[a]Total number of pregnant rabbits in each experimental stage.
[b]Total number of fetuses found in the uterine horns of pregnant rabbits in each experimental group.
[c]The weight of fetuses alive at birth in each experimental group.
[d]Results obtained from the final two trials (four pregnant rabbits) after consideration of each methodological factor listed in Table 1 during the preceding nine trials with 14 pregnant rabbits.
[e]Of the four pregnant rabbits, one rabbit was excluded due to poor maternal health.
[f]Of the six pregnant rabbits, two rabbits were excluded due to incidents during surgery, e.g. bleeding.
[g]IP includes both IP only and IP+IA (one injection administered partly to IP and then the rest to IA).
[h]There were only two fetuses alive at birth in the group. $^{*}P \leq 0.05$ when compared to Control+sham, $^{\#}P \leq 0.05$ when compared to Control+tx (corrected by Bonferroni method).
IA: intra-amniotic; IP: intraperitoneal; SD: standard deviation; US: ultrasound; tx: therapy.

survival (Table 2), we moved on to Phase II where we actually administered nutritional and sham solutions to IUGR and control fetuses, respectively. However, compared with the mortality of non-treated IUGR fetuses in our model that did not receive any injections,[24] the already compromised fetuses seemed to suffer even more after two IA injections of either sham or nutritional solution (Table 2). The survival of IUGR fetuses receiving injections was significantly lower than that of control fetuses administered the same solution. Additionally, the % kilogram loss in maternal weight during GD25-30 increased for rabbits subjected to two surgeries for two IA injections compared with those subjected to one surgery for one fetal IP injection (mean [SD]: −8.82 [4.01] vs. −4.30 [3.09], P [t-test]=0.0189). Regarding the birthweight of the four experimental groups (Control+sham, Control+tx [therapy], IUGR+sham, IUGR+tx), analysis by two-way ANOVA did not show any significant effect of IUGR, therapy, or their interaction.

Administration by two IA injections reaches the fetus and its internal organs

When EB dye was added to trace the solution administered by the two-injection method (Figure 4(a)), the technical success of the manual injections was immediately confirmed upon administration, because we were able to clearly see the blue-colored solution enter and spread evenly inside the amniotic cavity within the uterine wall. Upon delivery on GD30, the skin and internal organs of the fetuses were stained visibly blue by EB dye administered on GD27 (Figure 4(b) to (d)). Moreover, we observed variation in the extent of blue staining: all fetuses presented a blue-stained gastrointestinal tract, but the staining of skin and other organs ranged from those that looked completely blue (Figure 4(c) left picture) to those that looked partially blue (other organs did not look blue while the skin varied from light blue to pink, Figure 4(c) right picture).

Administration of nutrients by one fetal IP injection leads to overall high mortality

The technique itself was slightly more complicated than two IA injections, and IP+IA was even more complicated than IP only (Table 2). When administering the solution first by IP and then by IA, IA space was so limited that for one out of five injections, the needle being withdrawn from the fetus immediately left the amniotic cavity and the gestational sac had to be punctured for the second time to complete the IA administration of the remaining solution. The IP method in general was associated with increased mortality across all experimental groups, showing no difference in survival across groups by IUGR or therapy (Table 2). Regarding the birthweight of the four experimental groups (Control+sham, Control+tx, IUGR+sham, IUGR+tx of IP+IA), analysis by two-way ANOVA did not show any significant effect by IUGR, therapy, or their interaction.

Discussion

To develop an applicable and effective fetal therapy for IUGR, three strategies for IA administration were designed and compared: continuous IA infusion by osmotic pump, two IA injections, and one fetal IP injection. Our study demonstrated that despite the advantage of continuous infusion throughout the period of IUGR development in our animal model, inserting osmotic pumps into our rabbit model was not a technically feasible choice with limited resources. Treatment with two IA injections and one fetal IP injection were both technically feasible but with a negative effect on survival that seemed to outweigh the benefit of therapy.

