

RESEARCH ARTICLE

Nutritional intra-amniotic therapy increases survival in a rabbit model of fetal growth restriction

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Abstract

Objective

To evaluate the perinatal effects of a prenatal therapy based on intra-amniotic nutritional supplementation in a rabbit model of intrauterine growth restriction (IUGR).

Methods

IUGR was surgically induced in pregnant rabbits at gestational day 25 by ligating 40–50% of uteroplacental vessels of each gestational sac. At the same time, modified-parenteral nutrition solution (containing glucose, amino acids and electrolytes) was injected into the amniotic sac of nearly half of the IUGR fetuses (IUGR-T group n = 106), whereas sham injections were performed in the rest of fetuses (IUGR group n = 118). A control group without IUGR induction but sham injection was also included (n = 115). Five days after the ligation procedure, a cesarean section was performed to evaluate fetal cardiac function, survival and birth weight.

Results

Survival was significantly improved in the IUGR fetuses that were treated with intra-amniotic nutritional supplementation as compared to non-treated IUGR animals (survival rate: controls 71% vs. IUGR 44% p = 0.003 and IUGR-T 63% vs. IUGR 44% p = 0.02), whereas, birth weight (controls mean 43g ± SD 9 vs. IUGR 36g ± SD 9 vs. IUGR-T 35g ± SD 8, p = 0.001) and fetal cardiac function were similar among the IUGR groups.

Conclusion

Intra-amniotic injection of a modified-parenteral nutrient solution appears to be a promising therapy for reducing mortality among IUGR. These results provide an opportunity to develop new intra-amniotic nutritional strategies to reach the fetus by bypassing the placental insufficiency.

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Introduction

Intrauterine growth restriction (IUGR) is generally defined as a significant reduction in fetal growth rate resulting in a birth weight in the lowest 10th percentile. It affects 7–10% of all pregnancies [1] and is considered a major contributor to perinatal morbidity and mortality, responsible for about 20–50% of perinatal deaths. It is also associated with worse short and long-term outcomes as increased prevalence of intrapartum distress, neonatal complications [2], suboptimal neurodevelopment [3,4] and cardiovascular disease [5,6]. Currently, there is no effective nutritional therapy to improve fetal growth or to ameliorate the adverse outcomes associated with IUGR [7–10]. Thus, the assessment of fetal well-being and timely delivery remain as the main management strategy, outweighing fetal injury/stillbirth versus the risks of iatrogenic preterm delivery.

Placental insufficiency is the most common cause of IUGR where the nutrient transport to the fetus is compromised [11]. To date, several studies in humans [10,12–15] and animals [16–18] testing diverse therapies administrated to the mother have failed to demonstrate a substantial improvement in fetal outcomes related to placental insufficiency. The lack of effectiveness of these therapies that are only aimed at the mothers could most probably be explained by a failure of nutrient transport between the mother and the fetus in the presence of the placental disease [19–22], therefore the administrated therapies cannot cross the placenta and reach the fetus. Direct nutrient supply to the fetus could theoretically overcome this problem by bypassing the placenta. However, previous studies that attempted to supply carbohydrates, growth factors or amino acid mixtures through trans-amniotic catheter insertion or direct fetal injections led to inconclusive results [23–30]. Moreover, most studies used a single nutrient approach with an invasive trans-amniotic placement of a catheter for several days. We hypothesized now that the administration of a complete nutrient composition (combining essential nutrients such as glucose, amino acids and electrolytes) in a single intra-amniotic injection could improve the outcomes of IUGR. Thus, we planned to administrate this complete nutrient composition therapy by intra-amniotic injections based on the fetus capacity of swallowing amniotic fluid, by which essential nutrients delivered intra-amniotically would reach the gastrointestinal tract and be absorbed [31,32], potentially compensating the nutrient deficiency caused by placental insufficiency.

In this study, we used a rabbit model of placental insufficiency to test the hypothesis that intra-amniotic nutrient delivery would improve the perinatal outcome of IUGR fetuses, by analyzing survival, birth weight and fetal cardiac remodeling.

Material and methods

Animals and experimental procedure

The study has been reported according to the ARRIVE guidelines[33] for reporting the in vivo experiments. Animal handling and all procedures were performed in accordance with applicable regulations and guidelines and with the approval of the Animal Experimental Ethics Committee of the University of Barcelona (Permit no: 250/15) All efforts were made to reduce both animal suffering and the number of animals used.

Thirty-eight time-mated 24 months old New Zealand White pregnant rabbits were provided by a certified breeder at 18th day of gestation (full term is approximately 31 days). Dams were housed in separate cages on a reversed 12/12 h light cycle, with free access to water and standard chow. At 25th day of gestation, IUGR was induced surgically by uteroplacental vessel ligation and intra-amniotic injections were performed. At 30th day of gestation, an abdominal incision was made and the uterine horns were exteriorized to perform fetal echocardiography

in a subgroup of fetuses. Subsequently, fetuses were delivered by cesarean section. Experimental design and timeline are shown in Fig 1 and the description of all the procedures are detailed in the following sections.

