Docosahexaenoic Acid and Lactoferrin Effects on the Brain and Placenta in a Rabbit Model of Intrauterine Growth Restriction

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Abstract

Intrauterine growth restriction (IUGR) is associated with suboptimal perinatal outcomes and neurodevelopment in the offspring. We hypothesize that prenatal supplementation with docosahexaenoic acid (DHA) or lactoferrin (Lf) would ameliorate these consequences. At 25 days of gestation, IUGR was surgically induced in pregnant rabbits, which were randomized as follows: no treatment, or DHA or Lf administration. DHA or Lf were administrated orally once per day. Five days later, animals were delivered obtaining controls, untreated IUGR, IUGR treated with DHA and IUGR treated with Lf, and the associated placentas. At postnatal day 1, a functional evaluation was performed and, thereafter, brains were obtained. Neuronal arborization in the prefrontal cortex and the density of pre-oligodendrocytes in the corpus callosum were then evaluated.

Untreated IUGR pups presented a higher percentage of stillbirth, lower birth weight, and poorer neurobehavioral performance in comparison with control pups, and these are associated with structural changes in brain and placenta. Regarding treated IUGR animals, although no significant improvements were detected in perinatal data, functional and structural effects were observed in either the brain or the placenta. DHA and Lf supplements in a rabbit model of IUGR were related to neurodevelopmental improvements and an amelioration of the placental changes.

Introduction

Intrauterine growth restriction (IUGR) is defined as a significant reduction in the fetal growth rate affecting 7–10% of all pregnancies in developed countries[1]. One of the most prevalent causes of IUGR is placental insufficiency, which represents one of the major causes of perinatal morbidity and mortality[2]. IUGR has been related to poorer functional performance in the neonatal period[3,4], persisting during childhood and adolescence[5,6]. Although the exact structural substrate underlying neurodevelopmental impairments in IUGR is still under evaluation, different studies have suggested that abnormal oligodendrocyte maturation and neuronal arborization could be key processes[7–11]. Despite the clear impact of this condition, there are no effective therapies to either reverse IUGR or prevent its neurodevelopmental consequences.

Various clinical and experimental evidence suggests that early postnatal strategies such as breastfeeding[12], an individualized newborn developmental care and assessment program[13], and environmental enrichment[14] can partially ameliorate the neurodevelopmental impairment caused by IUGR. However, all these strategies have been applied after birth, when the adverse effects of IUGR on brain development have already been consolidated. We hypothesized that a strategy applied during the prenatal period, a “critical window of opportunity”[15], could have a more pronounced effect. Among potential therapies that could be applied during the prenatal period, docosahexaenoic acid (DHA) and lactoferrin (Lf) emerge as potential candidates. The selection of both supplements was made according to previous evidence demonstrating their neuroprotective role in several neurological disorders such as Parkinson’s disease and schizophrenia, and after perinatal insults such as acute hypoxic-ischemic

events[16–19]. DHA, a type of long-chain polyunsaturated fatty acid, plays a fundamental role in central nervous system development and transfers across the placenta to reach the fetal circulation[20,21] and fetal brain[22]. Lf is a sialic acid-rich glycoprotein that also crosses the placenta and blood–brain barrier and has been involved in modulating cell-to-cell interactions and neuronal outgrowth[23]. Likewise, preliminary evidence has linked both therapies with some positive effects on placental development. Reduced oxidative stress and placental apoptosis has been previously described in omega-3 supplemented pregnancies[24,25], whereas Lf has been involved in cytotrophoblast endothelial cell invasion and placental vasculature enhacement[26,27]. Furthermore, a key factor of DHA and lactoferrin supplements is that they are normal bioactive components of a great diversity of foods and maternal milk, and consequently can be safely used as supplements[28].

Despite all these evidences of the positive effect of DHA and Lf on brain and placental development, no previous studies have evaluated the potential neuroprotective role or placental effects of these two therapies in a well characterized model of placental insufficiency.

In this study, we aimed to evaluate the neuroprotective effects of DHA and Lf in the brain at the functional and structural level, including the density of pre-oligodendrocytes (pre-OLs) in the corpus callosum (CC) and neuronal arborization in the frontal cortex, in a validated model of IUGR in pregnant rabbits[29–32]. Finally, we explored the potential positive effects of DHA and Lf on placental structure by measuring the degree of ischemia, calcification, necrosis, vascular collapse, and vascular channel dilation.

Results

Perinatal data

In comparison with control animals, untreated IUGR animals presented a significantly higher percentage of stillbirth (51.7% vs 17.8%, $P < 0.01$), and a reduced birth weight (37.4 ± 8.64 vs 47.9 ± 6.86, $P < 0.01$). The offspring of animals that were treated for IUGR did not present statistical differences in these variables when compared with the animals with IUGR that were not treated, independently of the treatment group (stillbirth: IUGR-DHA 57.1%, $P = 0.58$; IUGR-Lf 53.5%, $P = 0.86$; birth weight: IUGR-DHA 40.4 ± 6.81, $P = 0.39$; IUGR-Lf 37.7 ± 7.93, $P = 1.00$).

Functional results

Untreated IUGR pups showed poorer results with statistical differences in righting reflex and circular motion in comparison with the control group (Table 1). Regarding the group with IUGR that was treated, only animals from mothers prenatally treated with DHA (IUGR-DHA) presented a significant improvement in the righting reflex variable when compared with untreated IUGR pups (Table 1).

Placental findings

No differences were observed in the placental weight across groups (Control: 6.28 g; untreated IUGR: 5.51 g;; IUGR-DHA: 4.96 g; IUGR-Lf: 5.05 g).

Table 2 and Figure 1 summarize the placental histological findings. Overall, the IUGR pups of untreated mothers presented an increase in the percentage of ischemia and a higher degree of calcification in comparison with controls, especially in the labyrinth zone of the placenta. Regarding treated placentas, although DHA and Lf placentas seemed to present a trend to a reduction of the percentage of ischemia in the decidua basalis, the most remarkable observation was in the labyrinth zone of the placentas of Lf treated mothers in which a significant decrease in vascular collapse accompanied by an increase in vascular channel dilation in comparison with the IUGR placentas of untreated mothers were observed.

O4-immunoreactive oligodendrocyte results

A non-significant decrease in O4-immunoreactive oligodendrocyte density (immune-reactive cells/mm^2) in the CC of the untreated IUGR group was observed when compared with control animals (untreated IUGR vs control: 0.81 cells/mm^2 ± 0.18 vs 1.05 cells/mm^2 ± 0.27, P = 0.13) (Fig. 2). Interestingly, a significant increase in the percentage of O4-immunoreactive oligodendrocytes was observed in the IUGR-DHA group in comparison with the untreated IUGR group (DHA vs untreated IUGR: 1.13 cells/mm^2 ± 0.18 vs 0.81 cells/mm^2 ± 0.18, P = 0.03) (Fig. 2).

Neuronal arborization

No differences were observed in the area of the soma, dendritic length, number of total intersections or number of intersections per Sholl ring between IUGR pups of untreated mothers vs control (Supplementary Table S1 and Supplementary Fig. S1). On the contrary, IUGR pups of untreated animals showed less dendritic basal complexity with decreased primary and secondary dendrites and a significant increase in the number of the more distal dendrites (quaternary and quinary dendrites) in comparison with the pups in the control group (Fig. 3 and Supplementary Table S1).