Though continuous IA therapy seemed to be a promising intervention that could directly deliver nutrients to the fetus throughout the development of IUGR in our model, the therapy failed to pass Phase I owing to the numerous technical difficulties of inserting osmotic pumps into a rabbit model. Several research teams across the world have performed IA administration in animal models, from daily or weekly injections in larger animals to catherization in smaller animals.[18,19,25,27,28] Of those studies, in the earlier ones catherization meant the catheter had one end inside the amniotic cavity and the other end passing through a subcutaneous tunnel of the mother to emerge from the maternal neck to be connected to an infusion pump, but in the later ones the catheters inside the amniotic cavity were connected to mini-osmotic pumps as in our study.[15,25,27–29] However, previous reports only describe the use of osmotic pumps in a sentence or two in the methods section with barely any details about technical complications.[28] Harrison *et al.* did elaborate further from a methodological perspective when exploring different animal models to give continuous IA therapy. The rabbit was chosen as the best model mainly owing to the final survival of operated mothers and catheterized fetuses, but technical issues during the study procedures were again not described.[25] Such reporting does not help to efficiently reproduce continuous IA administration in a rabbit model, where the anatomical structure of the rabbit allows catheterized gestational sacs made of amnion to physiologically slide within the uterine walls. The malalignment or the opposing force between the fixation of the catheter to the uterine wall and the movement of the gestational sac into which the catheter tip was inserted possibly contributed to the recurring incidence of catheter displacement and amnion rupture. Owing to those unresolved issues after 11 trials, we considered the setup of osmotic pumps for continuous IA infusion as an inefficient, unfeasible technique to be applied to the rabbit model.

An alternative strategy to continuous IA therapy was multiple IA injections during the period of IUGR development in our animal model; as we anticipated the negative impact of adding more surgeries on top of the IUGR induction on GD25, we decided to administer a total of two IA injections, one at the start and the next in the middle of the five-day period up to delivery.

Unlike continuous IA therapy, two-injection IA therapy was highly feasible as an administration technique, but there were repercussions from the additional surgery

required for the second injection on GD27. An aspect of its negative impact was reflected in the greater weight loss of mother rabbits allocated to the group receiving two IA injections compared with those allocated to the group receiving one fetal IP injection. Not only did the two-injection IA therapy fail to improve the survival of IUGR fetuses, but it also resulted in a further decrease when compared with the survival of IUGR fetuses with no injections.[14,24] In contrast, the survival of control fetuses did not differ from that of control fetuses that did not receive any injections.[14,24] This suggests that the already compromised IUGR fetuses are more affected by the negative impact of surgery than the control fetuses in terms of survival, whereas even control fetuses seem to be affected in terms of birthweight and thus no difference in birthweight is found across the groups. It would have been ideal to administer multiple IA injections without increasing the number of surgeries, i.e. by maternal percutaneous injection through the abdominal wall, which has been done in ovine models but remains extremely difficult in a rabbit model with smaller, multiple fetuses in a bicornuate uterus.[18,19] The anatomy and physiology of the specific animal demanded additional open surgery to correctly administer additional IA injection per gestational sac.

The two therapies discussed above both involved IA administration of nutritional solution by different methods, and fetal absorption and distribution were confirmed by BrdU staining and EB dye staining. This completely aligns with the results of past studies that traced radiolabeled nutrients after IA administration that was not necessarily given to the same animals with the same techniques. Pitkin *et al.* reported that injected protein underwent proteolysis in the fetal gut of rhesus monkeys and the derived amino acids were incorporated into protein in the fetal lung, liver, skeletal muscle, and brain.[30] Phillips *et al.* reported that infused glucose and proline were swallowed by rabbit fetuses and absorbed from the small intestine to reach other tissues such as the lung and liver.[15,20] In our study, BrdU was detected in the fetal organs sampled on GD30 after continuous IA infusion of the solution containing BrdU by osmotic pump during GD25-30. This was demonstrated in a fetus that had the catheter withdrawn on GD30 and in which it was unclear when the last BrdU entered the amniotic cavity. Nonetheless, BrdU had entered the amniotic cavity while the catheter was in place, then been swallowed and absorbed by the fetus into the fetal circulation, and finally been incorporated into the nuclei of proliferating fetal cells to be detected.