Rabbit model of IUGR and therapy administration

On gestational day 25, an abdominal midline laparotomy was performed and both uterine horns were exteriorized under endovenous anesthesia of Ketamine (Ketolar® 50mg/ml, Pfizer, 10 mg/kg) and Xylazine (Rompun® 2%, Bayer, 3mg/kg). Gestational sacs of both horns were counted and numbered, and each fetus was identified according to the fetal position within the bicornuate uterus. Prior to surgery, each uterine horn was randomly allocated to a group (control, IUGR or IUGR-T) based on a computer generated randomization number sequence. As each dam has two uterine horns, in order to obtain three experimental groups (control, IUGR and UGR-T), horns of each dam was assigned to a paired combination of these groups, resulting in three combinations (control and IUGR, control and IUGR-T, or IUGR and IUGR-T). Based on the ligation and nutrient injection, the experimental groups were created: Control (no IUGR induction and sham injection, n = 115), IUGR (IUGR induction and

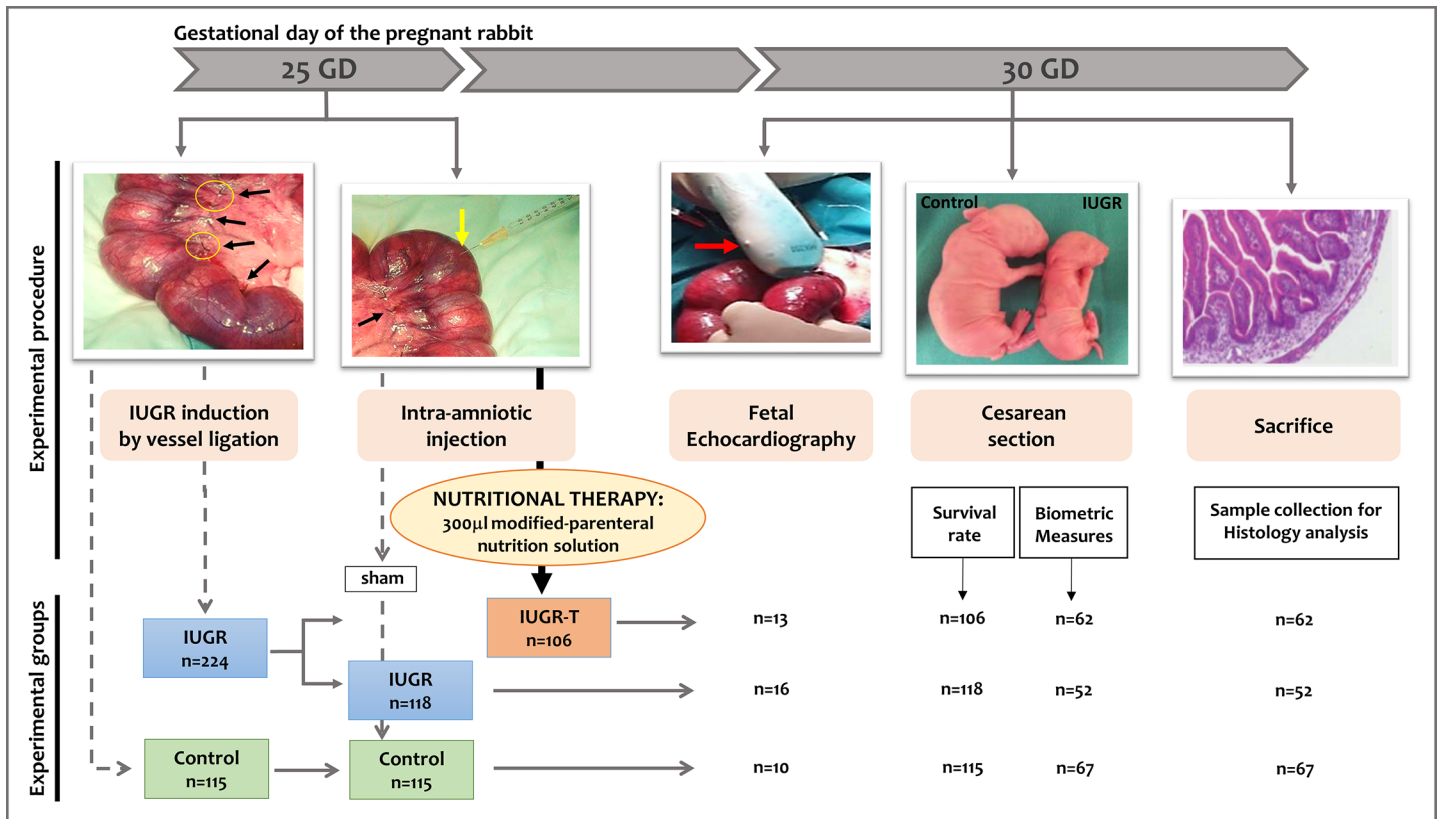


Fig 1. Experimental design. In pregnant rabbit at 25th day of gestation, IUGR was surgically induced by uteroplacental vessel ligation and intra-amniotic injections were performed. Control fetuses did not undergo vessel ligation and they had sham injection. IUGR fetuses underwent uteroplacental vessel ligation and sham injection, and finally IUGR-T fetuses underwent uteroplacental vessel ligation and therapy administration which is intra-amniotic injection of 300 µl of modified-parenteral nutrient solution. At 30th day of gestation, cesarean section was performed and uterine horns were exteriorized to perform fetal echocardiography in a subgroup of fetuses from each experimental group. The fetuses were then taken out for survival assessment and biometric measurements and then sacrificed for tissue sampling. Black arrows and yellow circles indicate ligated uteroplacental vessels of fetal sacs in the uterine horn, yellow arrow indicates intra-amniotic injection to the fetal sac and red arrow indicates the ultrasound transducer.