Regarding the pups of the treated animals, the IUGR-DHA group showed higher values in the total dendritic length, area of soma and number of intersections per Sholl ring (Supplementary Table S1 and Supplementary Fig. S1) and a significant increase in the number of primary and secondary dendrites, with no significant changes in the more distal dendrites when compared with the pups of untreated animals (Fig. 3 and Supplementary Table S1). The IUGR-Lf animals presented a decrease in the majority of the neuronal arborization parameters when compared with the pups of both untreated IUGR and control animals (Supplementary Table S1, Supplementary Fig. S1). Regarding dendrite complexity, the IUGR-Lf group showed a significant reduction in tertiary, quaternary and quinary dendrites when compared with the IUGR pups of untreated animals (Fig. 3).

Discussion

The present study explores for the first time the functional and structural effects on fetal neurodevelopment and placental insufficiency of the maternal administration of DHA and Lf in pregnancies complicated by IUGR. In this study we demonstrated that maternal supplementation with DHA or Lf presented some protective effects in the brain and in the placenta in the form of IUGR secondary to placental insufficiency.

Perinatal data and placental evaluation

In this study, the prenatal administration of DHA and Lf in an IUGR rabbit model, while related to other improvements, was not related to improvements in survival and birth weight. These results are in line with previous studies showing no improvements in birth weight, nor in fetal survival after Omega-3 supplementation in human IUGR pregnancies[33] and after Lf supplementation in an IUGR rat model[34].

Regarding the histological findings in the placenta, our findings in the placentas of the untreated IUGR animals are similar to previous findings in experimental models[7,35,36], but also to clinical studies[37]. Concerning the placentas of the treated animals, DHA supplementation produced no significant changes apart from a trend to reduce the proportion of ischemia in the decidua basalis. This is in contrast with previous literature that demonstrated some positive effect of DHA on the placenta. In normal pregnancies in rats supplemented with an omega-3 diet, a reduction in the placental oxidative stress was observed [24]. In the clinical setting, reduced placental apoptosis has been described in normal pregnancies after DHA administration[25]. A more remarkable effect was observed in the treated Lf placentas, in which a significant vascular channel dilation in comparison with the placentas of untreated IUGR animals was observed. This finding could be explained by the fact that Lf, especially apoLf, enhances matrix metalloproteinase-2 expression in the cytotrophoblast, which promotes endothelial cell invasion[26] and increases placental vasculature[27]. However, this improvement did not lead to an improvement in perinatal outcomes, perhaps due to the fact that Lf only improved the vasculature with no significant effects on fibrin deposition and necrosis, which could be determinants of perinatal outcomes. Further studies including fetal and maternal Doppler evaluations and specific vascular immunohistochemistry or matrix metalloproteinase-2 expression detection would be of help in evaluating the meaning of these findings.

Neurodevelopmental effects of the perinatal administration of DHA in IUGR

Overall, our data demonstrated for the first time the positive effect of DHA in ameliorating the functional and structural brain changes induced by IUGR in the neonatal period. Maternal administration of DHA was associated with functional improvements in righting reflex, similar to those previously reported in a neonatal acute hypoxic-ischemic[38] and in transient focal cerebral ischemia[39] mouse models. Regarding structural brain results, we observed a significant improvement of the pre-OLs in the DHA group. These results are in line with evidence that suggests a protective role of Omega 3 in preserving myelin and oligodendrocytes in a mouse model of traumatic brain injury[40], promoting remyelination in a rat model of periventricular leukomalacia[41] and also after neonatal hypoxia and ischemia in rats[18]. Concerning IUGR,

an enhanced postnatal nutrient supply including DHA increased white matter (WM) volume in IUGR piglets[42] and improved WM maturation measured by diffusion MRI[43] in human babies born prematurely after a pregnancy affected by IUGR. The improvement in pre-OLs observed after DHA supplementation might explain the functional improvement detected in the righting reflex. Pre-OLs act as a reservoir of cells for later differentiation and myelinization processes and these are essential for normal motor circuit function[44].

Regarding neuronal arboritzation, our results also suggest a protective role of DHA on these parameters with an increase in the area of neuronal soma, dendritic length, the number of primary and secondary dendrites, and the number of intersections per Sholl ring in comparison with the untreated IUGR group. These results are in line with previous data showing positive effects of Omega 3 fatty acids on dendritic arborization and new spine formation in the hippocampus during normal ageing[45,46] and on neurite length and branching in hippocampal embryonic neuronal cell cultures[47,48]. Interestingly, previous literature has demonstrated the link between enhanced neuronal dendritic growth in the motor cortex with motor functional improvements[49]. In this study, neuronal arboritzation has been evaluated in the frontal cortex, which has been involved in associative learning in rabbits[50]. Therefore, the functional correspondence of these neuronal arboritzation results in the frontal cortex might be assessed by evaluating other functional domains, rather than motor and reflex variables, such as social interaction, anxiety traits, or cognition.

Neurodevelopmental effects of the perinatal administration of Lf in IUGR

Overall, our data demonstrated the effect of Lf on neuronal arboritzation with no apparent effect on pre-OL density. Maternal administration of Lf to rabbits with pregnancies affected by IUGR was not associated with significant improvements in functional evaluation of the pups compared with IUGR pups born to untreated mothers. This could not be contrasted with previous evidence as there is a lack of functional data reported in hypoxic-ischemia and IUGR studies evaluating the neuroprotective role of Lf in this setting.

At a structural level, in our study Lf seemed to have no effect in the pre-OL cell population. This result goes against previous evidence in which a positive effect of Lf on the oligodendrocyte lineage was described, although the effect was in earlier stages of oligodendrocyte development: oligodendrocyte precursor NG2 + [51]. Regarding the neuronal arboritzation results, the prenatal administration of Lf also had a limited effect, with a significant reduction in the area of soma, total number of intersections, total dendritic length, and number of tertiary, quaternary, and quinary dendrites in comparison with IUGR pups born to untreated mothers. Although previous data regarding the specific effect of Lf on neuronal arboritzation at the same neonatal age as in our study are not available, the positive effects of Lf on neuroplasticity, cell migration, and the differentiation of neuronal progenitor cells during the postnatal period in piglets have been described[23,52]. Similarly, Lf demonstrated a tendency to improve synaptogenesis in a hypocaloric IUGR rat model supplemented with Lf during gestation and lactation at

postnatal day 7[51]. Regarding the decrease in the more distal dendrites observed in the IUGR-Lf, we speculate that Lf might avoid the compensatory mechanism observed in IUGR pups of untreated mothers in which a significant increase of the more distal dendrites was obtained in comparison with control pups[7,53]. The meaning of this effect is beyond the scope of this study and further research would be needed.

Strengths and limitations.

Among the strengths of the study was the use of a well described experimental model[29,30,32] that has consistently been shown to reproduce the neurodevelopmental effects of IUGR in humans. In addition, among all the animal species used in investigations of IUGR, the rabbit has been described as presenting higher similarities to humans in terms of brain maturation in comparison with other species, making this a reliable model for obtaining data that is translational to humans[54]. Also, the use of an experimental model allowed extensive functional and neurostructural analysis to span a significant life period of the animals. Another strength of the study is that the DHA and Lf effect has been evaluated at different levels: through histology of the placenta, in functional evaluation, and through WM and grey matter (GM) assessments, giving robustness to the conclusions of the effects of these therapies on our model.

