

Impact of postnatal nutrition on neurodevelopmental outcome in rat model of intrauterine growth restriction

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Abstract. – OBJECTIVE: There are limited data on nutritional management of infants with intrauterine growth restriction (IUGR). Postnatal protein supplementation for promoting growth is a common clinical practice in neonatology. The present study aims to investigate the consequences of protein supplementation on long-term growth, brain and body weight, brain histology and behavioral outcome in a rat model of IUGR.

MATERIALS AND METHODS: Twenty-four IUGR-formed rat puppies and 12 healthy puppies were included in the study. IUGR model was established by low (10%) protein diet throughout pregnancy together with intraperitoneal injection of lipopolysaccharide (LPS). Pups were started to be fed with either standard protein (SP), or high protein (HP) diet until postnatal day (PN) 35. Puppies in the control group were given SP diet for 35 days. Six pups from each group were sacrificed at PN7, remaining six were evaluated by Morris water maze test between PN 30 to 35 days and then sacrificed at PN35. Histologic evaluation of brain tissue was performed at PN7 and PN35.

RESULTS: IUGR group displayed lower body and brain weights at PN7 when compared with control. At PN35, SP group achieved similar brain/body weight ratios with control, whereas HP group displayed lowest brain/body weight ratio. The number of TUNEL positive cells was significantly higher and myelin basic protein and oligodendrocyte marker O4 immunoreactivity were significantly lower in HP group when compared with SP at PN35. Neuronal density in prefrontal cortex and hippocampus at PN7 were similar among SP and HP groups, but significantly lower in HP group when compared with SP at PN35. SP group displayed better results in the Morris water maze test when compared with HP group.

CONCLUSIONS: Although postnatal HP support is associated with increase in body weight at PN35, it did not result in better brain/body weight ratios in the rat model of IUGR. In IUGR rats, HP diet was associated with increased apoptosis in brain tissue with lower neuronal density and decreased myelination when compared to SP. Furthermore, better neurodevelopmental scores were achieved by SP diet rather than HP support in IUGR.

Key Words:

Intrauterine growth restriction (IUGR), Newborn, Brain, Neurodevelopment, Nutrition, Protein, Diet, Rat.

Introduction

Intrauterine growth restriction (IUGR) refers to a fetus growing slower than its expected potential due to maternal, placental, or fetal etiology¹⁻⁴. Postnatal management of IUGR remains as a challenging issue for clinicians despite advances in neonatal care². IUGR results in several long-term negative consequences including neurodevelopmental problems, motor delay and cognitive impairment¹⁻⁴. Establishing early optimal growth is of vital importance since these infants are also at high risk of experiencing poor growth through infancy and childhood, and poor catch-up growth is associated with motor and cognitive problems in IUGR infants¹. Although nutritional requirements in preterm infants are well defined, there are limited data on nutritional management of IUGR infants⁵. Postnatal protein supplementation

for promoting growth is a common clinical practice in neonatology, however, studies regarding its neurodevelopmental effects are scarce^{6,7}. Higher protein intake is reported to provide short-term growth in early infancy⁸, however its effects on neurodevelopmental outcome remains to be elucidated⁹. The study aims to investigate the consequences of protein supplementation on long-term growth, brain and body weight, brain histology and behavioral outcome in a rat model of IUGR.

Materials and Methods

Animals

This study was conducted in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the Dokuz Eylul University School of Medicine (Protocol number: 03/2015) and the ARRIVE guidelines¹⁰. Nine pregnant rats and 36 offspring were included to study. Timed pregnant Wistar rats were housed in a temperature and humidity-controlled animal facility with a 12-hour dark and light cycle. Presence of sperm in the vaginal smear was considered day 0 of pregnancy. All pregnant females and their offspring had free access to food and water throughout the experiments.