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Table 1. Composition of the modified-parenteral nutrition solution (per 30 mL).

Glucose (g)	3.7
Amino acids(g)	1.1
L-Isoleucine (g/L)	8
L-Leucine (g/L)	13
L-Lysine Monoacetate (g/L)	12
L-Lysine (g/L)	8.5
L-Methionine (g/L)	3.1
L-Phenylalanine (g/L)	3.8
L-Threonine (g/L)	4.4
L-Tryptophan (g/L)	2.0
L-Valine (g/L)	9
L-Arginine (g/L)	7.5
L-Histidine (g/L)	4.8
Glycine (g/L)	4.2
L-Alanine (g/L)	9.3
L-Proline (g/L)	9.7
L-Serine (g/L)	7.7
Taurine (g/L)	0.4
N-acetyl-L-tyrosine (g/L)	5.2
L-Tyrosine (g/L)	4.2
N-Acetyl-L-Cysteine (g/L)	0.7
L-Cysteine (g/L)	0.5
L-Malic Acid (g/L)	2.6
Sodium (mEq)	0.6
Calcium (mEq)	0.7
Magnesium (mEq)	0.2
Chlorine (mEq)	0.3
Phosphor (mmol)	0.2
Acetate (mEq)	0.7
Carnitine (mg)	3.7
Heparin (UI)	15.3

The solution has an osmolarity of 1.085 mOsm/L

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sham injection, n = 118) and IUGR-T (IUGR induction and therapy administration, n = 106). IUGR was surgically induced by ligation of 40–50% of the uteroplacental vessels of the assigned gestational sacs [34]. In addition, 300 µl of modified-parenteral nutrition solution (see Table 1 for composition details: a complete mixed composition containing glucose, amino acids and electrolytes, but excluding lipids, based on previous evidence of respiratory insufficiency in fetuses who received trans-amniotic lipid emulsion [26]), was injected to the amniotic sac of the IUGR-T fetuses via a 25G needle (B.Braun Sterican®). The IUGR and control groups received needle puncture without administrating any substance to the amniotic sac (sham injection) (Fig 1). Administration of Buprenorphine (Buprex injectable, 0.3 mg/ml; Schering-Ploug, Madrid, Spain) was used as a post-operative medication: The dams received a single dosage of Buprenorphine (0.01–0.05mg/kg), administrated subcutaneously after the induction of IUGR and administrated orally diluted in the water during the first 48 hours after the operation (0.03ml /5kg/8h).

Fetal echocardiography and cesarean section

At 30 days of pregnancy, an abdominal midline laparotomy was performed and uterine horns exteriorized under endovenous anesthesia of Ketamine (Ketolar® 50mg / ml, Pfizer) 10 mg/kg and Xylazine (Rompun® 2%, Bayer) 3mg/kg. After that, fetal echocardiography was performed in a subgroup of fetuses using vivid q ultrasound equipment (GE Healthcare, Little Chalfont Buckinghamshire, UK) with i12L-RS linear transducer, placed directly on each exteriorized gestational sac. Cardiac area, thoracic area, ventricular base-to-apex length and transverse diameters, and septal myocardial wall thickness were measured in end-diastole from a 2D image. Then, cardio-thoracic ratio was calculated by dividing cardiac area per thoracic area. Ventricular sphericity indexes were calculated as base-to-apex length divided by transverse diameter. Heart rate was also measured using Doppler applied on the left outflow tract.

Immediately after echocardiography, all live and stillborn fetuses were obtained by uterine horn incision and weighted. Dams were sacrificed by endovenous overdose of sodium pentobarbital (200 mg/kg), immediately after fetal extraction. All living newborns were sacrificed by immediate decapitation. Survival rate was determined by the ratio of live fetuses at the time of the cesarean section to all viable fetuses at the time of the ligature procedure. Intestine samples were collected after delivery for subsequent analysis.

Sampling and analysis of fetal intestine

After sacrificing the fetuses, one-centimeter tissue sample was collected from the proximal small intestine and fixed with 4% paraformaldehyde in PBS for 24 h at 4°C. Fixed intestinal samples were embedded in paraffin to obtain 5µm sections to be stained with hematoxylin and eosin. Histology images were acquired using a microscope (Leica, Bannockburn, IL) and software (Leica Application Suite, version 3.4). Quantification of intestine diameter, villus height and muscular and sub-mucosal layer thickness was performed using Image J software (<http://rsbweb.nih.gov/ij>) in order to evaluate intestinal structure.