We acknowledge that there are some limitations in the study. Regarding the functional test used, we applied the only test validated in rabbits for the neonatal period. This test was described in an acute and severe hypoxia-ischemia rabbit model in the neonatal period[55] that presented severe hypertonia, locomotion, and reflex deficits, mimicking cerebral palsy in humans[56]. In this regard, the paucity of functional impairments detected in our model demonstrates the low suitability of this test in the evaluation of subtle and chronic IUGR. We consider that a more refined functional test capable of evaluating subtle motor impairments along with other neurodevelopmental dimensions such as behavior and cognition might be more appropriate for this study and model. Regarding the effects of Lf supplementation on the offspring born after IUGR, no apparent effect on the WM was observed. It should be noted that we only included the evaluation of O4 + immunoreactivity oligodendrocytes. The evaluation of earlier oligodendrocyte stages (such as NG2+) would be required in future studies to further evaluate the real role of Lf in the WM in our animal model. Similarly, the evaluation of more mature stages of neuronal connectivity such as dendrite spine formation and synaptogenesis would provide additional insight regarding the mechanisms by which Lf exerts its neuroprotective role as suggested in previous studies. Another limitation of the study was that histological analyses were restricted to the frontal cortex and CC. Other brain regions such as the hippocampus and other structures such as WM in the cerebellum have been documented to be key structures altered in IUGR[57,58] and would therefore be interesting to evaluate. Finally, although the molecular mechanisms and pathways involved in the effects of DHA and Lf are beyond the scope of this study, the description of the molecular mechanisms by which both therapies exert their neuroprotective role would be useful in understanding the differences in the structural effects of both therapies. Future in vitro culture studies might be of help in the evaluation of the molecular mechanism of action of both therapies on brain neurodevelopment. This investigation would

be critical especially for Lf as it would be of help in evaluating its real effect in the brain avoiding the interference in neurodevelopment secondary to the mitigation of placental insufficiency due to Lf.

Conclusions

In summary, our study provides novel insights into the potential protective effects of DHA and Lf administered to mothers with pregnancies affected by IUGR during the prenatal period. We demonstrated for the first time that DHA supplementation of pregnant rabbits with surgically induced placental insufficiency had a positive effect on brain development ameliorating the brain changes induced by IUGR. Regarding the Lf results, the paucity of brain results along with the changes observed in the placenta warrants further studies. Likewise, the experimental results here described justify the design and implementation of clinical studies testing the potential role of DHA and lactoferrin as neuroprotective strategies in IUGR.

Methods

IUGR induction, experimental groups, and therapy administration

All procedures were performed following all the applicable regulations and guidelines of the Animal Experimental Ethics Committee (CEEA) of the University of Barcelona and were approved with the license number 03/17. Also, animal work has been conducted fulfilling ARRIVE's guidelines and reported accordingly[59].

At 25 days of gestation, a total of 19 pregnant rabbits were subjected to the IUGR induction protocol. The selection of the sample size was made taking into account the mortality related to the IUGR model following previous experience in the group[7,14,29,60]. This sample size would allow us to include the required number of animals for each analysis of the study following previous evidence (see each paragraph for detailed information). The IUGR induction protocol was performed as previously described[29]. Briefly, IUGR was induced by ligating 40–50% of the uteroplacental vessels of gestational sacs, whereas non-ligated gestational sacs provided normally-grown controls. At the time of IUGR induction, the pregnant rabbits were randomly assigned to 3 groups: no treatment (n = 9), treatment with DHA (n = 5) and treatment with Lf (n = 5). In the mothers that had been randomized to receive DHA or Lf treatment at the time of the IUGR induction all the gestational sacs in both horns were ligated, whereas in the untreated mothers sacs from one horn were ligated while sacs from the contralateral horn were not ligated, providing normally grown controls. At 30 days of gestation, cesarean section was performed obtaining living and stillborn animals and their placentas. Control and untreated IUGR pups were obtained from untreated mothers, whereas IUGR-DHA and IUGR-Lf pups were obtained from mothers treated with DHA or Lf, respectively.

DHA or Lf were orally administered to the pregnant rabbits once per day from day 25 until the day of the cesarean section. The therapy was administered using a syringe to cautiously release the solution into

the mouth. The specific dose of DHA (37 mg/kg/day) and Lf (166 mg/kg/day) was calculated taking into account previous evidence[19,61]. Doses were adjusted with the formula for interspecies translation based on the body surface area (BSA)[62], obtained as follows:

$$BSA(\mathrm{m}^2) = 9.9x\frac{rabbit\ weight\ (g)}{10\ 000}$$

and on the K_m value, obtained by:

$$k_m = \frac{body\ weight\ (kg)}{BSA}$$

Finally, the formula used for interspecies translation was as follows[63]:

$$HED\ (mg/kg) = known\ dose\ (mg/kg)_x\frac{k_m\ animal\ of\ known\ dose}{rabbit\ K_m}$$

The DHA was obtained from Rendon Europe Laboratories and was presented in 3 g 100% pure powder from Microalga oil *Schizochytrium* sp. The Lf administrated was bovine Lf containing a low concentration of iron (9.2 mg of iron per 100 g of protein) and was obtained from Farmalabor.

Functional evaluation

Due to differences in the mortality rate obtained in the experimental groups, the final sample differed between groups: 15 normally-grown controls, 15 untreated IUGR, 18 IUGR-DHA, and 19 IUGR-Lf. At postnatal day 1, general motor skills, tone, reflexes, and olfactory sensitivity were evaluated in all offspring following the previous methodology described by Derrick et al.[55]. For each animal, the testing was videotaped and variables were scored on a scale of 0 to 3 (0 = worst and 3 = best), except for tone that was scored 0 to 4 according to the Ashworth scale[64] by 2 blinded observers (MI, LP). A detailed explanation of how each variable was assessed can be reviewed in the supplementary material (Supplementary methods and Supplementary Table S2).

Sample collection

After the cesarean section, placentas were obtained, carefully washed in saline solution, weighed, fixed for 24 hours in 10% formalin and embedded in paraffin.

After functional evaluation, newborns were weighed and sacrificed by decapitation after the administration of ketamine (35 mg/kg) and xylazine (5mg/kg) intramuscularly. All brains were carefully dissected and fixed according to the analysis performed. Four brains from each group were randomly selected for the Golgi-Cox staining protocol and were processed according to it (detailed below). The other brains (*n* = 11 control, 11 untreated IUGR, 14 IUGR-DHA, 15 IUGR-DHA) were fixed for 24 hours in 10% formalin, dehydrated for 48 hours with sucrose 30% and finally frozen at −80 ºC.

Histological procedures

Placenta

Five placentas from each group were randomly selected for histological evaluation based on findings from previous work in pregnant rabbits[7]. All the placentas were evaluated except one from the IUGR-DHA group, in which the analysis could not be performed owing to technical problems with the processing. Two consecutive slices (4 µm) of each placenta from paraffin blocks were stained following a hematoxylin and eosin standard protocol. The pathologist was blinded to the experimental groups. The analyses were performed on two different regions of the placenta: the decidua basalis (maternal part) and the labyrinth zone (fetal part). Ischemia, necrosis, fibrin, and calcifications were assessed in the decidua, while ischemia, vascular collapse, congestion, and calcifications were evaluated in the labyrinth zone. Ischemia and fibrin were expressed as a percentage, necrosis was assessed by the presence of karyorrhexis and karyolysis, and the remaining variables were evaluated with a semiquantitative system that graded each lesion from 0 to 5. The grading criteria followed were: 0) Unremarkable; 1) Minimal; 2) Mild; 3) Moderate; 4) Marked; 5) Severe.