The number of pregnant animals selected for the IUGR model was 6. These animals were fed a 10% low protein diet throughout pregnancy¹¹. On the 18th and 19th days of pregnancy, the animals were injected with 300 µg/kg intraperitoneal lipopolysaccharide. Thus, it was ensured that the IUGR clinic developed¹²⁻¹⁶. At the end of the experiment, a total of 24 puppies with IUGR were born. These pups were divided into 2 equal groups with 12 rats in each group. Animals in Group 1 were given SP diet (IUGR+SP), while animals in Group 2 were given a 45% HP diet (IUGR+HP). The protein diet lasted 35 days. Three pregnant rats were selected for the control group. These rats were fed with SP diet (20%) throughout pregnancy. 12 healthy (non-IUGR) pups born from these rats were taken as the control group and fed with SP diet for 35 days¹⁷. Six puppies from the IUGR and control groups were sacrificed on day PN7 and their brains were removed. The remaining puppies were sacrificed at PN35 days, and their brains were removed. Except the amount of protein, the content of the chows in all groups was similar, including 2850 kcal energy, ions (sodium, calcium, potassium, phosphorus, and chlorine),

vitamin complex, essential amino acids (lysine, methionine, arginine, threonine, leucine, isoleucine, valine, tryptophan) and essential elements (iron, zinc, copper, selenium).

Histomorphological Evaluation

In rodents, PN7 is considered as equivalent to the term human infant, and PN35 corresponds to adolescence regarding brain development¹⁸. Six offspring from each group were sacrificed at PN7 and PN35, body and brain weights were measured after sacrifice. After removal, brain tissues were fixed in 10% formalin. Routine histological methods were applied to tissues embedded in paraffin blocks. Coronal sections of 5 µm were obtained using a rotary microtome (Leica RM2135, Germany). Sections were taken from the periventricular area for white matter examination (Plates 13-17). Other sections were taken from the prefrontal cortex (PFC) (Plates 8,9) and hippocampus (CA1, dentate gyrus (DG)) (Plates 22,23) for neuronal density, respectively in rat atlas of Paxinos and Watson¹⁹.

TUNEL Staining

To detect DNA fragmentation in cell nuclei, TUNEL (The DeadEnd Colorimetric TUNEL system kit, Roche, Mannheim, Germany) staining was used. Sections were deparaffinized, rehydrated and pretreated in proteinase K for 15 minutes. After washing, samples were incubated TdT at 37°C for 60 minutes. Converter POD (Detection of Peroxidase) solution was then applied to the slides. Sections were stained with diaminobenzidine and counter-stained with Mayer's hematoxylin²⁰.

Immunohistochemistry

After deparaffinization and rehydration, sections were treated with 10 mM citrate buffer (Cat No. AP-9003-125 Labvision). Endogenous peroxidase was inactivated in 3% H₂O₂, and then incubated with normal serum blocking solution. Sections were incubated in 18 hours at +4°C overnight with MBP (myelin basic protein, SC-71546, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and O4 (Oligodendrocyte marker, Neuromics MO15002, Edina) primer antibodies. After washing, sections were then incubated with biotinylated IgG and then with streptavidin conjugated to horseradish peroxidase (Invitrogen, Carlsbad, CA, USA; 85-9043). Sections were stained with DAB and counter-stained with Mayer hematoxylin and analyzed a light microscope²¹.

Image Analysis Methods

After the staining process, sections were examined with a light microscope (Olympus BX-51, Tokyo, Japan) and images were transferred to the computer using a high-resolution camera (Olympus DP-71, Japan). All sections were digitally photographed. Measurements were made using the UTHSCA Image Tool (software version 3.0, University of Texas Health Science Center, TX, USA)²².

Quantification of Immunohistochemical Data

For immunohistochemical scoring, positive cells were counted in five fields containing 100 cells in each preparation. The average of the scores was calculated²³.

Neuronal Density

The images were analyzed by using a computer assisted image analyzer system. The counting frame was placed randomly three times on the image analyzer system monitor, the neurons were counted (UTHSCA Image Tool for windows, software version 3.0) and the average was calculated in prefrontal cortex, CA1 and dentate gyrus. All counting and measurement procedures were performed blindly.

Morris Water Maze Test

Six animals from each group were evaluated between PN 30 to 35 days with Morris water maze test. This test is for assessment of spatial learning and reference memory in rats. It was performed in a tank with a diameter of 122 cm. The temperature of the water within the tank is maintained at 21°C. The water was tinted with a white paint to obscure the platform. A platform with a size of 10 cm × 10 cm was hidden 1 cm below the surface of water. Entry points were varied. Each trial lasted until either the rats had found the fixed platform or for a maximum of three minutes. All

rats were allowed to rest on the platform for 20 seconds and they were allowed four trials per day for four days. One day after training, the test was performed again, and the examiner determined the time of swimming until the rats reached the platform. Total distance to find the platform and escape latency periods were recorded²⁴.