Statistical analysis

The STATA14.0 package was used for statistical analyses. Qualitative variables were compared by Pearson's Chi Square test. Normal distribution of quantitative variables was assessed by Shapiro-Wilk test. Normally-distributed variables were expressed as mean and standard deviation and analyzed by one-way ANOVA followed by a Bonferroni's Multiple Comparison post hoc test. Non-normal distributed parameters were shown as median and interquartile range and compared by non-parametric Kruskal-Wallis. Statistical significance was declared at $p < 0.05$.

Results

Intra-amniotic nutrient supplementation increases IUGR survival with no improvement in birth weight

A total of 339 fetuses were obtained (115 control, 106 IUGR and 108 IUGR-T fetuses, respectively) from 38 dams. Of these 339 fetuses, 201 were alive at the day 30 of cesarean section (82 controls, 52 IUGR and 67 IUGR-T). The mean litter size was 11.4 ± 2.3 .

Non-treated IUGR fetuses presented a significantly lower survival rate (IUGR 44% vs. control 71% $p = 0.003$) and lower birth weight as compared to controls (Figs 2A and 3). However, under therapy, IUGR-T fetuses showed a significantly higher rate of survival (IUGR-T 63% vs. IUGR 44%, $p = 0.02$) despite the birth weight was similar to non-treated IUGR (Figs 2A and 3). A further analysis associated with fetuses' uterine position revealed that birth weight of

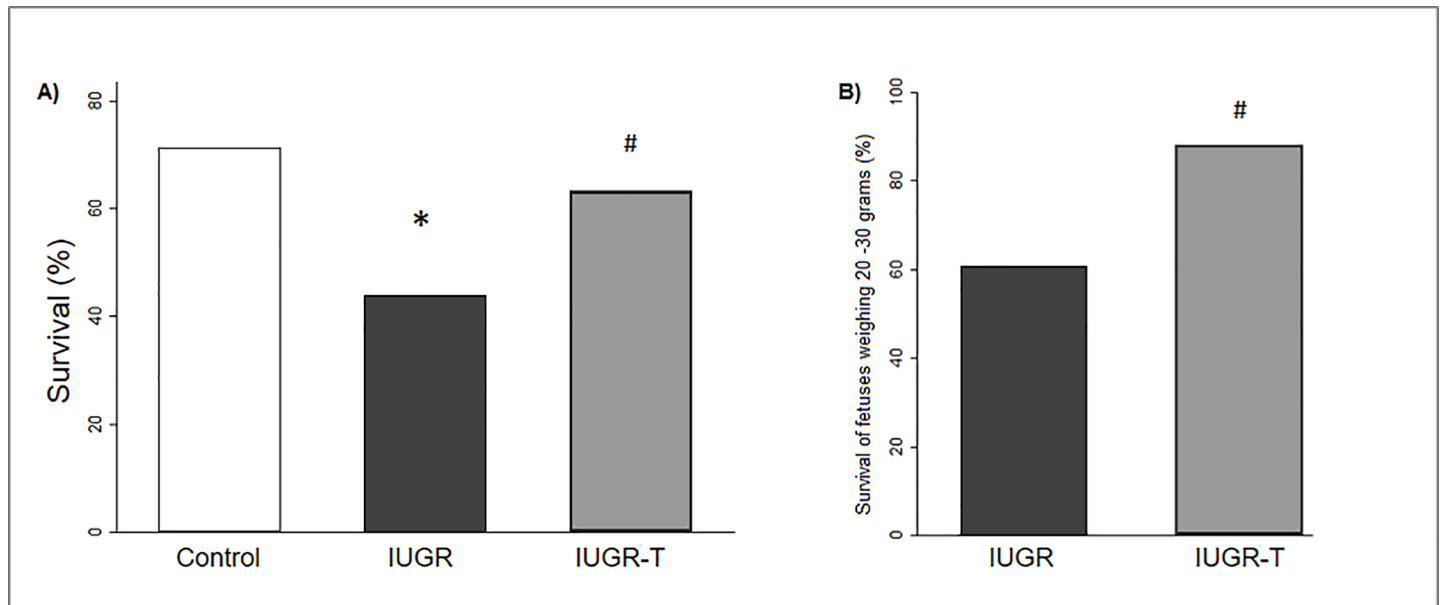


Fig 2. Survival rate. Survival rate among controls, intrauterine growth restricted cases (IUGR) or IUGR cases with therapy (IUGR-T) in the overall population (Control: 82 surviving out of 115; IUGR: 52 surviving out of 118; IUGR-T: 67 surviving out of 106) (A) and among newborns weighing 20-30g (IUGR: 14 surviving out of 23; IUGR-T: 22 surviving out of 25) (B). * $p < 0.05$ as compared to controls; # $p < 0.05$ as compared to IUGR.

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control fetuses was significantly higher as compared to both IUGR and IUGR-T groups independently from the uterine position (Fig 4). As expected, fetuses in extreme positions (ovarian and cervical ends) had higher survival rate as compared to the fetuses in intermediate positions (control 78% versus 68%; IUGR 50% versus 41%; IUGR-T 66% versus 62%, Fig 5) as compared to the fetuses in the intermediate positions. This observation was detected in all the experimental groups. For both positions, control fetuses had significantly higher birth weight than IUGR and IUGR-T fetuses, while birth weight of the latter two groups did not differ from each other (Fig 4).