O4-immunoreactive oligodendrocytes

Eight formalin-fixed brains per group were selected for the O4-immunoreactive oligodendrocyte evaluation. The sample size for oligodendrocyte evaluation was calculated taking into consideration previous studies with the same animal model[7,60]. Three coronal sections of 40 µm at the level of the genu CC were obtained and processed as previously described[7]. Briefly, slices were incubated overnight with anti-oligodendrocyte marker O4 (1:50, Chemicon) followed by 1% Hoechst 33258 (1:1000, Thermofischer) incubation for cellular nucleus visualization. Finally, slices were incubated with the specific secondary antibody conjugated to 488 Alexa Fluor (1:400, MoBitec). Immunoreactive sites were observed under the confocal microscope and images were taken as previously described[7]. The number of the total cell nuclei (stained with Hoechst) and the number of cells with fluorescent staining around the nucleus (O4-immunoreactive oligodendrocytes) were counted using the software ImageJ. O4-immunoreactive

oligodendrocyte density (immune-reactive cells/mm^2) was then calculated. The evaluation for each experimental group was blinded for the evaluator (LP). The anti-O4 antibody was selected as a marker for the latter stages of oligodendrocyte maturation (O4-OL), which include pre-OLs, pre-myelinating OL, and myelinating OL[65]. Since pre-OL cells are the most predominant oligodendrocytes in rabbits at the day of evaluation, we assumed that the O4 marker used mainly stained the pre-OL cells[56].

Neuronal arborization (Golgi-Cox staining)

Four brains per group were included in the neuronal arborization analyses, similarly to previous studies[7,8]. Neuronal arborization evaluation was performed as previously described[7]. Briefly, vibratome was used to obtain 100 µm serial and coronal sections that were processed for Golgi-Cox impregnation with the FD Rapid Golgi Stain kit (FD Neurotechnologies Inc.). Five pyramidal neurons that fulfilled the inclusion criteria from the frontal cortex per brain hemisphere were selected randomly and one image per neuron was obtained. The inclusion criteria were: pyramidal neurons within layer II and III and complete filling of the dendritic tree with well-defined endings. The evaluation for each experimental group was blinded for the evaluators (LP, MM). The parameters evaluated were: i) area of the soma; ii) total basal dendritic length; iii) basal dendritic complexity, including number of dendrites and number of intersections for each Sholl ring. The study design is summarized in Figure 4.

Statistical analyses

The software packages STATA14.0 and GraphPad 5 were used for statistical analyses and graphical representation. For quantitative variables, normality was assessed by the Shapiro-Wilk test and homoscedasticity was determined by Levene's Test, except for variables with more than 30 observations (Golgi-Cox variables) for which normal distribution was already assumed. For ordinal variables, non-normal distribution was assumed. The descriptives of the variables were expressed as mean and standard deviation (SD) for normal distributions, whereas median and interquartile range (IQR) were used for non-normal distributions and ordinal variables. Appropriate statistical tests were used according to the variable: ANOVA with Dunnett's multiple comparison test was used for continuous variables, Kruskal-Wallis with Dunn's multiple comparison test for ordinal variables and chi-squared for binary variables. Statistical significance was declared at $P < 0.05$ in all variables evaluated.

Declarations

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

MI and LP contributed to the analyses, the interpretation of data, and the writing of the original draft. CL, LP, and MI contributed to the animal surgery and functional evaluation. CM, LP, and MM contributed to the immunochemistry analyses. BK and MB contributed in reviewing and editing the original draft. MI, EE, and EG participated in conceptualization and supervision.

Competing interests

The authors declare no competing interests.

References

1. Kady, S. M. & Gardosi, J. Perinatal mortality and fetal growth restriction. *Best Pract. Res. Clin. Obstet. Gynaecol.***18**, 397–410 (2004).
2. Malhotra, A. *et al.* Neonatal morbidities of fetal growth restriction: Pathophysiology and impact. *Front. Endocrinol. (Lausanne).***10**, 1–18 (2019).
3. Figueras, F. *et al.* Neurobehavior in term, small-for-gestational age infants with normal placental function. *Pediatrics***124**, e934–e941 (2009).
4. Feldman, R. & Eidelman, A. I. Neonatal state organization, neuromaturation, mother-infant interaction, and cognitive development in small-for-gestational-age premature infants. *Pediatrics***118**, e869-78 (2006).
5. Leitner, Y. *et al.* Neurodevelopmental outcome of children with intrauterine growth retardation: A longitudinal, 10-Year prospective study. *J. Child Neurol.***22**, 580–587 (2007).
6. Tideman, E., Mar, K. S. & Ley, D. Cognitive function in young adults following intrauterine growth restriction with abnormal fetal aortic blood flow. *Ultrasound Obs. Gynecol***29**, 614–618 (2007).
7. Pla, L. *et al.* Structural brain changes at the neonatal period in a rabbit model of Intrauterine Growth Restriction. *Dev. Neurosci.* (2020). (Accepted)
8. Dean, J. M. *et al.* Prenatal Cerebral Ischemia Disrupts MRI-Defined Cortical Microstructure Through Disturbances in Neuronal Arborization. *Sci. Transl. Med.***5**, 168ra7-168ra7 (2013).
9. Dieni, S. & Rees, S. Dendritic morphology is altered in hippocampal neurons following prenatal compromise. *J. Neurobiol.***55**, 41–52 (2003).