In contrast to BrdU, which labeled cells, EB dye stained the whole organs visibly blue to the naked eye. EB dye was added to the solution on GD27 because we anticipated that absorbed EB dye in fetal plasma would disappear after a few days.[31] EB dye binds strongly to serum albumin and preferentially enters damaged cells; thus, the visible staining of the fetal organs is probably due to the protein-bound dye in the blood vessels.[32] Moreover, this convenient marker also allowed us to immediately confirm the technical success of IA injections by clearly visualizing the solution administered into the amniotic cavity. The variation in the extent of macroscopic staining observed in the fetuses could be possibly explained by variation in fetal swallowing and absorption; it is reasonable to assume that the frequency and amount of fetal swallowing vary between fetuses, and thus the amount of staining that was absorbed from the gut and into the blood flow would vary to some extent.[33]

The third strategy for IA administration added a new element: the fetal IP route, including both IP only and IP+IA administration. The administration technique required ultrasound guidance to differentiate between the IP and IA spaces and was slightly more sophisticated than the simple manual IA injections of the two-injection therapy. The principal issue was not the feasibility of the technique but the high overall mortality of both IUGR and control fetuses. As the IP route does not depend on fetal swallowing for the nutrients to enter the fetus, we considered this administration method to be a more direct fetal delivery than IA therapy. Several studies have shown that vital nutrients such as glucose, amino acids, and lipids could be absorbed adequately by IP administration to rabbits, and the rabbits were even able to survive solely on IP nutrition without apparent adverse events for a month.[34–37] Although we did decrease the dose via the IP route compared with that via IA route for fear of the rapid fluid infusion affecting fetal circulation, we cannot deny the possibility that the single dose of 1–2 mL (about 5–10% of fetal weight) into the peritoneal cavity did more harm than good. DeAlvaro *et al.* demonstrated that filling the peritoneal cavity of adult rabbits with a volume of 10% of the animal weight was bearable,[35] but the immature organs of fetuses should probably not be expected to similarly withstand the osmolarity and volume of the IP administration. This could possibly explain why survival was lowest for IP only administration of nutritional solution to both control and IUGR fetuses. Furthermore, direct injection into the fetus poses higher risk of fetal bleeding from the intraperitoneal organs, which could negatively affect survival even with no additional maternal surgery.

A major strength of this study is that it is one of the few studies so far to compare different IA administration strategies in an animal model from a methodological point of view, and the first study to do so in our IUGR rabbit model. Our focus and comprehensive description of methodology differentiate our study from the majority of past studies: their often brief comments on the methods do not easily allow reproduction. The animal model we have chosen is one that has been characterized well and used in many studies on IUGR.[11] The interventions were all fetal therapies that bypass the placenta, which is often dysfunctional in human IUGR.[38] By focusing on the actual procedures performed on animals, whose inherent characteristics are different from humans, we were able to highlight the many factors influencing the development of a new fetal therapy.

There are some limitations to note. First of all, we focused on only one animal, the rabbit, because this is the animal in which our research team had established an IUGR model that we have been studying for more than a decade,[14] and to prove an effective strategy for fetal therapy in this model would be a crucial step toward testing potential therapies for human IUGR. Though representing the

same fetal condition of IUGR, different animal models would obviously not reproduce the features of the human condition in exactly the same way. Moreover, the animal species themselves are different in anatomy, physiology, growth, and development.[39,40] Rabbits are more similar to humans than other animals such as rodents in regard to the perinatal pattern of brain development and the type of placentation (hemochorial placenta).[41,42] The characteristics unique to the animal model would affect not only the disease condition it represents but also the various aspects of the study design and execution, including efficiency and feasibility. The choice of animal model is a crucial decision that should be based on a thorough understanding of all the available options and their implications for the research. For example, a sheep model would not have posed the issue of the gestational sacs moving inside the uterus; owing to its greater anatomical size and fewer number of fetuses, we could have inserted pumps directly into the amniotic cavity without the catheter or could have given daily IA injections through the maternal abdominal wall without surgery. Including IUGR models of different animal species would have broadened the range of their characteristics and changed our results in developing a new therapy, but that was beyond the scope of our present study.