An analysis performed for a subgroup of fetuses that weighed less than 30 grams (which correspond to the 10th centile of normally distributed weight at birth [3,6,34–36]) revealed that a significantly higher proportion of IUGR-T animals with that weight were alive compared to IUGR animals (Fig 2B).

Intra-amniotic nutrient supplementation does not compensate for fetal cardiac adaptation

Fetal echocardiography revealed similar cardiac alterations in both IUGR and IUGR-T fetuses with larger hearts, thicker myocardial walls and a more spherical left ventricle as compared to controls (Table 2).

Intra-amniotic nutrient supplementation ameliorates IUGR intestine structural changes

Regardless of the absence of any positive change in fetal cardiac functions and birth weight among IUGR-T treated fetuses, the notable improvement in the survival rate in this group versus IUGR fetuses may suggest that the nutritional supplementation administered was able to reach circulation in IUGR fetuses, very likely through swallowing and intestinal absorption. Actually, performed histological analyses of the small intestine (Fig 6) provide some evidence

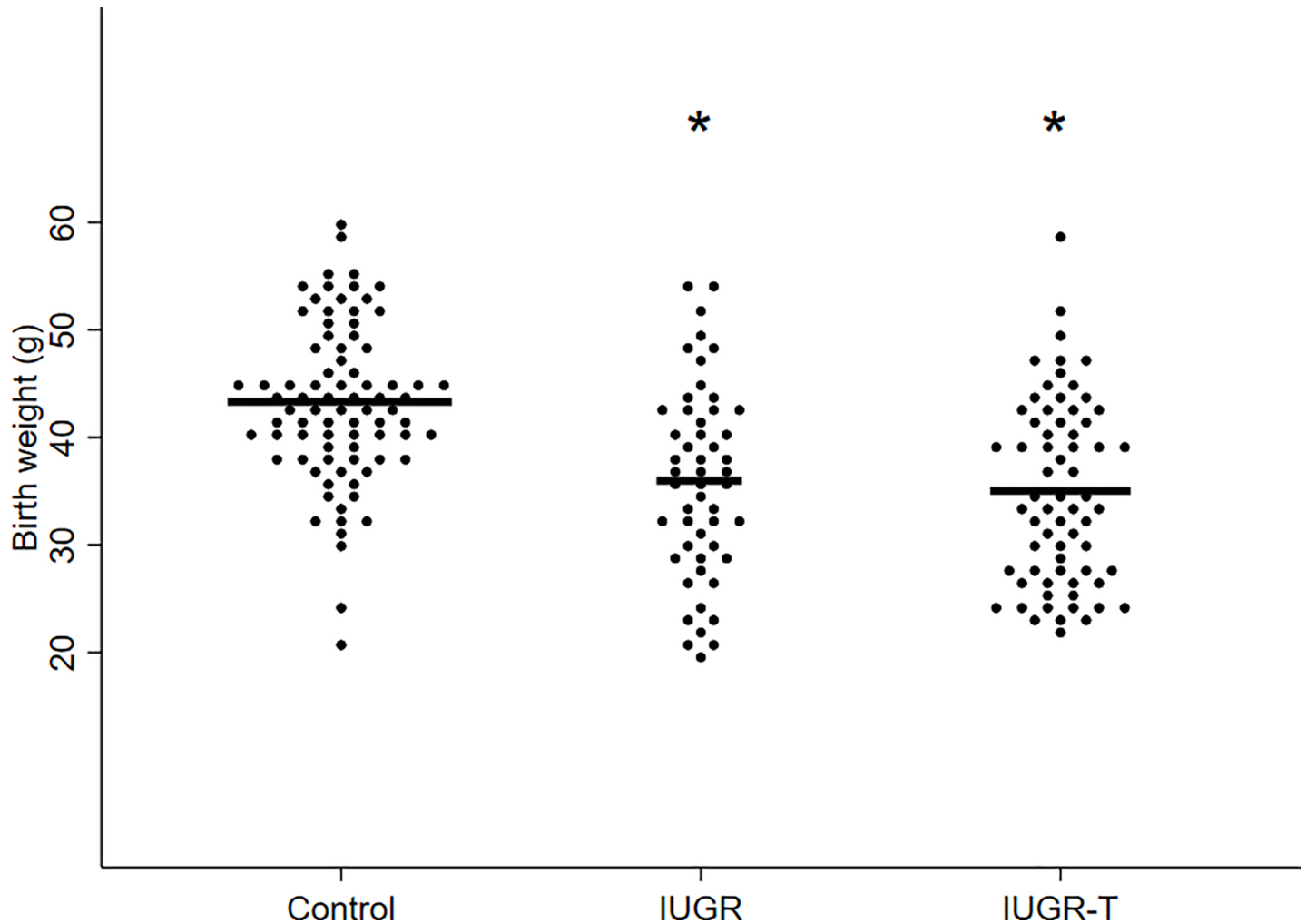


Fig 3. Birth weight. Scatter dot plot of birth weight among controls, intrauterine growth restricted cases (IUGR) or IUGR cases with therapy (IUGR-T). The horizontal line is the mean. * $p < 0.05$ as compared to controls.