10. Back, S. A. *et al.* Selective vulnerability of late oligodendrocyte progenitors to hypoxia- ischemia. *J. Neurosci.***22**, 455–463 (2002).
11. Tolcos, M. *et al.* Intrauterine growth restriction affects the maturation of myelin. *Exp. Neurol.***232**, 53–65 (2011).
12. Rao, M. R., Hediger, M. L., Levine, R. J., Naficy, a B. & Vik, T. Effect of breastfeeding on cognitive development of infants born small for gestational age. *Acta Paediatr.***91**, 267–74 (2002).
13. Als, H. *et al.* NIDCAP improves brain function and structure in preterm infants with severe intrauterine growth restriction. *J. Perinatol.***32**, 797–803 (2012).
14. Illa, M. *et al.* Early Environmental Enrichment Enhances Abnormal Brain Connectivity in a Rabbit Model of Intrauterine Growth Restriction. *Fetal Diagn. Ther.***44**, 184–193 (2018).
15. Andersen, S. L. Trajectories of brain development : point of vulnerability or window of opportunity? *Neurosci. Biobehav. Rev.***27**, 3–18 (2003).
16. Rousseau, E., Michel, P. P. & Hirsch, E. C. The Iron-Binding Protein Lactoferrin Protects Vulnerable Dopamine Neurons from Degeneration by Preserving Mitochondrial Calcium Homeostasis. *Mol. Pharmacol.***84**, 888–898 (2013).
17. Zugno, A. I. *et al.* Omega-3 prevents behavior response and brain oxidative damage in the ketamine model of schizophrenia. *Neuroscience***259**, 223–231 (2014).
18. Arteaga, O. *et al.* Docosahexaenoic Acid Reduces Cerebral Damage and Ameliorates Long-Term Cognitive Impairments Caused by Neonatal Hypoxia-Ischemia in Rats. *Mol. Neurobiol.***54**, 7137–7155 (2016).
19. Looij, Y. Van De *et al.* Lactoferrin during lactation protects the immature hypoxic- ischemic rat brain. *Ann. Clin. Transl. Neurol.***1**, 955–967 (2014).
20. Innis, S. M. Essential Fatty Acid Transfer and Fetal Development. *Placenta***26**, S70–S75 (2005).
21. Gil-Sánchez, A. *et al.* Maternal-fetal in vivo transfer of [13C]docosahexaenoic and other fatty acids across the human placenta 12 h after maternal oral intake. *Am. J. Clin. Nutr.***92**, 115–122 (2010).
22. Ikeno, M. *et al.* Fatty acid composition of the brain of intrauterine growth retardation rats and the effect of maternal docosahexaenoic acid enriched diet. *Early Hum. Dev.***85**, 733–735 (2009).
23. Wang, B. Molecular Determinants of Milk Lactoferrin as a Bioactive Compound in Early Neurodevelopment and Cognition. *J. Pediatr.***173**, S29–S36 (2016).
24. Jones, M. L., Mark, P. J., Mori, T. A., Keelan, J. A. & Waddell, B. J. Maternal Dietary Omega-3 Fatty Acid Supplementation Reduces Placental Oxidative Stress and Increases Fetal and Placental Growth in the Rat. *Biol. Reprod.***88**, 37, 1–8 (2013).
25. Wietrak, E., Kamiński, K., Leszczyńska-Gorzelak, B. & Oleszczuk, J. Effect of Docosahexaenoic Acid on Apoptosis and Proliferation in the Placenta: Preliminary Report. *Biomed Res. Int.***2015**, (2015).
26. Lopez, V., Kelleher, S. L. & Lönnerdal, B. Lactoferrin receptor mediates apo- but not holo-lactoferrin internalization via clathrin-mediated endocytosis in trophoblasts. *Biochem. J.***411**, 271–278 (2008).

27. Chen, J. & Khalil, R. A. Matrix Metalloproteinases in Normal Pregnancy and Preeclampsia. *Prog. Mol. Biol. Transl. Sci.***148**, 87–165 (2017).
28. Boquien, C. Y. Human milk: An ideal food for nutrition of preterm newborn. *Front. Pediatr.***6**, 1–9 (2018).
29. Eixarch, E. *et al.* An experimental model of fetal growth restriction based on selective ligature of uteroplacental vessels in the pregnant rabbit. *Fetal Diagn. Ther.***26**, 203–211 (2009).
30. Eixarch, E. *et al.* Neonatal neurobehavior and diffusion MRI changes in brain reorganization due to intrauterine growth restriction in a rabbit model. *PLoS One***7**, e31497 (2012).
31. Illa, M. *et al.* Long-term functional outcomes and correlation with regional brain connectivity by MRI diffusion tractography metrics in a near-term rabbit model of intrauterine growth restriction. *PLoS One***8**, e76453 (2013).
32. Illa Armengol, M. *et al.* Neurodevelopmental Effects of Undernutrition and Placental Underperfusion in Fetal Growth Restriction Rabbit Models. *Fetal Diagn. Ther.***42**, 189–197 (2017).
33. Saccone, G., Berghella, V., Maruotti, G. M., Sarno, L. & Martinelli, P. Omega-3 supplementation during pregnancy to prevent recurrent intrauterine growth restriction: Systematic review and meta-analysis of randomized controlled trials. *Ultrasound Obstet. Gynecol.***46**, 659–664 (2015).
34. Somm, E. *et al.* Protective effects of maternal nutritional supplementation with lactoferrin on growth and brain metabolism. *Pediatr. Res.***75**, 51–61 (2014).
35. López-Tello, J. *et al.* The effects of sildenafil citrate on feto-placental development and haemodynamics in a rabbit model of intrauterine growth restriction. *Reprod. Fertil. Dev.***29**, 1239–1248 (2016).
36. Lopez-Tello, J. *et al.* Competition for materno-fetal resource partitioning in a rabbit model of undernourished pregnancy. *PLoS One***12**, e0169194 (2017).
37. Mardi, K. & Sharma, J. Histopathological evaluation of placentas in IUGR pregnancies. *Indian J. Pathol. Microbiol.***46**, 551–554 (2003).
38. Mayurasakorn, K. *et al.* DHA but Not EPA Emulsions Preserve Neurological and Mitochondrial Function after Brain Hypoxia-Ischemia in Neonatal Mice. *PLoS One***11**, e0160870 (2016).
39. Jian, X. post-stroke therapeutic regimen with omega-3 polyunsaturated fatty acids that promotes white matter integrity and beneficial microglial responses after cerebral ischemia *et al.* A post-stroke therapeutic regimen with omega-3 polyunsaturated fatty acids that promotes white matter integrity and beneficial microglial responses after cerebral ischemia. *Transl. Stroke Res.***7**, 548–561 (2016).
40. Pu, H. *et al.* Omega-3 polyunsaturated fatty acid supplementation improves neurologic recovery and attenuates white matter injury after experimental traumatic brain injury. *J. Cereb. Blood Flow Metab.***33**, 1474–1484 (2013).
41. Tuzun, F. *et al.* Maternal omega-3 fatty acid supplementation protects against lipopolysaccharide-induced white matter injury in the neonatal rat brain. *J. Matern. Neonatal Med.***25**, 849–854 (2012).

42. Caputo, M. P. *et al.* Hydrolyzed Fat Formula Increases Brain White Matter in Small for Gestational Age and Appropriate for Gestational Age Neonatal Piglets. *Front. Pediatr.***8**, 32 (2020).
43. Strømmen, K. *et al.* Enhanced nutrient supply to very low birth weight infants is associated with improved white matter maturation and head growth. *Neonatology***107**, 68–75 (2015).
44. Pepper, R. E., Pitman, K. A., Cullen, C. L. & Young, K. M. How do cells of the oligodendrocyte lineage affect neuronal circuits to influence motor function, memory and mood? *Front. Cell. Neurosci.***12**, 1–14 (2018).
45. Cutuli, D. Functional and Structural Benefits Induced by Omega-3 Polyunsaturated Fatty Acids During Aging. *Curr. Neuropharmacol.***15**, 534–542 (2017).
46. Crupi, R., Marino, A. & Cuzzocrea, S. n-3 Fatty Acids : Role in Neurogenesis and Neuroplasticity. *Curr. Med. Chem.***20**, 2953–2963 (2013).
47. Calderon, F. & Kim, H. Y. Docosahexaenoic acid promotes neurite growth in hippocampal neurons. *J. Neurochem.***90**, 979–988 (2004).
48. Carbone, B. E. *et al.* Synaptic Connectivity and Cortical Maturation Are Promoted by the ω-3 Fatty Acid Docosahexaenoic Acid. *Cereb. cortex***30**, 226–240 (2020).
49. Biernaskie, J. & Corbett, D. Enriched Rehabilitative Training Promotes Improved Forelimb Motor Function and Enhanced Dendritic Growth after Focal Ischemic Injury. *J. Neurosci.***21**, 5272–5280 (2001).
50. Woodruff-Pak, D. S., Agelan, A. & Valle, L. Del. A rabbit model of Alzheimer's disease: Valid at neuropathological, cognitive, and therapeutic levels. *J. Alzheimer's Dis.***11**, 371–383 (2011).
51. Looij, Y. Van De *et al.* Nutritional intervention for developmental brain damage: Effects of lactoferrin supplementation in hypocaloric induced intrauterine growth restriction rat pups. *Front. Endocrinol. (Lausanne).***10**, 1–14 (2019).
52. Chen, Y. *et al.* Lactoferrin Promotes Early Neurodevelopment and Cognition in Postnatal Piglets by Upregulating the BDNF Signaling Pathway and Polysialylation. *Mol. Neurobiol.***52**, 256–269 (2015).
53. Ruan, Y. *et al.* Dendritic plasticity of CA1 pyramidal neurons after transient global ischemia. *Neuroscience***140**, 191–201 (2006).
54. Workman, A. D., Charvet, C. J., Clancy, B., Darlington, R. B. & Finlay, B. L. Modeling transformations of neurodevelopmental sequences across mammalian species. *J. Neurosci.***33**, 7368–7383 (2013).
55. Derrick, M. *et al.* Preterm Fetal Hypoxia–Ischemia Causes Hypertonia and Motor Deficits in the Neonatal Rabbit: A Model for Human Cerebral Palsy? *J. Neurosci.***24**, 24–34 (2004).
56. Saadani-makki, F. *et al.* Intrauterine administration of endotoxin leads to motor deficits in a rabbit model: a link between prenatal infection and cerebral palsy. *Am. J. Obstet. Gynecol.***199**, 651. e1-651. e7. (2008).
57. Lodygensky, G. A. *et al.* Intrauterine growth restriction affects the preterm infant's hippocampus. *Pediatr. Res.***63**, 438–443 (2008).