Statistical Analysis

Data were analyzed using SPSS version 22.0 (IBM, Armonk, NY, USA). Values are presented as mean±SD. Normality of the distribution was evaluated with the Shapiro–Wilk test. One Way ANOVA was used for parametric group comparison with post-hoc Games Hovel test for pairwise comparisons. Differences among groups for histologic data were analyzed with Kruskal Wallis test for non-parametric data and the post-hoc pairwise comparisons were examined with the Dunn's test. *p*-values less than 0.05 were considered significant.

Results

Effects of Postnatal Diet on Body and Brain Weights

Both IUGR groups displayed lower body and brain weights at PN7 when compared with control group (*p*<0.05). Among three groups, HP group had highest, SP group had lowest body weights at PN35, and however the differences were not statistically significant. Brain/body weight ratios at PN7 were similar among SP and HP groups (*p*=0.9) and both are lower than that of control group (*p*=0.012 and 0.009, respectively). At PN35, SP group achieved similar brain/body weight ratios with control. Although statistically insignificant, HP group displayed lowest brain/body weight ratio (*p*=0.271), (Table I).

Table I. Effects of postnatal diet on body and brain weight.

	1. IUGR+SP* (n=12)	2. IUGR+HP (n=12)	3. Control (n=12)	<i>p</i> ** KW test	<i>p</i> *** post-hoc Dunn'test		
					1 vs. 2;	1 vs. 3;	2 vs. 3
Brain weight PN7 (g)	0.4 (0.3-0.4)	0.4 (0.3-0.4)	0.75 (0.7-0.4)	0.002	0.991;	0.006;	0.006
Body weight PN7 (g)	9.45 (9.2-9.5)	9.4 (9.2-10)	11.7 (11.3-13.2)	0.003	0.985;	0.011;	0.009
Brain/body weight PN7	0.042 (0.032-0.043)	0.042 (0.030-0.043)	0.062 (0.058-0.070)	0.003	0.914;	0.012;	0.009
Brain weight PN35 (g)	1.5 (1.3-1.6)	1.55 (1.4-1.7)	1.65 (1.5-1.8)	0.151	0.284;	0.070;	0.310
Body weight PN35 (g)	215.5 (179-246)	244.5 (214-313)	244 (212-275)	0.172	0.180;	0.078;	0.818
Brain/body weight PN35	0.007 (0.006 - 0.008)	0.006 (0.005-0.007)	0.007 (0.006-0.008)	0.271	0.180;	0.818;	0.240

*Values are expressed as median (min-max); **KW: Kruskal Wallis; ****p*<0.05, PN: Postnatal.

Table II. Effects of postnatal diet on apoptosis and myelinization in periventricular white matter.

Groups	TUNEL*		MBP		O4	
	PN7	PN35	PN7	PN35	PN7	PN35
1. IUGR+SP (n=6)	41.1±2.80	26.4±2.79	32.1±2.51	27.7±2.94	29.2±3.35	26.3±2.71
2. IUGR+HP (n=6)	39.9±2.55	30.2±3.48	31.7±2.79	22.9±2.02	28.9±2.76	21.4±3.09
3. Control (n=6)	4.0±0.94	3.9±0.73	48.3±4.69	37.2±4.02	47.1±4.60	34.2±2.65
<i>p</i> -value**	1 vs. 3: <0.001 2 vs. 3: <0.001	1 vs. 2: 0.010 1 vs. 3: <0.001 2 vs. 3: <0.001	1 vs. 3: <0.001 2 vs. 3: <0.001	1 vs. 2: <0.001 1 vs. 3: <0.001 2 vs. 3: <0.001	1 vs. 3: <0.001 2 vs. 3: <0.001	1 vs. 2: <0.001 1 vs. 3: <0.001 2 vs. 3: <0.001

*Values are expressed as mean±standard deviation, ***p*<0.05. MBP: Myelin basic protein, TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling, O4: Oligodendrocyte marker O4

Immunohistochemistry

The number of TUNEL positive cells (indicating apoptosis), in periventricular white matter were higher in the IUGR groups both at PN7 and PN35 when compared with control (Table I, Figure 1). The number of TUNEL positive cells at PN7 was similar among SP and HP groups, but significantly higher in HP group when compared with SP group at PN35 (*p*=0,010). MBP and O4 immunoreactivity (indicating myelination) were lower in the IUGR groups both at PN7 and PN35 when compared with control. MBP and O4 immunoreactivity at PN7 were similar among SP and HP groups. At PN35, MBP and O4 immunoreactivity were significantly lower in HP group when compared with SP group (*p*<0.001), (Table II, Figure 2). Neuronal density in PFC and CA1, and DG re-

gions of hippocampus were lower in IUGR group both at PN7 and PN35. Neuronal density at PN7 was similar among SP and HP groups and significantly lower in HP group when compared with SP group at PN35 (*p*<0.001), (Table III, Figure 1).