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for that hypothesis; revealing shorter villus height and a less organized structure of the absorbent surface in IUGR than IUGR-T fetuses, which appears to be partially ameliorated in IUGR-T intestines (Fig 6).

Discussion

Our results support intra-amniotic injection of nutrients as a promising therapy for reducing mortality among IUGR, regardless of no apparent effects on birth weight. These results open opportunities for intra-amniotic nutritional strategies to reach the fetus bypassing the placenta.

The striking finding of our study is the improvement of survival rate in IUGR fetuses receiving intra-amniotic nutritional supplementation. Our results demonstrate that intra-amniotic injection of a modified-parenteral nutrition notably reduces mortality in an animal model of placental insufficiency. In contrast, intra-amniotic nutrition was not able to ameliorate birth weight or fetal cardiac adaptation. Several studies in the early 90s also attempted to supply nutrients in the amniotic cavity with dissimilar results. Mulvihill et al. demonstrated a

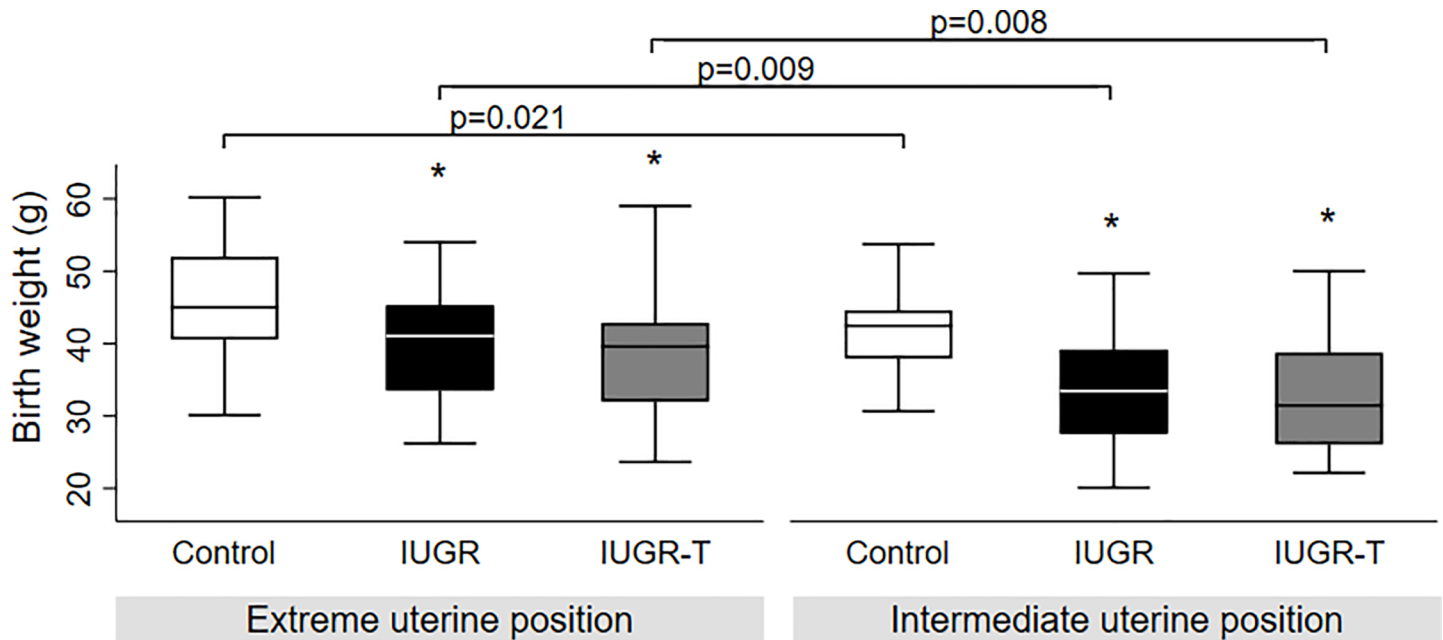


Fig 4. Birth weight in relation to uterine position. Birth weight among controls, intrauterine growth restricted cases without (IUGR) or with therapy (IUGR-T) in fetuses that are in extreme positions (ovarian and cervical ends) (Control n = 32; IUGR n = 20; IUGR-T n = 25) (A) and intermediate positions (Control n = 50; IUGR n = 32; IUGR-T n = 42) (B), * p<0.05.

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positive effect on fetal growth with similar mortality by 5-days intra-amniotic continuous infusion of bovine amniotic fluid or dextrose plus amino acids in rabbits [27,28]. In contrast, Flake et al. could not demonstrate any improvement in birth weight by 6-days continuous amniotic infusion of dextrose, dextrose-amino acid mixture or lipids in a ‘natural runting’ IUGR rabbit model [26]. Actually, the infusion of lipid emulsion resulted in chronic lipid aspiration and further growth retardation. Phillips et al. used 4-days continuous intra-amniotic infusion of