58. Padilla, N. *et al.* Differential effects of intrauterine growth restriction on brain structure and development in preterm infants: A magnetic resonance imaging study. *Brain Res.***1382**, 98–108 (2011).
59. du Sert, N. P. *et al.* The arrive guidelines 2.0: Updated guidelines for reporting animal research. *PLoS Biol.***18**, e300041 (2020).
60. Barenys, M. *et al.* Rabbit neurospheres as a novel in vitro tool for studying neurodevelopmental effects induced by intrauterine growth restriction (IUGR). *Stem Cells Transl. Med.***10**, 1–13 (2020).
61. Greenberg, J. A., Bell, S. J. & Ausdal, W. Van. Omega-3 Fatty Acid Supplementation During Pregnancy. *Rev. Obstet. Gynecol.***1**, 162–169 (2008).
62. Zehnder, A. M., Hawkins, M. G., Trestrail, E. A., Holt, R. W. & Kent, M. S. Calculation of body surface area via computed tomography–guided modeling in domestic rabbits (Oryctolagus cuniculus). *Am. J. Vet. Res.***73**, 1859–1863 (2012).
63. Anroop B. Nair, S. J. A simple practice guide for dose conversion between animals and human. *J. Basic Clin. Pharm.***7**, 27–31 (2016).
64. Damiano, D. L. *et al.* What does the Ashworth scale really measure and are instrumented measures more valid and precise? *Dev. Med. Child Neurol.***44**, 112–118 (2002).
65. Kuhn, S., Gritti, L., Crooks, D. & Dombrowski, Y. Oligodendrocytes in Development, Myelin Generation and Beyond. *Cells***8**, 1424 (2019).

Tables

Table 1. Functional results in the study groups.

Variables	Control *N* = 15	Untreated IUGR *N* = 15	IUGR-DHA *N* = 18	IUGR-Lf *N* = 19
Posture, score[α]	3 (0)	3 (0)	3 (0)	3(0)
Righting reflex, no. of turns[β]	9.47 (1.25)	7.44 (2.92)*	9.67 (0.69)**	5.47 (3.13)*,**
Tone, score[α]	4 (0)	4 (0)	4 (0)	4 (1)*, **
Circular motion, score[α]	3 (1)	2 (1)*	2 (1)	2 (1)*
Hindlimb locomotion, score[α]	3 (1)	2 (2)	2 (0)	2 (1)
Intensity, score[α]	3 (0)	2.5 (1)	3 (0)	2 (1)*
Duration, score[α]	3 (0)	3 (0)	3 (0)	3 (1)
Lineal movement, no.[β]	3.27 (1.58)	2.22 (1.70)	3.06 (1.80)	1.32 (1.60)*
Fore–hindpaw distance, mm[β]	0.4 (1.06)	2.28 (3.83)	1.22 (2.73)	0.67 (1.34)
Sucking and swallowing, score[α]	3 (0)	3 (2)	3 (1)	3 (0)**
Head turning, score[α]	3 (0)	3 (1)	3 (0)	3 (1)
Olfaction, score[α]	3 (1)	3 (1)	3 (1)	2 (1)
Olfaction time, seconds[β]	3.0 (2.3)	3.67 (2.43)	3.72 (2.82)	3.58 (1.51)

Results are given as a median and interquartile range (median(IQR)) for ordinal variables ([α]) and mean and standard deviation (mean(SD)) for the continuous variables ([β]). Statistical significance was declared at $P < 0.05$ between the control and the rest of the experimental groups (*) and between untreated IUGR and IUGR-DHA or IUGR-Lf (**).

Abbreviations: untreated IUGR = intrauterine growth restriction with no treatment; IUGR-DHA = intrauterine growth restriction treated with DHA; IUGR-Lf= intrauterine growth restriction treated with Lf.

Table 2. Placental histopathological findings in the study groups

DECIDUA BASALIS				
Study groups	Ischemia (%)	Necrosis (Y/N)	Fibrin (%)	Calcification (0-5)
Control	93.8 (10.7)	Y	3.60 (1.14)	1 (2.5)
Untreated IUGR	99.8 (0.45)	Y	4.40 (0.55)	3 (3.5)
IUGR-DHA	88.3 (5.69)	Y	4.25 (0.50)	3 (1.5)
IUGR-Lf	89.0 (9.62)	Y	4.40 (0.55)	2 (1.5)
LABYRINTH ZONE				
Study Groups	Ischemia (%)	Collapse (0–5)	Congestion (0–5)	Calcification (0–5)
Control	16.8 (5.77)	1 (1.5)	0 (0)	1 (2)
Untreated IUGR	96.0 (1.73)*	3 (0)	0 (0)	4 (4)
IUGR-DHA	97.0 (2.45)*	2 (1.5)	0 (1.5)	3 (1.5)
IUGR-Lf	89.0 (4.18)*	0 (1.5)**	4 (1.5)	4 (1.5)*,**

Results are mean and standard deviation (mean (SD)) for continuous variables (expressed as a percentage) and median and interquartile range (median(IQR)) for ordinal variables (expressed as a semiquantitative grading system, score = 0–5). The nomenclature of Y (yes) or N (no) was used for the variable of necrosis. Statistical significance was declared at $P < 0.05$ between control and the rest of the experimental groups (*) and between untreated IUGR and IUGR-DHA or IUGR-Lf (**).

Abbreviations: untreated IUGR = intrauterine growth restriction with no treatment; IUGR-DHA = intrauterine growth restriction treated with DHA; IUGR-Lf = intrauterine growth restriction treated with Lf.