Morris Water Maze Behavior Test

Both IUGR groups displayed longer total distances (the total distance traveled by the animal to find the platform) when compared with control (*p*=0.036). Total distances were significantly shorter in SP group when compared with HP (*p*=0.019). Both IUGR groups demonstrated prolonged escape latencies when compared with control group. Escape latencies were shorter in SP group when compared with HP but not statistically significant (*p*=0.580), (Table IV).

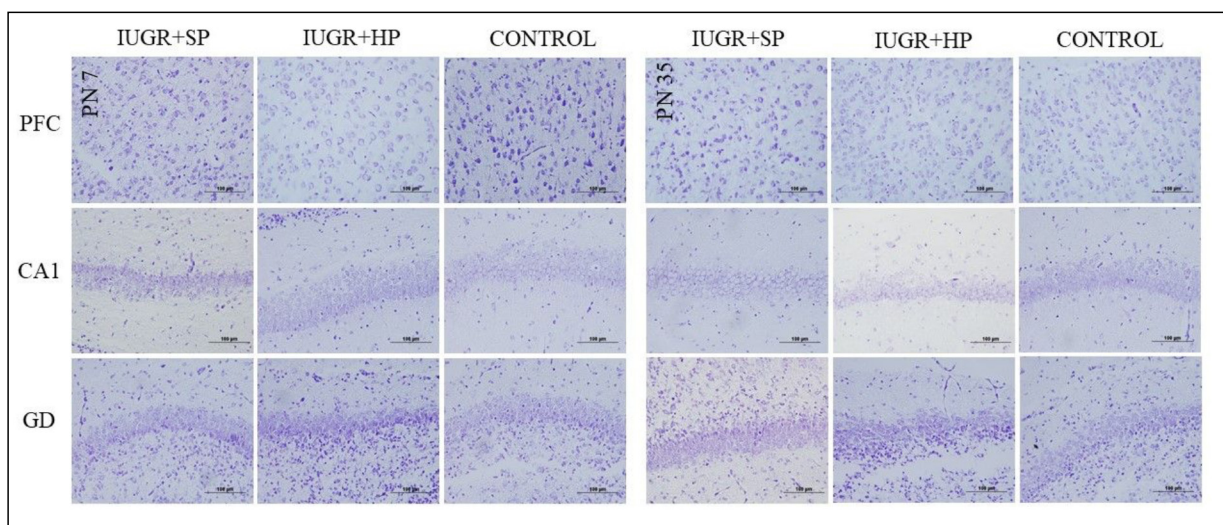


Figure 1. Crystal violet stain of Nissle positive neurons (x40). Neuronal density in the PFC, CA1, DG regions were lower in IUGR groups when compared to control (*p*<0.05). Neuronal density at PN7 were similar among SP and HP groups and significantly lower in HP group when compared with SP group at PN35 (*p*<0.001).

Table III. Effects of postnatal diet on neuronal density in PFC, CA1, and DG regions.

Groups	PFC* PN7	PN35	CA1 PN7	PN35	DG PN7	PN35
1. IUGR+SP (n=6)	31.4±4.8	37.3±3.05	73±7.7	67.2±1.87	80.5±25.0	47.8±3.04
2. IUGR+HP (n=6)	32.1±5.7	26.5±4.70	73.1±5.7	60.6±6.3	80.4±9.2	39.6±2.10
3. Control (n=6)	66.5±12.3	41.5±3.86	85.7±12.1	73.6±3.63	86.41±11.8	58.0±2.52
<i>p</i> -value**	1 vs. 3: <0.001 2 vs. 3: <0.001	1 vs. 2: 0.001 1 vs. 3: <0.001 2 vs. 3: <0.001	1 vs. 3: <0.001 2 vs. 3: <0.001	1 vs. 2: <0.001 1 vs. 3: <0.001 2 vs. 3: <0.001	1 vs. 3: <0.001 2 vs. 3: <0.001	1 vs. 2: <0.010 1 vs. 3: <0.001 2 vs. 3: <0.001

*Values are expressed as mean±standard deviation, ***p*< 0.05.