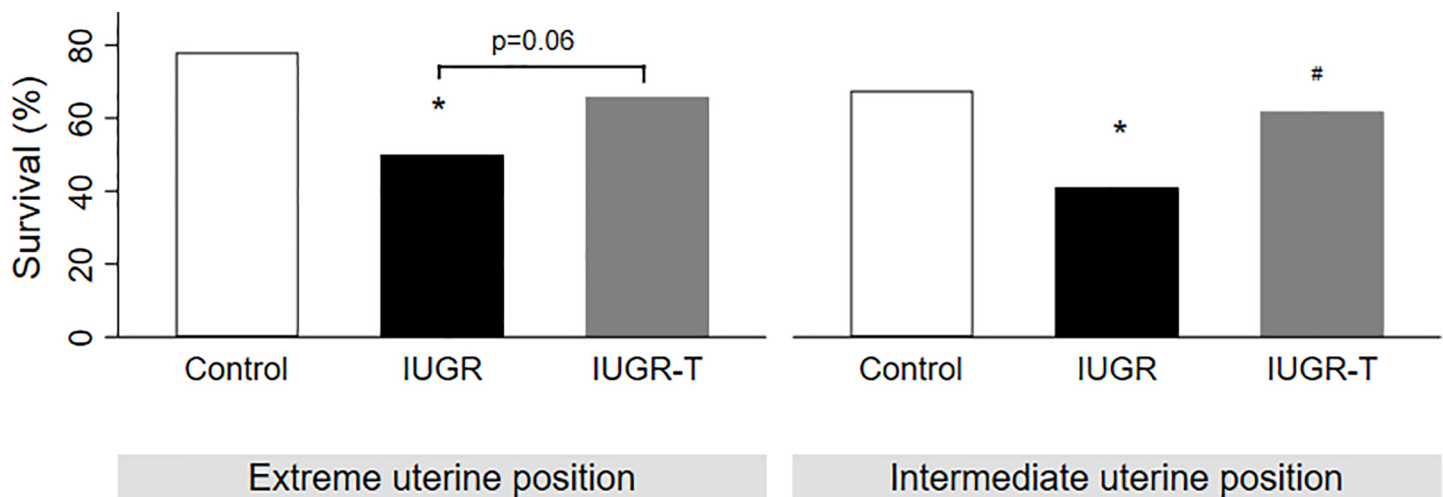


Fig 5. Survival rate in relation to uterine position. Survival rate among controls, intrauterine growth restricted cases without (IUGR) or with therapy (IUGR-T) in fetuses that are in extreme positions (Control: 32 surviving out of 41; IUGR 20 surviving out of 40; IUGR-T 25 surviving out of 38) and intermediate positions (Control: 50 surviving out of 74; IUGR: 32 surviving out of 78; IUGR-T: 42 surviving out of 68) positions. * p<0.05 as compared to controls; # p<0.05 as compared to IUGR.

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Table 2. Fetal echocardiographic results in the studied groups.

	Control (n = 10)	IUGR (n = 16)	IUGR-T (n = 13)
Cardio-thoracic area	0.35 ± 0.03	0.40 ± 0.06*	0.39 ± 0.06*
Myocardial wall thickness [†]	0.17 ± 1.01	0.21 ± 0.05*	0.20 ± 0.07*
LV sphericity index	1.43 ± 0.05	1.32 ± 0.05*	1.22 ± 0.06*
RV sphericity index	1.22 ± 0.17	1.26 ± 0.21	1.19 ± 0.09

Values are mean ± standard deviation.

*p<0.05 as compared to the control group.

†Myocardial wall thickness normalized by cardiac area. LV, left ventricular; RV, right ventricular.

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radioactive glucose and proline to demonstrate fetal nutrients absorption but failed to show changes in survival and birth weight [30]. Buchmiller et al. showed unchanged body weight and mortality after 4-days intra-amniotic infusion of galactose [37]. Finally, in the present study a combination of carbohydrates, amino acids and electrolytes were administered by a single amniotic injection in a rabbit model of uteroplacental vessel ligation showing improvement of survival despite no improvement in birth weight. Overall, the contradictory results from different studies could be explained by differences in therapy duration (single administration vs. 4–6 days of continuous infusion) and timing, type of nutrients administered (including or not including electrolytes), IUGR models (naturally vs. uteroplacental ligation) and sample size. Nutrient administration by a single injection might associate less mortality than a more invasive procedure such as a catheter insertion required for continuous infusion during several days. While intra-amniotic lipids seem deleterious, carbohydrates and amino acids appear essential for fetal development and growth. In addition, electrolytes such as