Figures

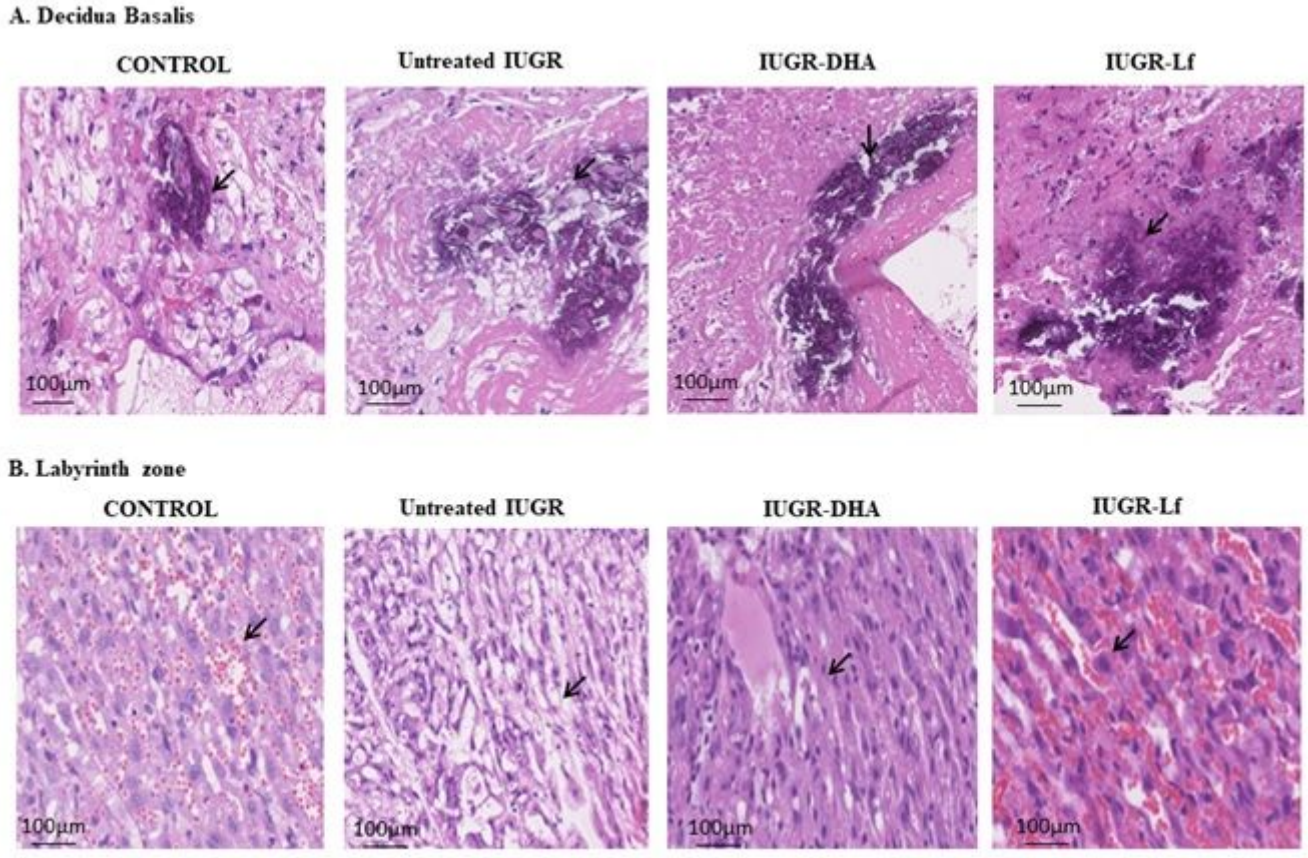

Figure 1

Placenta histological findings in the study groups. A. Representative images of calcification (arrow) in decidua basalis among the study groups. B. Representative images of vascular channel congestion (arrow) in the labyrinth zone among the study groups. Abbreviations: untreated IUGR = intrauterine growth restriction with no treatment; IUGR-DHA = intrauterine growth restriction treated with DHA; IUGR-Lf = intrauterine growth restriction treated with Lf.

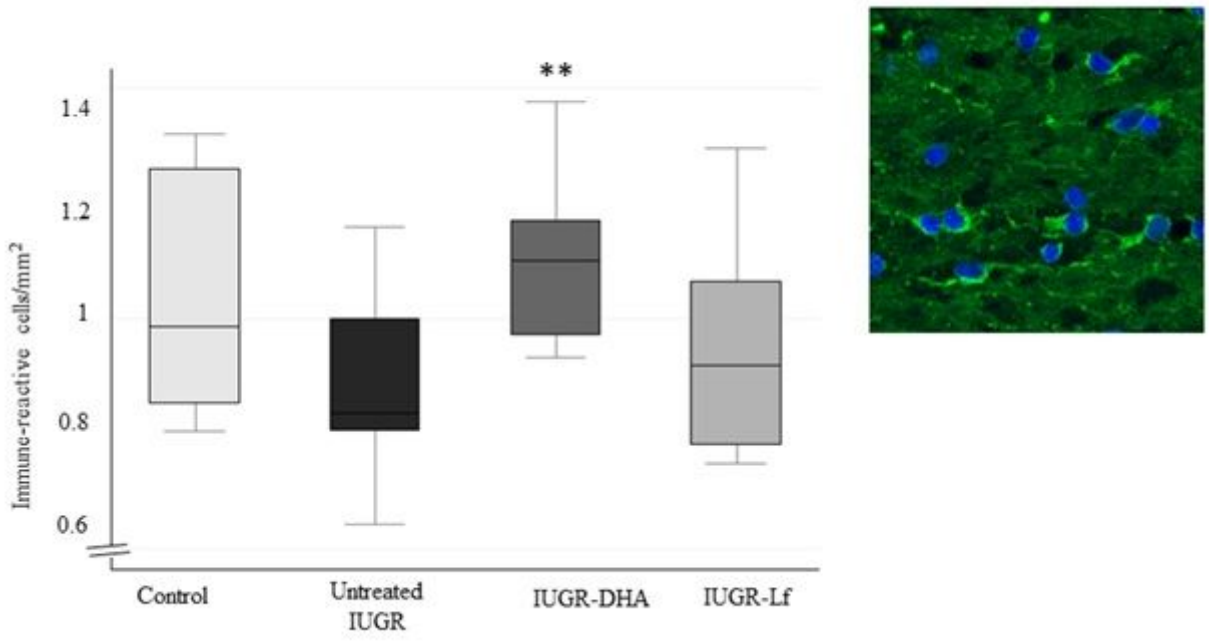

Figure 2

O4-immunoreactive oligodendrocytes in the CC in the study groups. Statistical significance was declared at $P < 0.05$ between the control and the rest of the experimental groups (*) and between untreated IUGR and IUGR-DHA or IUGR-Lf (**). Abbreviations: untreated IUGR = intrauterine growth restriction with no treatment; IUGR-DHA = intrauterine growth restriction treated with DHA; IUGR-Lf = intrauterine growth restriction treated with Lf.