Discussion

IUGR complicates approximately one out of ten pregnancies, affecting 30 million infants per year²⁵. It is associated with reduced total brain volume with structural alterations leading to long-term functional impairments and cognitive problems². IUGR infants can present with low school performance and one-third of IUGR infants reported to have lower IQ scores in the adulthood²⁶. Degree of cognitive impairment in IUGR is strongly associated with lower anthropometric measures at birth². Catch-up growth has been identified as one of the principal determinants for improved neurodevelopment in IUGR infants²⁷. To prevent growth deficits in infants with low birth weight, either protein supplementation of breast milk or use of high protein post discharge formula is a common practice in neonatal care^{6,7}. However, evidence regarding long-term effects of protein supplementation is scarce. In the present study, the consequences of protein supplementation on long-term growth, brain and body weight, brain histology and behavioral outcomes in a rat model of IUGR are investigated. Our results show that, although postnatal protein supplementation in IUGR when compared with SP diet resulted in higher body weight in adolescence, brain/body weight ratios were not better. Furthermore, histologic evaluation of brain at PN35 regarding apoptosis, myelinization and neuronal density revealed that

HP resulted in poor scores. In accordance with histology, HP group displayed worse results in the Morris Water Maze test when compared with SP group.

Recently, catch-up growth in IUGR is reported to improve learning and memory skills in a mouse model²⁸. On the other hand, experimental data regarding neurodevelopmental results of a HP diet intervention after IUGR is lacking. The consequences of HP diet on brain tissue in adult rats are also controversial. Despite the reports regarding its beneficial neurological effects after a certain insult²⁹⁻³¹, it was reported to increase lipid peroxidation in brain³², and induce oxidative stress in cerebral cortex and hypothalamus in adult rats³³. Authors conclude that HP provides the induction of antioxidant defense system as well; however, this mechanism could not protect the brain from oxidative damage³³. Parallel with these studies, our results also revealed negative histologic consequences of HP diet on brain tissue of IUGR animals. The mechanism and signaling pathways behind these results remains to be illuminated by further molecular studies.

Clinical studies regarding nutritional management of IUGR infants are also controversial. Establishing early optimal growth is of vital importance since poor catch-up has been associated with worse neurologic outcomes². Nevertheless, nutritional management of IUGR presents as a major challenge for clinicians with a lot of unk-

Table IV. Water Maze behavior test results.

Groups	IUGR+SP (n=6)	IUGR+HP (n=6)	Control (n=6)	<i>p</i> -ANOVA	post-hoc
Total distance (cm)	1364.57± 165.15	1470.21± 189.10	1085.78±262.75	0.010	1 vs. 2: 0,019 1 vs. 3: 0,100 2 vs. 3: 1,000
Escape latency (s)	13.75 ± 11.93	18.4± 15.71	10.46± 10.94	0.580	