Second, unlike continuous IA infusion by osmotic pump and two IA injections, for one fetal IP injection we went straight to Phase II instead of starting with Phase I, thus working with the fewest number of animals and trials for this method. This decision was based on our experience with the Vevo3100 ultrasound system as well as with single injections in our previous study;[24] we did not expect concerning complications with the technique. Moreover, more trials for establishing the technique in Phase I or for increasing the sample size in Phase II are unlikely to have changed the final outcome of high mortality as IP administration is inevitably a more invasive method than IA administration.

Third, we had limited scientific evidence regarding the dose to be administered for each therapy. A previous study from our research group showed that a single IA injection (0.3 mL/dose) of nutritional solution to our surgical IUGR rabbit model improved survival at birth but not birthweight.[24] Based on our hypothesis that supplying sufficient nutrients directly to the fetus could promote not only survival but attenuate the negative effects of IUGR, we aimed to increase the dose of the same nutritional solution from our previous study. Since our previous study was the only study using the same nutritional solution, we had to combine the knowledge extrapolated from past reports (regarding maximum concentration and quantity in IA and IP administration) with our own observation of immediate effect after administration to the fetus and amniotic cavity.[25,34] A few points to contemplate when critically adjusting the administration schedule were as follows: continuous IA infusion of 10 μL/h for one day possibly being comparable to the single dose of 0.3 mL in our previous study; two IA injections of 1 mL each during the five-day period possibly being comparable to repeating a daily dose of 0.3 mL for five days; and our observation that one IA injection of 3 mL did not make the gestational sac feel tense.

To conclude, out of the three different methods that were evaluated, we did not succeed in developing an effective strategy for IA fetal therapy in the IUGR rabbit model. New development is always about finding the balance between cost and benefit. For an intervention of a translational study to be judged "feasible", it needs to be reasonably easy to perform by anyone without putting too much burden on the animal (i.e. resulting in minimal mortality), and all three therapies failed to meet those requirements in our model. Considering the many variables affecting IA fetal therapy in an IUGR animal model of specific type and species, the truly optimal method for fetal administration, with low technical failure and positive fetal impact on survival and birthweight, is yet to be developed.

AUTHORS' CONTRIBUTIONS

All authors participated in the conception and design of the study, and the analysis and interpretation of the data, and reviewed the final version of the manuscript for approval. Specifically, MK, CL, MI, MZ conducted the experiments, MK, CL, MI, MZ drafted the article, and FC, EG critically revised the manuscript for important intellectual content.

ACKNOWLEDGMENTS

The authors thank Dr Alexander Engels and Dr Elisenda Eixarch for their technical support during the Phase I trials using osmotic pumps.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

FUNDING

This work was supported by the Erasmus + Programme of the European Union (Framework Agreement number: 2013–0040; this publication reflects the views only of the author, and the Commission cannot be held responsible for any use that may be made of the information contained therein); the La Caixa Foundation (grant agreement LCF/PR/GN18/10310003); the Instituto de Salud Carlos III (PI15/00130, PI17/00675) integrados en el Plan Nacional de I + D+I y cofinanciados por el ISCIII-Subdirección General de Evaluación y el Fondo Europeo de Desarrollo Regional (FEDER) "Una manera de hacer Europa"; the AGAUR 2017 SGR grant no. 1531; and the Departament de Salut grant no. SLT006/17/00325.

ORCID iD

Mari Kinoshita https://orcid.org/0000-0002-0692-2941

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(Received December 18, 2020, Accepted February 27, 2021)