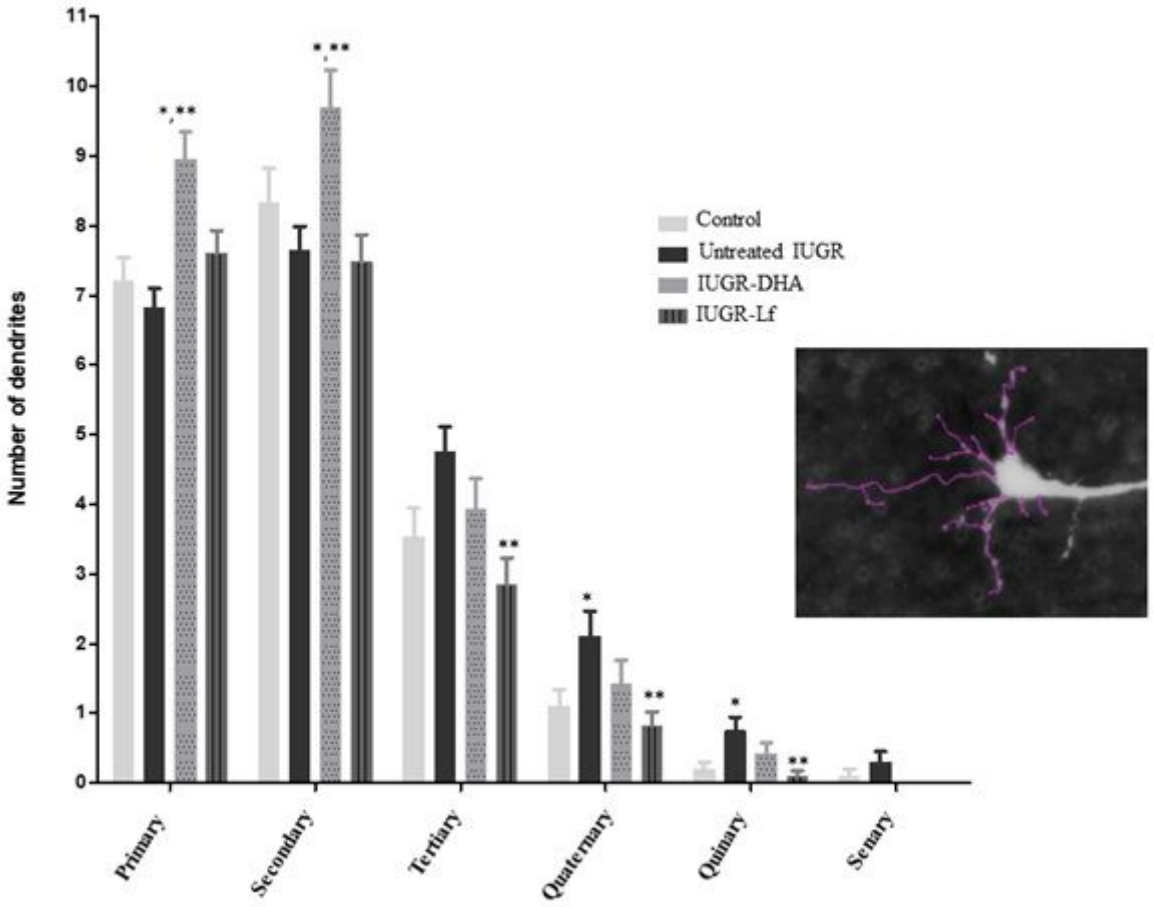

Figure 3

Number of each basal dendritic branch in the frontal cortex in the study groups. Statistical significance was declared at $P < 0.05$ between control and the rest of the experimental groups (*) and between untreated IUGR and IUGR-DHA or IUGR-Lf (**). Abbreviations: untreated IUGR = intrauterine growth restriction with no treatment; IUGR-DHA = intrauterine growth restriction treated with DHA; IUGR-Lf = intrauterine growth restriction treated with Lf.

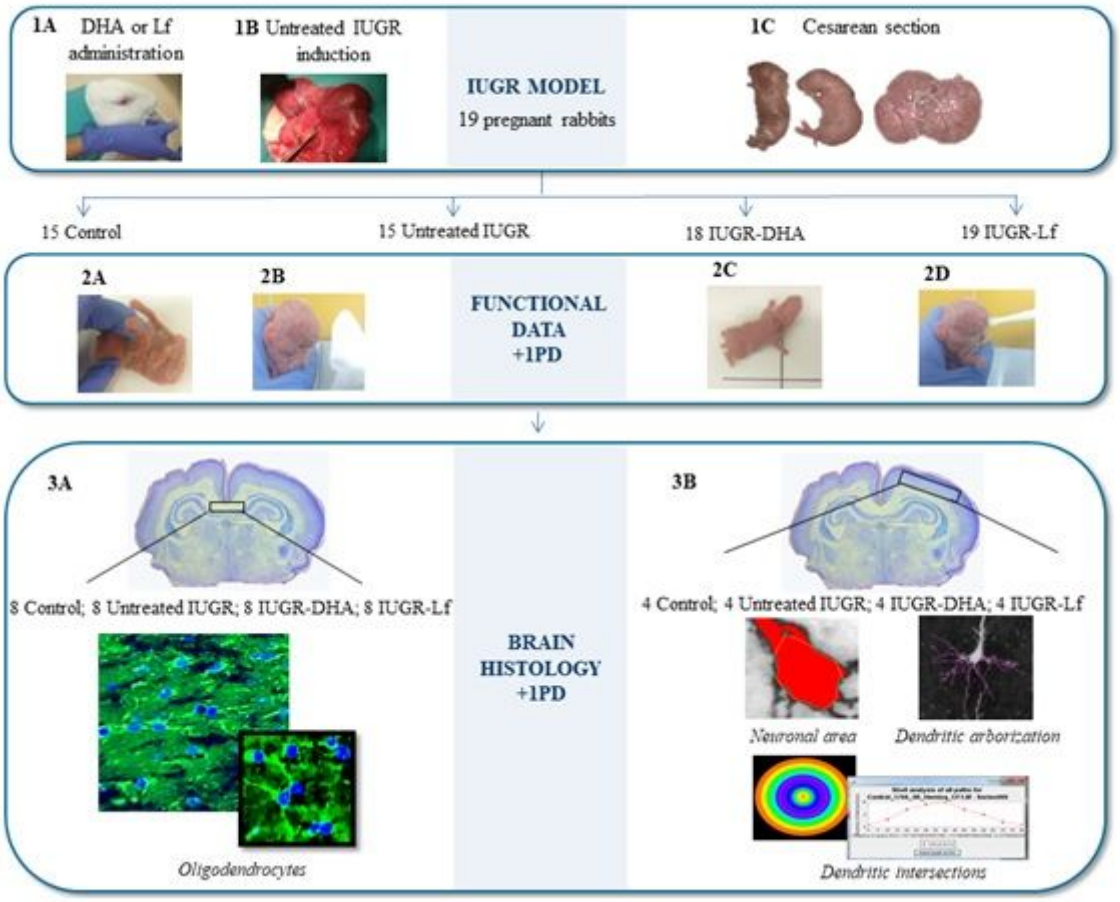

Figure 4

1A Illustrative image of DHA or Lf administration to the pregnant rabbits from 25 days of gestation until the day of the cesarean section; 1B Illustrative image of unilateral ligation of 40–50% of uteroplacental vessels at 25 days of pregnancy; 1C Illustrative images of living newborns and placenta at 30 days of pregnancy on the day of the cesarean section; Illustrative images of the neurobehavioral evaluation of righting reflex (A), smelling test (B), locomotion (C) and sucking and swallowing (D) performed at +1 postnatal day; 3A Illustrative images of O4-immunoreactive oligodendrocyte density evaluation in the CC; 3B Illustrative images of neuronal arborization analyses in the frontal cortex.

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