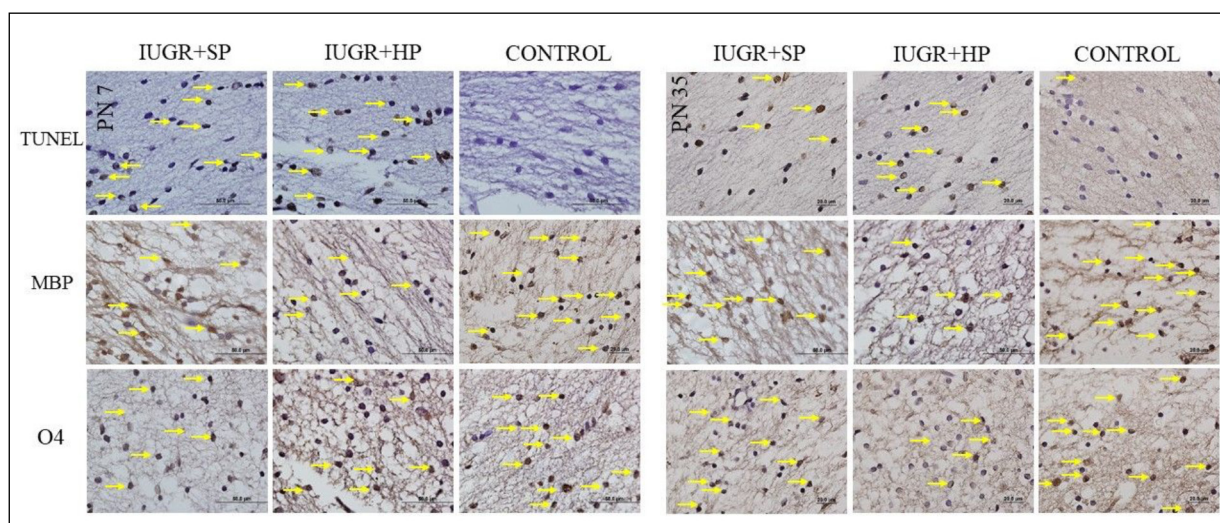


Figure 2. TUNEL, MBP and O4 immunohistochemistry of neurons (x10). The number of TUNEL positive cells were increased, MBP and O4 signaling were decreased in IUGR groups when compared with control group (both at PN7 and PN35) ($p < 0.05$). The number of TUNEL positive cells at PN7 were similar among SP and HP groups, but significantly higher in HP group when compared with SP group at PN35 ($p = 0.010$). MBP and O4 staining at PN7 were similar among SP and HP groups. At PN35, MBP and O4 were significantly lower in HP group when compared with SP group ($p < 0.001$). Staining cells are shown by yellow arrows.

nouns. Currently, either fortification of breast-milk with protein or high protein post discharge formula is recommended in small-for-gestational age (SGA) infants^{6,7}. There are still many controversies on this issue and clinical data regarding the effects of protein supplementation on neurodevelopmental outcome are limited⁵. A recent clinical systematic review suggests that, although nutrient enriched diets with higher protein to energy ratios provide increased lean mass and head circumference in preterm infants at one year, they exert subtle effect on neurodevelopmental outcome³⁴. Furthermore, a randomized controlled trial investigating the effects of high protein post discharge formula on growth pattern of SGA infants did not report any significant differences even in terms of fat mass, fat free mass and the quality of growth⁵.

A recent Cochrane meta-analysis investigated the effects of low (< 3.0 g/kg/d), high (≥ 3.0 < 4.0 g/kg/d), or very high (≥ 4.0 g/kg/d) protein in formula-fed hospitalized neonates weighing less than 2.5 kg³⁵. Evidence from six eligible trials that enrolled 218 infants reveal that early nutrition with high protein formula provides accelerated weight gain. Neurodevelopmental outcomes were evaluated by three out of the six studies and authors signify that the current state of evidence is not enough to make conclusion regarding the neurodevelopmental effects of early high protein diet whereas uncertainty remains about effectiveness

as well as safety and of protein intake ≥ 4.0 g/kg/d. Considering our results of brain histology in the HP group, we also propose that further studies should be made in order to clarify the protein requirements of IUGR infants and long-term effects of protein supplementation on brain.

Certain anatomical, physiological, biochemical, and behavioral effects of prenatal and postnatal protein malnutrition on the developing rat brain are previously reported^{36,37}. We think that, if there would be another IUGR group fed with low protein diet postnatally (representing the poor feeding IUGR infants), overall, the worst histologic and neurodevelopmental outcome will be noted in this group. IUGR infants inherently possess high risk for postnatal growth failure due to poor feeding, so they may experience more prominent postnatal weight loss and it may take much longer to regain birth weight when compared to appropriate-for-gestational-age infants³⁸. Close follow-up and prompt intervention is required in IUGR infants³⁸. Nevertheless, postnatal nutritional management of infants with IUGR requires a fine balance. Neither insufficient catch-up growth as a result of inadequate nutrient supply, nor promoting rapid fat tissue gain with potential long-term negative consequences is preferred⁶. Generally, too fast postnatal catch-up growth has been linked to chronic diseases of adult life such as insulin resistance and metabolic syndrome

whereas poor catch-up growth is associated with low IQ and reduced final height³⁹. Despite enough cases, the short duration of protein administration is an important limitation such as the fact that it has not been clearly tested whether the offspring develops IUGR. Lack of evaluation on the development of organs other than the brain is another shortcoming.

Conclusions

Current evidence on protein requirements in term IUGR infants remain scarce³⁹. According to present study, although postnatal HP support is associated with increase in body weight in adolescence period, it did not result in better brain/body weight ratio in the rat model of IUGR. Furthermore, in the presence of IUGR, better neurodevelopmental scores were achieved by SP diet rather than HP support, whereas HP diet was associated with increased apoptosis, lower neuronal density and decreased myelination. Future experimental and clinical studies are warranted in order to determine the precise protein requirements of IUGR infants and long-term risks or benefits of protein supplementation on brain.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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