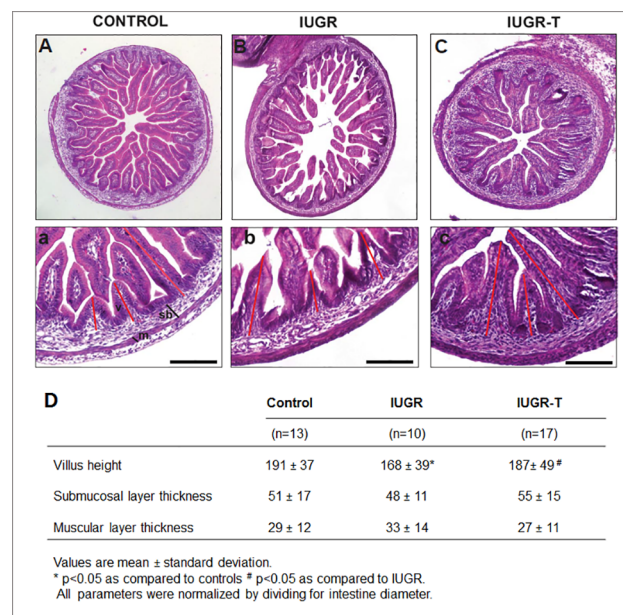


Fig 6. Histological analysis of the small intestine. Hematoxylin and eosin stained sections from controls (A), intrauterine growth restricted cases (IUGR, B) or IUGR cases with therapy (IUGR-T, C), illustrating similar villus height in controls and IUGR-T, with shorter height in IUGR. (a,b,c) 200 x magnification of stained sections, scale bars correspond to 100µm. sb denotes sub-mucosa and m mucosa. (D) Intestinal morphometric measurements of controls, intrauterine growth restricted cases without (IUGR) or with therapy (IUGR-T).

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potassium, calcium, magnesium could also be essential for fetal survival by regulating nutrient uptake [38]. The use of large sample size in a severe IUGR model with high perinatal mortality enabled us to demonstrate an improvement in survival rate among IUGR-T fetuses. We speculate that the specific mixture of glucose, amino acids and electrolytes (without lipids) administered by a single amniotic injection in the present study is enough to improve the fetal nutritional status subsequently increasing survival. Our data also suggest that nutritional status seems to be more critical than hypoxia for survival. A possible explanation for the lack of birth weight improvement in IUGR-T fetuses could be that survival of mainly the more severely restricted animals (fetuses with birth weight between 20 and 30 grams that would otherwise have died) pulled down the mean birth weight. Another potential explanation is that single administration of nutrients could only partially counteract the effect of placental insufficiency. Placental insufficiency is usually associated with a complex pathophysiologic adaptation leading to nutrient and oxygen restriction to the fetus, but also increased placental resistance inducing pressure overload to the fetal heart (that has to pump against a more resistant placenta). Most likely, intra-amniotic injection of modified-parenteral nutrition permits to ameliorate the critical fetal nutritional deficiency, but not the fetal hypoxia or pressure overload (that would explain the maintained low birth weight and fetal cardiac remodeling).

Uterine horn position seems to be a relevant factor for birthweight in rabbit model. Bautista and colleagues [39] demonstrated that animals closer to the extremities of the uterine horn had higher weight and survival compared to the animals in intermediate position. In correlation with the results reported by Bautista et al., we have also found that fetuses in the extreme positions had significantly higher birth weight than the fetuses in the intermediate position in all groups (Fig 4). Moreover, the birth weight difference of the fetuses between subgroups was significant which is consistent with our results of the whole population (control fetuses had significantly higher birth weight than IUGR and IUGR-T fetuses, while the birth weight was similar in IUGR and IUGR-T), independently of the position. We have also observed a non-significant trend for higher survival rate in extreme positions (Fig 5) which is also consistent with previously reported data [39]. Taken together, the position analysis indicates that the therapy is effective to counteract IUGR, independent of the uterine position.

The present study also showed that IUGR induction by uteroplacental vessels ligation had a negative impact on the gut structure that seems to be ameliorated by intra-amniotic injection of nutrients. Similarly, previous studies exhibited improved small intestine growth in IUGR animals by esophageal infusions of nutrients to fetal rabbit and fetal sheep [28,29], demonstrating the nutritive value of fetal swallowing in fetal intestine. In addition, previous data suggest that intra-amniotic infusion of nutrients swallowed by the fetus are transported through the gastrointestinal tract, absorbed and concreted into fetal tissues [30], suggesting an active transport of nutrients in the fetal small intestine. Taken together, our findings correlate with these studies and provide additional support for the hypothesis that intra-amniotic infusion of nutrients has a trophic effect in fetal small intestine which might provide an additional explanation for the increase in survival with IUGR-T fetuses in our study.

We acknowledge gender issues as one potential limitation for our study as sex of the animals could not be determined at the time of birth, therefore it was not possible to analyze differences in survival rate and birth weight by gender. As it was stated in the study of Tarrade et al. [40], sexual dimorphism can be often observed in rabbits in the HFD model. Further studies are needed to assess the difference between male and female birth weight and survival rate in the nutritional intra-amniotic therapy model. We also acknowledge limited information on the weights of placenta of newborn rabbits, therefore we could not calculate the fetal-placental weight ratio (F:P). Future studies are warranted to examine the impact of intra-amniotic therapies in placental development.

In conclusion, our study demonstrates that intra-amniotic nutrient supplementation increases survival rates of IUGR fetuses remarkably, particularly among those more severe IUGR animals. The use of a mixed nutritional solution containing essential carbohydrates, amino acids and electrolytes seems as an appropriate approach for reducing the mortality in IUGR. The findings from our study could be considered as a potential advance to fetal intervention of IUGR. This would raise the possibility of therapeutic strategies to improve survival particularly in those more severely restricted fetuses. Future studies are warranted to evaluate different fetal nutritional supplementation in IUGR outcomes in order to find the optimal mode, dose and timing of administration and nutritional composition.

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Writing – review & editing: Miriam Illa, Monica Zamora, Fatima Crispi, Eduard Gratacos.

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